ARTICLE

# Effect of a Low-Fat Diet Enriched with Oleic Acid on Postprandial Lipemia in Patients with Type 2 Diabetes Mellitus

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**ABSTRACT:** The aim of the present study was to compare the effects of a low-fat diet enriched with oleic acid to those of a low-fat diet enriched with linoleic acid on fasting lipids, postprandial lipemia, and oxidative susceptibility of low-density lipoprotein (LDL) in patients with type 2 diabetes mellitus (DM). In a 3-wk randomized crossover study, eight patients with type 2 DM were given an experimental low-fat diet enriched with either oleic acid or linoleic acid. The oleic-acid-enriched diet contained 5, 15, and 5% energy from saturated, monounsaturated, and polyunsaturated fatty acids, and the linoleic-acid-enriched diet contained 5, 5, and 15% energy from saturated, monounsaturated, and polyunsaturated fatty acids, respectively. In addition to evaluating the fasting lipids and oxidative susceptibility of LDL, we evaluated postprandial lipemia using an oral fat load at the end of each 3-wk dietary phase. There were no significant differences in fasting lipid profile or lag time of LDL oxidation between the two experimental dietary phases. The average and maximal increments of remnant-like particle (RLP) cholesterol levels during oral fat load were significantly higher after the oleic-acid-enriched dietary phase than after the linoleic-acid-enriched dietary phase. The area under the curve of RLP cholesterol was also significantly larger after the oleic-acid-enriched dietary phase than after the linoleic-acid-enriched dietary phase. These results suggest that the oleic-acid-enriched diet was associated with increased formation of postprandial chylomicron remnants compared with the linoleic-acid-enriched diet.

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Postprandial lipemia is a potential risk factor for atherosclerosis that has been reported to be enhanced in patients with coronary heart disease (CHD) or diabetes mellitus (DM) (1–4). In particular, intestinal chylomicron remnants, which increase in circulating blood after fat intake, are thought to be atherogenic lipoproteins (5–9). Recently, these remnants have been reevaluated as remnant-like particles (RLP) that are not bound to immunoaffinity-mixed gels containing apolipoprotein (apo) A-I and apo B-100 monoclonal antibodies (10). Clinical studies using RLP have reported that RLP cholesterol in the fasting state is associated with atherosclerosis (11–13). RLP cholesterol has been known to increase in the postprandial state, so it is a useful and suitable marker of remnant lipoproteins after an oral fat-loading test (14). Nakamura *et al.* (15) reported that responses of RLP cholesterol after an oral fat load in CHD patients were higher than those in control patients with similar fasting lipid profiles. It is possible that optimizing diet reduces RLP cholesterol after a fat load could have beneficial effects in vascular disease.

The importance of dietary therapy in patients with type 2 DM is well known. Recent studies have suggested that oleic acid has several beneficial effects, such as decreasing low density lipoprotein (LDL) cholesterol without decreasing high density lipoprotein (HDL) cholesterol and providing protection from oxidation of the LDL particles (16–18). However, we previously reported that postprandial chylomicron remnants increase after an olive oil load compared to milk fat or safflower oil loads among healthy men using the measurement of RLP as intestinal chylomicron remnants (19). A high monounsaturated fatty acid-enriched diet is reportedly not preferable to a high-carbohydrate diet in patients with type 1 DM with regard to the occurrence of postprandial lipemia (20); however, it has been unclear whether this is also true for patients with type 2 DM.

In the Japanese population, the percentage of total daily calories from fat is lower than 30%, a lower percentage than most Western diets. To date, how various compositions of fatty acids in relatively low-fat diets might affect fasting and postprandial lipoproteins has been unclear. In the present study, we investigated the effects of oleic-acid-enriched and the linoleic-acid-enriched low-fat diets on fasting, postprandial lipemia, and susceptibility to LDL oxidation in patients with type 2 DM.

## MATERIALS AND METHODS

*Subjects*. We studied eight patients (two men and six postmenopausal women; mean age: 62.0 ± 6.9 yr; mean body mass index: 24.7 ± 2.5 kg/m$^2$) who were admitted to our hospital for control of hyperglycemia. The mean ± SD of fasting glucose, glycosylated hemoglobin, total cholesterol, triglyceride (TG), and HDL cholesterol levels were 170 ± 58 mg/dL, 8.8 ± 0.9%, 5.28 ± 0.78 mmol/L, 1.52 ± 0.40 mmol/L, and

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Abbreviations: apo, apolipoprotein; AUC, area under the curve; CHD, coronary heart disease; DM, diabetes mellitus; FFA, free fatty acid; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; LPL, lipoprotein lipase; LPO, lipid peroxidation; PBS, phosphate-buffered saline; RLP, remnant-like particles; TG, triglyceride.

1.14 ± 0.18 mmol/L, respectively. Four of the subjects were taking sulfonylureas, but none of the subjects was taking other medications that affect lipoprotein metabolism before or throughout the present study. All subjects gave written informed consent for participation in the present study. The experimental protocol was approved by the Ethical Committee of the National Defense Medical College of Japan.

*Experimental protocol.* A randomized crossover design was used with patients following each of the two experimental diets for 3 wk following admission to our hospital. The experimental diets consisted of 25, 58, and 17% energy from fat, carbohydrate, and protein, respectively. The oleic-acid-enriched diet contained 5, 15, and 5% energy from saturated, monounsaturated, and polyunsaturated fatty acid, and the linoleic-acid-enriched diet contained 5, 5, and 15% energy from saturated, monounsaturated, and polyunsaturated fatty acid, respectively. All food was prepared in the nutritional department of the National Defense Medical College Hospital. The number of calories and the amounts of cholesterol and fiber were about the same in both diets. All participants remained in the hospital during the experiment, and they consumed only food that was supplied. The total calories consumed and the amount of exercise each patient performed were continued and were not changed during the experiment.

To evaluate postprandial lipemia, all subjects underwent an oral fat-loading test after a 12-h fast at the end of each 3-wk dietary phase. The test drink was 25 g/$m^2$ body surface area of bovine milk fat emulsion (Fresh Cream; Meiji Milk Co., Tokyo, Japan) and 20 g/$m^2$ body surface area of sucrose. Blood samples were drawn before and at 2, 3, 4, 5, and 7 h after ingestion of the test drink. TG and RLP cholesterol levels of all samples were measured as markers of postprandial lipemia. Intake of food or drink was prohibited throughout the postprandial study. On another day, subjects received an intravenous injection of heparin (30 IU/kg body wt) after an overnight fast. A blood sample was collected 15 min later for determination of post-heparin lipoprotein lipase (LPL) mass.

*Biochemical analysis.* Blood was collected in tubes containing a final concentration of 0.1% EDTA. Plasma was immediately separated by centrifugation (2,000 rpm) for 20 min at 4°C. The analysis of TG, total cholesterol, and HDL cholesterol was performed using an enzymatic method (Determiner L; Kyowa, Tokyo, Japan). RLP particles were isolated from other lipoproteins as the unbound fraction in immunoaffinity mixed gels containing monoclonal apoA-I and apoB-100 antibodies within 48 h, following the method of Nakajima *et al.* (10). In brief, 5 μL of plasma was added to the mixture of 50 μL of CNBr Sepharose 4B (Amersham Pharmacia Biotec, Tokyo, Japan), which contained 125 μg of anti-apoA-I monoclonal antibody, 125 μg anti-apoB-100 antibody (JI-H), and 300 μL of reaction suspension. The mixture was shaken gently for 60 min to mix and then allowed to sit for 10 min. Thirty microliters of clear supernatant was taken, and the cholesterol level of RLP was determined enzymatically. Lipid peroxidation (LPO) in plasma was determined by using a commercially available kit (Determiner LPO; Kyowa) of a leukomethylene blue derivative with lipid peroxides in the presence of heme compounds. The oxidative susceptibility of LDL was evaluated by the modified method of Esterbauer *et al.* (21). LDL, adjusted to 50 μg protein/mL with phosphate-buffered saline (PBS), was incubated with 2 mmol/L $CuSO_4$ in PBS (final volume, 2 mL) at 37°C. Conjugated diene formation during LDL oxidation was monitored by changes in absorbance at 234 nm in a spectrophotometer (Shimadzu 160A; Shimadzu, Tokyo, Japan) equipped with a six-position automatic changer. The lag time of LDL oxidation was defined as the intercept of the tangent of the slope in the absorbance curve during the propagation phase. The LPL mass of post-heparin plasma was determined by sandwich enzyme-linked immunosorbant assay (Markit F LPL; Dainippon, Osaka, Japan). Plasma fatty acid composition was determined by gas chromatography (22). Plasma glucose and free fatty acid (FFA) levels were measured enzymatically, and insulin level was determined by radioimmunoassay (23). Vitamin E in LDL was determined by high-performance liquid chromatography (HPLC). In brief, LDL was precipitated with ethanol, and vitamin E was subsequently extracted with hexane. The hexane phase was evaporated under $N_2$ gas and the residue dissolved in ethanol. Vitamin E was separated by reversed-phase HPLC on C18 columns (TSK gel ODS-80Ts; Tohso, Tokyo, Japan) that were eluted with ethanol/distilled water (92:8, vol/vol) at 1.0 mL/min as the mobile phase and monitored at 295 nm in an ultraviolet detector (UV-8000; Tohso).

*Statistical analysis.* Statistical analyses were carried out using the Macintosh for Expert StatView 5.0 software. The fasting values between the pre-experiment levels and those detected at the end of each 3-wk dietary phase were compared by paired *t*-test. The fasting and postprandial values at the end of each 3-wk dietary phase were compared by repeated measures analysis of variance. The average level, maximal increment, and area under the curve (AUC) of TG and RLP cholesterol between groups during a fat load were compared by paired *t*-test. Values of $P < 0.05$ were considered significant.

## RESULTS

*Fasting parameters*. The total calories, amount of exercise, and medication dose(s) for each patient did not change throughout the study, and all subjects completed this experiment with good compliance. The change of fatty acid composition after each dietary phase is shown in Table 1. The percentage of 18:1n-9 in total fatty acids after the oleic-acid-enriched dietary phase and that of 18:2n-6 after the linoleic-acid-enriched dietary phase were significantly higher than the counterpart diets, respectively. The fasting parameters in blood at the end of each 3-wk dietary phase are shown in Table 2. Fasting blood glucose after both the oleic-acid-enriched dietary phase and the linoleic-acid-enriched dietary phase was significantly decreased compared to the pre-experiment value ($P < 0.05$, $P < 0.05$, respectively). The levels of TG and HDL cholesterol after each dietary phase did not change significantly compared to the pre-experiment value.

**TABLE 1**
**Change of Plasma Fatty Acid Composition at the End of Each Dietary Phase[a]**

| | Linoleic-acid-enriched diet | Oleic-acid-enriched diet |
|---|---|---|
| 16:0 | 23.3 ± 0.9 | 23.4 ± 2.2 |
| 18:0 | 6.3 ± 0.5 | 6.3 ± 0.6 |
| 18:1n-9 | 18.3 ± 0.9 | 20.4 ± 2.4* |
| 18:2n-6 | 30.5 ± 1.5 | 27.1 ± 3.2* |
| 18:3n-3 | 0.2 ± 0.1 | 0.2 ± 0.2 |
| 18:3n-6 | 0.7 ± 0.3 | 0.7 ± 0.1 |

[a]Values are mean ± SD. *, $P < 0.05$ difference from linoleic-acid-enriched diet.

**TABLE 2**
**Fasting Various Parameters at the End of Each Dietary Phase[a]**

| | Linoleic-acid-enriched diet | Oleic-acid-enriched diet |
|---|---|---|
| Blood glucose (mg/dL) | 121 ± 16 | 107 ± 12 |
| Insulin (μU/mL) | 5.9 ± 3.7 | 7.3 ± 5.9 |
| NEFA (meq/L) | 0.6 ± 0.2 | 0.7 ± 0.2 |
| Total cholesterol (mmol/L) | 4.68 ± 0.50 | 4.69 ± 0.66 |
| Triglyceride (mmol/L) | 1.22 ± 0.31 | 1.32 ± 0.71 |
| HDL cholesterol (mmol/L) | 1.19 ± 0.25 | 1.21 ± 0.25 |
| LDL cholesterol (mmol/L) | 2.96 ± 0.39 | 2.89 ± 0.46 |
| RLP cholesterol (mmol/L) | 7.3 ± 2.0 | 9.8 ± 6.3 |
| Lipoprotein lipase (ng/mL) | 216 ± 45 | 201 ± 52 |
| Lipid peroxide (nmol/L) | 0.9 ± 0.1 | 0.8 ± 0.4 |
| Vitamin E in LDL (μmol/g) | 8.3 ± 1.4 | 8.6 ± 2.3 |
| Lag time (min) | 61.9 ± 8.5 | 61.0 ± 9.2 |

[a]Values are presented as mean ± SD. There was no significant difference between the two dietary phases. NEFA, nonesterified free fatty acid; HDL, high density lipoprotein; LDL, low density lipoprotein; RLP, remnant-like particles.

The levels of total cholesterol and LDL cholesterol after the oleic-acid-enriched dietary phase were insignificantly decreased ($P = 0.06$, $P = 0.08$, respectively); however, those after the linoleic-acid-enriched dietary phase were significantly decreased ($P < 0.01$, $P < 0.05$, respectively). Fasting blood glucose was slightly lower after the oleic-acid-enriched dietary phase than after the linoleic-acid-enriched dietary phase, but the difference was not statistically significant ($P = 0.09$). There were no differences between the two diets in fasting insulin or FFA. The levels of total cholesterol, TG, HDL cholesterol, LDL cholesterol, RLP cholesterol and LPL mass, lipid peroxide, lag time of LDL oxidation, and vitamin E concentration in LDL were not significantly different between the two dietary phases.

*Postprandial parameters.* The increment in TG and RLP cholesterol levels after an oral fat load is shown in Figure 1.

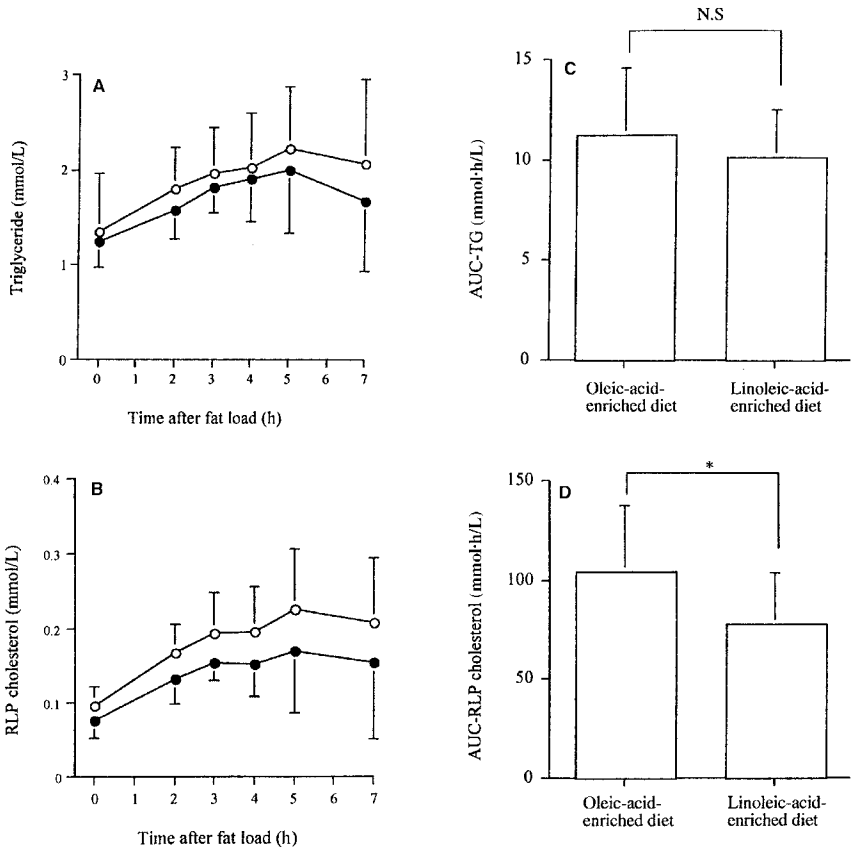


**FIG. 1.** The increment of triglyceride (TG) (A) and remnant-like particle (RLP) cholesterol (B) levels in plasma, and the area under the curve (AUC) of TG (C) and RLP cholesterol (D) during an oral fat load at the end of each 3-wk dietary phase ($n = 8$). Open circles indicate the oleic-acid-enriched dietary phase; closed circles, the linoleic-acid-enriched dietary phase.

**TABLE 3**
**Postprandial Parameters During Oral Fat Load at the End of Each Dietary Phase**

| | Linoleic-acid-enriched diet | Oleic-acid-enriched diet |
|---|---|---|
| Average of triglyceride | 150 ± 37 | 167 ± 51 |
| Maximal increment of triglyceride | 63 ± 40 | 76 ± 19 |
| AUC of triglyceride | 893 ± 202 | 992 ± 289 |
| Average of RLP cholesterol | 5.3 ± 1.9 | 6.9 ± 2.0* |
| Maximal increment of RLP cholesterol | 3.5 ± 2.6 | 4.9 ± 2.2* |
| AUC of RLP cholesterol | 31.5 ± 10.0 | 40.7 ± 11.3* |

*Values are presented as mean ± SD (mmol/L or mmol·h/L). AUC indicates area under the curve; see Table 2 for other abbreviation. *$P < 0.05$ difference from linoleic-acid-enriched diet.

The increment in plasma TG concentration was maximal 5 h after an oral fat load. The increment in TG during a fat load was not significantly different between the oleic-acid-enriched dietary phase and the linoleic-acid-enriched dietary phase. The increment in RLP cholesterol was also maximal 5 h after a fat load. RLP cholesterol levels during an oral fat load were not significantly higher after the oleic-acid-enriched dietary phase than after the linoleic-acid-enriched dietary phase. The average level, maximal increment, and the AUC of TG and RLP cholesterol during an oral fat load at the end of each dietary phase are shown in Table 3. The average level, maximal increment, and AUC of TG were similar between the two dietary phases. However, the average level, maximal increment, and AUC of RLP cholesterol during an oral fat load were significantly higher ($P < 0.05$) after the oleic-acid-enriched dietary phase than after the linoleic-acid-enriched dietary phase.

## DISCUSSION

We could confirm that all subjects completed the dietary study with good compliance, because all subjects consumed the experimental diets while staying in the hospital. Moreover, the expected change of plasma fatty acid after each dietary phase showed good compliance. In the present study, the oleic-acid-enriched diet increased the maximal increment of postprandial chylomicron remnants represented by RLP cholesterol after an oral fat load more than did the linoleic-acid-enriched diet. Although both diets improved fasting glucose levels, there was no statistically significant difference in fasting glucose, lipid profile, or susceptibility to LDL oxidation between the two experimental dietary phases.

It has been reported that linoleic acid decreases LDL cholesterol and HDL cholesterol in healthy subjects but that oleic acid decreases LDL cholesterol without decreasing HDL cholesterol (16). On the other hand, it was reported that lipid-lowering diets enriched with monounsaturated or polyunsaturated fatty acids have similar effects on serum lipid concentrations in hyperlipidemic patients (24). The latter findings might be in agreement with our present study, because the improvement in fasting lipid parameters was similar after the oleic-acid-enriched dietary phase and the linoleic-acid-enriched dietary phase. In the present study, the HDL cholesterol level did not decrease in the linoleic-acid-enriched diet. The initial HDL cholesterol level might be low and the initial LDL cholesterol level might be high in the present subjects without well-controlled DM, or the effect of fatty acid composition in the low-fat diets in the present study might not be enough to affect these lipoproteins. At the least, these lipoprotein levels might slightly improve if the patient follows a strict diet plan, although the effect of the different fatty acid composition in a low-fat diet on fasting glucose level was not clear in this study. Therefore, glucose metabolism might need to be controlled to gain an improved lipid profile.

Remnant lipoproteins, which appear in the postprandial state, have been thought to be one of the atherogenic lipoproteins because they are easily absorbed and increase the esterified cholesterol of cells (5–9). Therefore, a reduction of the usual increase in RLP in the postprandial state could be beneficial and might counter the development of atherosclerosis or its complications. In the present study, dietary intervention for 3 wk in the form of a diet enriched with oleic acid increased the appearance of postprandial remnants compared to a diet enriched with linoleic acid. The present result is similar to the findings of Georgopoulos *et al.* (20) in patients with type 1 DM. They reported that a high monounsaturated fatty acid-enriched diet might not be preferable to a high-carbohydrate diet in patients with type 1 DM with regard to the effect on postprandial lipemia. Moreover, two other recent studies reported increase postprandial lipemia by dietary oleic acid, despite the beneficial effects on hemostatic factors in healthy subjects (25,26). We consider these findings to be in agreement with our present study.

The mechanism of increased postprandial lipemia with the oleic-acid-enriched diet was not investigated in the present study. However, this finding could be due to increased influx or to delayed clearance of chylomicron remnants. We previously reported that the level of RLP cholesterol after an acute fat load enriched with olive oil increased more than that with milk fat and safflower oil (19). As chylomicron remnants increase in the postprandial state during consumption of the oleic-acid-enriched diet, they might remain steady during a fasting state. Several reports have described the effects of various fatty acids on the secretion of apoB-containing lipoproteins by Caco-2 cells and HepG2 cells, which have been used as experimental models of intestinal and hepatic lipoprotein synthesis. Both Caco-2 cells and HepG2 cells incubated with oleic acid secrete more particles than do cells incubated with palmitic or linoleic acid (27,28). Thus, oleic acid has been thought to have a strong affinity to substrates of acylcoenzyme A:cholesterol acyltransferase. In that case, oleic acid increases synthesis and secretion of TG-rich lipoproteins derived from the intestine and liver in patients and leads to an increase in chylomicron remnants in the blood by competition as a receptor to the TG-rich lipoproteins.

On the other hand, it has been suggested that linoleic acid activates the LDL receptor in the liver (29,30), which requires apo B100 or apo E in LDL for binding. Some researchers believe that chylomicron remnants, which are enriched with apo E, are taken up by the LDL receptor (31). Therefore, a linoleic-acid-enriched diet might activate the LDL receptor and accelerate the clearance of chylomicron remnants by this receptor. This process may be one reason that less RLP appears after a fat load when following a linoleic-acid-enriched diet than when following an oleic-acid-enriched diet.

It has been shown that oleic acid protects against LDL oxidation, which has been suggested to play an important role in atherogenesis (17,18). In contrast, Schwab *et al.* (32) reported that the dietary fatty-acid composition in reduced-fat diets has no significant differences in LDL lag time among olive oil, canola oil, corn oil, and rice bran oil. Our present study is in agreement with the latter study. The difference in fatty acid composition of low-fat diets (25 energy %) in the latter study, which is similar to the present study, might be too weak to affect susceptibility to LDL oxidation. Moreover, the similar level of vitamin E in LDL might be due to a similar susceptibility to LDL oxidation.

In conclusion, both the oleic-acid-enriched and the linoleic-acid-enriched diets we studied are low-fat diets with adequate calories to improve fasting glucose among patients with poorly controlled type 2 DM. However, the former diet might increase the appearance of postprandial chylomicron remnants more than the latter diet.

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# A Long-Term Seal- and Cod-Liver-Oil Supplementation in Hypercholesterolemic Subjects

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**ABSTRACT:** In this long-term study, we wanted to explore the effect of dietary supplementation of seal oil (SO) as compared cod-liver oil (CLO) on subjects with moderate hypercholesterolemia. The test parameters included fatty acid composition in serum, blood lipids, platelet aggregation, and the activity of blood monocytes. After a run-in period of 6 mon, 120 clinically healthy hypercholesterolemic (7.0–9.5 mmol/L; 270–366 mg/dL) subjects were randomly selected to consume either 15 mL of SO or CLO daily for 14 mon followed by a 4-mon wash-out period. A third group was not given any dietary supplement (control). Consumption of marine oils (SO and CLO) changed the fatty acid composition of serum significantly. Maximal levels were achieved after 10 mon. No further changes were seen after 14 mon. A wash-out period of 4 mon hardly altered the level of n-3 fatty acids in serum. Addition of SO gave 30% higher level of eicosapentaenoic acid, as compared to CLO. Subjects taking SO or CLO had lower whole-blood platelet aggregation than the control group. Neither SO nor CLO had any effects on the levels of serum total cholesterol, high-density lipoprotein cholesterol, postprandial triacylglycerol, apolipoproteins A1 and B100, lipoprotein (a), monocyte function expressed as monocyte-derived tissue factor expression, and tumor necrosis factor.

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During the last two decades it has been established that Greenland Eskimos living on their traditional diet have a lower incidence of coronary heart disease than Danes living on a Western diet (1,2).

The Eskimos have significantly prolonged primary bleeding time, a mild clinical bleeding tendency, increased level of n-3 fatty acids in blood plasma and cell membranes, lower serum total cholesterol, high density lipoprotein (HDL)-cholesterol, lower apolipoprotein (Apo) B100 and higher Apo A1 levels as compared to those regarding the Danes (3–6). These findings have been attributed to their diet, particularly the marine fatty acids.

Dietary intake of fish, fish oil, and n-3 fatty acid-containing capsules does not give similar findings of blood n-3/n-6 ratio, total cholesterol, HDL-cholesterol, Apo A1, and Apo B100 as in Greenland Eskimos.

The Eskimo diet consists mainly of meat and blubber of seal and whale, containing high amounts of monounsaturated acids (MUFA) and n-3 polyunsaturated fatty acids (PUFA) and relatively small amounts of saturated fatty acids (SFA) and fish (7,8).

Eskimos consume the bulk of their food raw or dried, seldom boiled or exposed to excessive heat. The fatty acid composition in seal and whale blubber also deviates from fish oil in the positioning of the n-3 fatty acids in the glycerol (9–11). It has also been shown that PUFA in marine mammals are quite well-protected from oxidation (12).

Seal oil (SO) contains slightly more MUFA as compared to cod-liver oil (CLO); the eicosapentaenoic acid (EPA; 20:5n-3) molecule is mainly in the 1,3-position of the glycerol, and SO also contains a strong natural antioxidant, not yet fully identified. If the positioning of the fatty acids in the glycerol molecule or the antioxidative abilities of seal fat are significant in the prevention of arteriosclerosis, it might be possible to detect effects on parameters related to the development of this condition, by ingesting SO or blubber.

The oils served in the present study were subjected to a traditional CLO refining process. The main objectives of such processes are to remove pesticides and to make an edible and stable product. Removal of molecules that cause off-flavors or taste by "steam stripping" (exposure to excessive heat) to improve sensory attributes may destroy potent antioxidants. Other steps in the process designed to remove impurities (protein, water, and polar lipids) may as well remove components with potential beneficial effects (13,14).

Reduced tendency of developing arteriosclerosis has been related to the lower reactivity of platelets and less production of proinflammatory products, e.g., cytokines, prostaglandins,

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Abbreviations: ALAT, alanine amino transferase; ALP, alkaline phosphatase; Apo, apolipoprotein; AST, aspartate aminotransferase; CLO, cod-liver oil; CRP, C reactive protein; CV, coefficient of variation; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, high density lipoprotein; Lp(a), lipoprotein (a); MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SO, seal oil; TF, tissue factor; TNF, tumor necrosis factor.

and leukotrienes (15,16), and the maintenance of prostacyclin production in the endothelial cells (17). Various studies have verified that dietary intake of n-3 fatty acids (fish, fish oil, or fish oil capsules) increases the level of these fatty acids in blood plasma and cellular compartments and prolongs the bleeding time.

The objective of the present work is, by mimicking an aspect of the traditional Eskimo diet, to explore therapeutic and prophylactic possibilities of SO on parameters related to development of cardiovascular disease [n-3 fatty acids in serum, blood cholesterol, platelet activity, blood monocyte activity (tissue factor: TF), inflammatory activity (tumor necrosis factor: TNF)], as compared to CLO.

## MATERIALS AND METHODS

*Subjects.* The study was carried out at Hammerfest General Hospital (situated at 70° N latitude) in northern Norway. It included 120 clinically healthy volunteers from 43 to 66 yr of age (median age 55), 60 males (median age 54) and 60 females (median age 56); 102 subjects finished the study. The inclusion criteria were serum cholesterol 7.0–9.5 mmol/L (270–366 mg/dL), clinically healthy, and no lipid-lowering drugs. They were given general dietary advice (consume more vegetables, fruit and fish, and less saturated fat). All participants were asked to continue their daily habits. The study was approved by the Regional Board of Research Ethics.

*Study design.* The subjects were randomly divided into three groups. One group received 15 mL/d SO (about 1.1 g EPA, 20:5n-3 and 1.5 g DHA, 22:6n-3); another group received 15 mL CLO (about 1.5 g EPA and 1.8 g DHA); and a third group received no oil (control). The fatty acid composition of the oils is given in Table 1. The study was double-blinded for the groups given the dietary oils. No placebo was given in the control group, and the study of this group could thus not be blinded to test subjects or investigators. The groups were in parallel for a period of 2 yr.

Before the intake of the oils, there was a run-in period of 6 mon where the serum cholesterol level was monitored. The period of oil supplementation lasted 14 mon, followed by a 4 mon wash-out period.

*Oil supplements.* The oils were processed by a standard procedure. This included heating to minimum 150°C, removal of pesticides and dioxins, vitamins A and D, molecules that cause off-flavor or taste, and addition of vitamin E (tocopherol, 1 mg/mL). The oils were kept frozen until used by the participants. Each test subject was delivered one 250-mL bottle of oil at regular intervals from the start of the study.

Both SO and CLO are relatively rich in n-3 fatty acids (Table 1). SO contains higher amounts of MUFA (16:1, 18:1, 20:1, 22:1). The fatty acid content of both oils, kept in control bottles in a refrigerator at the test laboratory, were controlled several times during the study and did not change (Table 1).

*Diet.* The test subjects consumed an ordinary Norwegian diet throughout the study (in general about 40% fat with P/S ratio of 0.5, 15% proteins, and 45% carbohydrates). The dietary content was evaluated by a clinical dietician by means of a questionnaire at the entry of the study and repeated twice during the study to discover possible dietary changes. The test subjects were asked to report their weekly physical activity in the same questionnaire.

**TABLE 1**
**The Fatty Acid Composition (weight percent) of Cod-Liver Oil and Seal Oil**

| | Cod-liver oil[a] | | Seal oil[a] | |
|---|---|---|---|---|
| | Entry | 14 mon | Entry | 14 mon |
| Fatty acid | | | | |
| 14:0 | 3.8 | 3.6 | 5 | 5 |
| 16:0 | 9.4 | 9.2 | 7.6 | 7.6 |
| 18:0 | 2.1 | 1.9 | 0.5 | 1.1 |
| Sum | 15.3 | 14.7 | 13.1 | 13.7 |
| 16:1n-7 | 7.9 | 7.2 | 12.1 | 11.6 |
| 18:1n-9 | 17.1 | 17.2 | 19.6 | 19.6 |
| 18.1n-7 | 4.2 | 3.6 | 3.2 | 2.9 |
| 20:1n-9 | 10.9 | 11.5 | 9.6 | 10.2 |
| 22:1n-11 | 5.3 | 5.9 | 3.5 | 3.9 |
| 22:1n-9 | 0.5 | 0.5 | 0.4 | 0.4 |
| Sum | 45.9 | 45.9 | 48.4 | 48.6 |
| 18:2n-6 | 1.8 | 1.7 | 1.9 | 1.9 |
| 18:3n-3 | 1 | 0.9 | 1 | 0.9 |
| 18:4n-3 | 3 | 2.7 | 3.5 | 3.2 |
| 20:5n-3 | 10.1 | 9.7 | 7.4 | 7.2 |
| 22:5n-3 | 1.2 | 1.2 | 3.9 | 3.9 |
| 22:6n-3 | 11.9 | 12.1 | 10.5 | 10.5 |
| Sum | 27.2 | 26.6 | 26.3 | 25.7 |
| n-3/n-6 | 15.1 | 15.6 | 13.8 | 13.5 |
| Rest (unidentified) | 9.8 | 11.1 | 10.3 | 10.1 |

[a]The oils were kept at 4°C.

*Body weight.* Each of the participants was asked to report their body weight three times during the study (6 wk before oil intake, and after 2 and 6 mon of dietary oil supplementation).

*Blood sampling.* Blood samples were collected at 6 and 3 mon before the start of the oil supplementation (run-in period), at the start after 4, 10, and 14 mon of supplementation, and after the wash-out period (4 mon). The test subjects were informed not to drink alcohol or perform any strenuous exercise for 48 h prior to each blood sampling, and the food intake prior to the sampling (breakfast) in general contained coffee and bread. Blood samples were collected between 9:00 and 11:00 A.M. from nonfasted subjects. This implies that the variation/imprecision in measured triacylglycerol is considerable, and results are difficult to interpret.

*Lipids in serum.* Serum was prepared by whole blood clotted in a glass tube for 2 h at room temperature, followed by centrifugation (2,500 × *g* for 15 min). Serum was tested for total cholesterol (enzymatic colorimetric test, cholesterol esterase and cholesterol oxidase), HDL-cholesterol [after precipitating chylomicron, very low density lipoprotein (VLDL), low density lipoprotein (LDL) with phosphotungstic acid, and $Mg^{2+}$], triacylglycerol (enzymatic colorimetric test, lipoprotein lipase/oxidation/-peroxidase). The analyses were done in Axon autoanalyzer, Technicon/Bayer. Apo A1 and Apo B100

were determined in a Beckman Nephelometer Immunonephelometric method, with reagents from Beckman. Lipoprotein (a) [Lp(a)] was measured by enzyme-linked immunosorbent assay-technique, TintElize (Biopool, Ventura, CA).

*Fatty acids.* Total fatty acids in serum were determined after extraction by modified Folch method (chloroform/methanol/KCl aq., 8:4:3) and methylation ($BF_3$ in methanol) as described earlier (18,19). The fatty acids were analyzed by capillary gas–liquid chromatography (Hewlett-Packard 5880, SP 2380 capillary column; Palo Alto, CA), at an injection temperature of 250°C, column temperature of 180–225°C, and hydrogen flame-ionization detector temperature of 270°C. The signal was analyzed by a disc integrator and compared with those of assays on mixtures of fatty acids supplied by Nu-Chek-Prep (Elysian, MN). The fatty acid compositions of CLO and SO were expressed as relative percentage (area), and the individual fatty acids in serum were calculated by use of 17:0 as internal standard. The identification was based on retention times. Results were expressed as mmol/L.

*Standard clinical chemistry analysis.* Creatinine (modified Jaffe method), bilirubin (total), total protein, aspartate aminotransferase (AST) and alanine aminotransferase (ALAT), measured as photometrically determined NADH decrease (international standardized procedure), alkaline phosphatase (ALP; colorimetric assay), and C reactive protein (CRP) in serum were analyzed by Axon Autoanalyser, Technicon, with reagents from Technicon/Bayer. The CRP method did not measure "ultrasensitive" CRP.

*Standard hematological parameters.* Hemoglobin (modified cyanmethemoglobin-method), white cells, red cells, platelets, and hematocrit were determined by use of Cobas Argos, Roche Diagnostics Systems (Nutley, NJ).

TF and TNF were analyzed as earlier described (13).

*Platelet aggregation.* Fifteen subjects from each group (control, SO, CLO) were randomly selected, and after a period of 14 mon of oil supplementation, the subjects were tested for platelet aggregation using collagen as platelet agonist. Two concentrations of collagen (Collagen-reagent Horm) were used: 1.0 μg/mL and 10 μg/mL. Platelet aggregation was measured in citrated whole blood using a dual channel Crono-Log aggregometer model 540, Coulter Electronic Ltd., which operates by impedance method (20).

*Statistical analyses.* To compare the various fatty acids and platelet aggregation in the three groups, paired and two-sample *t*-tests were used. Handling of data was done using Microsoft Excel (Redmond, WA) and SAS statistical system (Cary, NC).

## RESULTS

The participants maintained their diet throughout the study, which on average contained 1–2 fish meals per week. The subjects tolerated oil supplementation well, and no adverse effects were reported. There were no effects on liver and kidney function or hematological parameters. The level of CRP remained unchanged. The body weight did not change significantly in any of the groups during the study (Table 2).

**TABLE 2**
**Body Weight (kg) at 6 wk Before and 2 and 6 mon After Dietary Addition of 15 mL Cod-Liver Oil (CLO) and Seal Oil (S)[a]**

| | Before | 2 mon | 6 mon | *n* |
|---|---|---|---|---|
| CLO | 70.8 (13.6) | 71.3 (13.8) | 71.3 (14.0) | 38 |
| SO | 72.6 (13.8) | 72.6 (13.2) | 72.4 (13.1) | 37 |
| Control | 69.8 (10.2) | 69.6 (9.9) | 69.0 (8.5) | 37 |

[a]Mean and SD.

*Lipids in serum.* The levels of serum total-cholesterol were unaffected both by CLO [mean (SD); 8.3 (0.8) mmol/L (320 mg/dL) before and 7.8 (0.9) mmol/L (301 mg/dL) after 14 mon of oil supplementation], and by SO [8.2 (0.9) mmol/L (316 mg/dL) before and 8.0 (0.7) mmol/L (309 mg/dL) after] as were HDL-cholesterol [CLO; 1.3 (0.4) mmol/L (50 mg/dL) before and 1.3 (0.4) mmol/L (50 mg/dL) after, SO; 1.3 (0.3) mmol/L (50 mg/dL) before and 1.4 (0.3) mmol/l (54 mg/dL) after], A1 [CLO; 1.6 (0.2) g/L before and 1.7 (0.3) g/L after; SO, 1.6 (0.3) g/L before and 1.8 (0.4) g/L after], B100 [CLO; 2.0 (0.3) g/L before and 2.0 (0.3) g/L after, SO; 2.0 (0.3) before and 2.0 (0.3) g/L after] and Lp(a) [CLO; 185 (181) mg/L before and 185 (194) mg/L after 14 mon of oil supplementation, SO; 163 (170) before and 183 (168) mg/L after]. The great SD of Lp(a) measurements is due to considerable interindividual differences in the Lp(a) level (50–800 mg/L). The intraindividual variation was relatively small (<20%), and the coefficient of variation of the assay (CV) was 4%.

The triacylglycerol level (nonfasting) was analyzed during and after the period of oil supplementation (Table 3). There were no significant differences between the controls and the subjects given the oil supplementation, and the wash-out period had no effect. The intraindividual variation was relatively great (10–40%), and the CV of the assay was 5%.

*Fatty acids.* The three groups (CLO, SO, control) did not differ in the level of any fatty acid, at the entry of the study (Table 4). No significant changes in the serum fatty acid pattern in the control group during the study were observed. Both the SO and the CLO had a significant effect on the composition of fatty acids in serum. This was more pronounced during the course of the study. The maximum effect was achieved after 10 mon. Analysis after 14 mon of oil supplementation showed no further changes.

In both in the CLO and SO groups, the level of several fatty acids was raised significantly including MUFA and the PUFA (Table 4). There was no significant fall in the absolute levels

**TABLE 3**
**Nonfasting Triacylglycerol Levels (mg/dL) in Hypercholesterolemic Subjects During Dietary Addition of 15 mL of CLO and SO and After a 4-mon Wash-out Period[a]**

| | Oil supplementation period | | | Wash-out, | |
|---|---|---|---|---|---|
| | 4 mon | 10 mon | 14 mon | 4 mon | *n* |
| CLO | 177 (79) | 203 (115) | 195 (124) | 195 (97) | 38 |
| SO | 186 (97) | 195 (88) | 168 (79) | 212 (159) | 38 |
| Control | 186 (79) | 195 (88) | 195 (79) | 204 (115) | 37 |

[a]Mean and SD. For abbreviations see Table 2.

**TABLE 4**
**The Fatty Acid Composition (mmol/L) of Serum**

| | Before, during, and after daily addition of 15 mL cod-liver oil | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Entry | | 14 mon with oil | | | Wash-out, 4 mon | | |
| Fatty acid | Mean ($n$ = 40) | SD | Mean ($n$ = 36) | SD | $P^a$ | Mean ($n$ = 36) | SD | $P^a$ |
| 14:0 | 0.36 | 0.16 | 0.42 | 0.21 | | 0.36 | 0.13 | |
| 16:0 | 3.61 | 1.13 | 5.14 | 1.51 | 0.0001 | 4.19 | 0.93 | 0.0003 |
| 18:0 | 1.14 | 0.3 | 1.53 | 0.37 | 0.0001 | 1.55 | 0.34 | 0.0001 |
| 20.0 | 0.05 | 0.05 | 0.04 | 0.05 | | 0.15 | 0.07 | 0.0001 |
| 22:0 | 0.1 | 0.03 | 0.15 | 0.03 | 0.0001 | 0.15 | 0.03 | 0.0001 |
| 24.0 | 0.02 | 0.03 | 0.06 | 0.05 | 0.0001 | 0.07 | 0.03 | 0.0001 |
| Sum | 5.28 | | 7.34 | | | 6.47 | | |
| 16:1n-7 | 0.37 | 0.23 | 0.52 | 0.31 | 0.0001 | 0.42 | | |
| 18:1n-9 | 2.83 | 0.9 | 4.06 | 1.39 | 0.0001 | 3.77 | | |
| 20:1n-9 | 0.03 | 0.11 | 0.17 | 0.13 | 0.0001 | 0.13 | | |
| Sum | 3.23 | | 4.75 | | | 4.32 | | |
| 18:2n-6 | 4.66 | 0.84 | 6.45 | 1.51 | 0.0001 | 6.04 | 1.03 | 0.0001 |
| 20:2n-6 | 0.06 | 0.06 | 0.08 | 0.08 | | 0.09 | 0.08 | |
| 20:3n-6 | 0.16 | 0.07 | 0.21 | 0.09 | 0.0001 | 0.21 | 0.07 | 0.0001 |
| 20:4n-6 | 0.63 | 0.15 | 0.84 | 0.21 | 0.0001 | 0.91 | 0.22 | 0.0001 |
| 22:4n-6 | 0.07 | 0.07 | 0.25 | 0.08 | | 0.48 | 0.11 | 0.0001 |
| Sum | 5.58 | | 7.83 | | | 7.73 | | |
| 18:3n-3 | 0.19 | 0.12 | 0.19 | 0.09 | | 0.18 | 0.07 | |
| 20:5n-3 | 0.31 | 0.2 | 0.73 | 0.32 | 0.0001 | 0.61 | 0.3 | 0.0001 |
| 22:5n-3 | 0.07 | 0.04 | 0.13 | 0.05 | 0.0001 | 0.11 | 0.04 | 0.0001 |
| 22:6n-3 | 0.47 | 0.17 | 1.01 | 0.29 | 0.0001 | 1.03 | 0.26 | 0.0001 |
| Sum | 1.04 | | 2.05 | | | 1.93 | | |
| n-3/n-6 | 0.19 | | 0.26 | | | 0.27 | | |

| | Before, during, and after daily addition of 15 mL seal oil | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Entry | | 14 mon with oil | | | Wash-out, 4 mon | | |
| Fatty acid | Mean ($n$ = 40) | SD | Mean ($n$ = 36) | SD | $P^a$ | Mean ($n$ = 36) | SD | $P^a$ |
| 14:0 | 0.35 | 0.14 | 0.41 | 0.14 | | 0.39 | 0.27 | |
| 16:0 | 3.8 | 1.06 | 5.15 | 1.32 | 0.0001 | 4.2 | 1.76 | |
| 18:0 | 1.19 | 0.31 | 1.60 | 0.33 | 0.0001 | 1.5 | 0.48 | 0.0045 |
| 20.0 | 0.02 | 0.04 | 0.04 | 0.05 | | 0.17 | 0.1 | 0.0001 |
| 22:0 | 0.09 | 0.03 | 0.15 | 0.03 | 0.0001 | 0.14 | 0.03 | 0.0001 |
| 24.0 | 0.03 | 0.04 | 0.05 | 0.04 | | 0.06 | 0.05 | 0.0054 |
| Sum | 5.48 | | 7.4 | | | 6.46 | | |
| 16:1n-7 | 0.37 | 0.18 | 0.47 | 0.16 | 0.0011 | 0.45 | 0.21 | 0.0367 |
| 18:1n-9 | 3.01 | 0.96 | 3.83 | 1.09 | 0.005 | 3.76 | 1.83 | |
| 20:1n-9 | 0.03 | 0.09 | 0.14 | 0.13 | 0.0003 | 0.16 | 0.16 | 0.0003 |
| Sum | 3.41 | | 4.44 | | | 4.37 | | |

(*continued*)

of any fatty acid. Although SO is almost 30% (relative) lower in EPA as compared to CLO, the serum level of EPA (20:5n-3), after 14 mon of oil supplementation, is about 30% higher in the SO group (0.96 vs. 0.73 mmol/L).

After the wash-out period of 4 mon, the fatty acid changes, compared to the entry values, of both the SO and CLO groups were not so prominent, but they were still significantly different from the start of the study. For example the level of DHA (22:6n-3) did not fall during the wash-out period.

*Platelet aggregation.* Collagen-induced platelet aggregation was compared between 15 subjects of each group after ingestion of oils for 14 mon. The CLO group showed the lowest platelet aggregation, statistically significant lower than the control group ($P$ = 0.03) (Fig. 1, collagen 1 μg/mL). The aggregation in the SO

Before, during, and after daily addition of 15 mL seal oil (*cont.*)

| | Entry | | 14 mon with oil | | | Wash-out, 4 mon | | |
|---|---|---|---|---|---|---|---|---|
| Fatty acid | Mean ($n$ = 40) | SD | Mean ($n$ = 36) | SD | $P^a$ | Mean ($n$ = 36) | SD | $P^a$ |
| 18:2n-6 | 4.9 | 0.98 | 6.53 | 1.49 | 0.0001 | 5.59 | 1.13 | 0.0257 |
| 20:2n-6 | 0.05 | 0.05 | 0.08 | 0.08 | | 0.12 | 0.12 | 0.0114 |
| 20:3n-6 | 0.16 | 0.07 | 0.19 | 0.09 | | 0.19 | 0.08 | |
| 20:4n-6 | 0.6 | 0.19 | 0.88 | 0.25 | 0.0001 | 0.83 | 0.24 | 0.0001 |
| 22:4n-6 | 0.07 | 0.04 | 0.26 | 0.08 | 0.0001 | 0.45 | 0.13 | 0.0001 |
| Sum | 5.78 | | 7.94 | | | 7.18 | | |
| 18:3n-3 | 0.19 | 0.11 | 0.19 | 0.07 | | 0.19 | 0.08 | |
| 20:5n-3 | 0.27 | 0.18 | 0.96 | 0.44 | 0.0001 | 0.74 | 0.29 | 0.0001 |
| 22:5n-3 | 0.07 | 0.04 | 0.15 | 0.44 | 0.0001 | 0.15 | 0.07 | 0.0001 |
| 22:6n-3 | 0.48 | 0.19 | 1.08 | 0.31 | 0.0001 | 1.09 | 0.32 | 0.0001 |
| Sum | 1.01 | | 2.38 | | | 2.17 | | |
| n-3/n-6 | 0.17 | | 0.3 | | | 0.3 | | |

Control group (no dietary addition)

| | Entry | | 14 mon | | |
|---|---|---|---|---|---|
| Fatty acid | Mean ($n$ = 36) | SD | Mean ($n$ = 36) | SD | $P^a$ |
| 14:0 | 0.34 | 0.13 | 0.31 | 0.12 | |
| 16:0 | 3.78 | 1.18 | 3.76 | 0.79 | |
| 18:0 | 1.19 | 0.31 | 1.28 | 0.25 | |
| 20.0 | 0.03 | 0.04 | 0.07 | 0.07 | 0.0022 |
| 22:0 | 0.09 | 0.04 | 0.1 | 0.05 | |
| 24.0 | 0.02 | 0.03 | 0.05 | 0.07 | 0.0075 |
| Sum | 5.45 | | 5.57 | | |
| 16:1n-7 | 0.42 | 0.24 | 0.43 | 0.21 | |
| 18:1n-9 | 3.1 | 1.27 | 3.02 | 0.78 | |
| 20:1n-9 | 0.05 | 0.12 | 0.13 | 0.14 | |
| Sum | 3.99 | | 3.58 | | |
| 18:2n-6 | 4.71 | 1.03 | 5 | 0.83 | |
| 20:2n-6 | 0.07 | 0.05 | 0.08 | 0.08 | |
| 20:3n-6 | 0.16 | 0.06 | 0.2 | 0.1 | |
| 20:4n-6 | 0.62 | 0.15 | 0.67 | 0.14 | |
| 22:4n-6 | 0.11 | 0.1 | 0.1 | 0.05 | |
| Sum | 5.67 | | 6.05 | | |
| 18:3n-3 | 0.2 | 0.12 | 0.17 | 0.06 | |
| 20:5n-3 | 0.3 | 0.18 | 0.31 | 0.22 | |
| 22:5n-3 | 0.07 | 0.05 | 0.07 | 0.05 | |
| 22:6n-3 | 0.53 | 0.2 | 0.67 | 0.31 | |
| Sum | 1.1 | | 1.22 | | |
| n-3/n-6 | 0.19 | | 0.2 | | |

[a]Versus entry values.

group was also reduced as compared with the control group, but the change was not statistically significant ($P$ = 0.07).

No effect of SO or CLO was seen on either TF or TNF (Table 5) after 10 mon of oil supplementation, when compared to the control group. The wash-out period of 4 mon had no effect in any of the groups (control, CLO,SO).

## DISCUSSION

The present study focused on subjects with hypercholesterolemia and included a run-in period of 6 mon, and an oil supplementation period of 14 mon, which should be sufficient to observe effect on the parameters studied, and a 4-mon wash-out period.

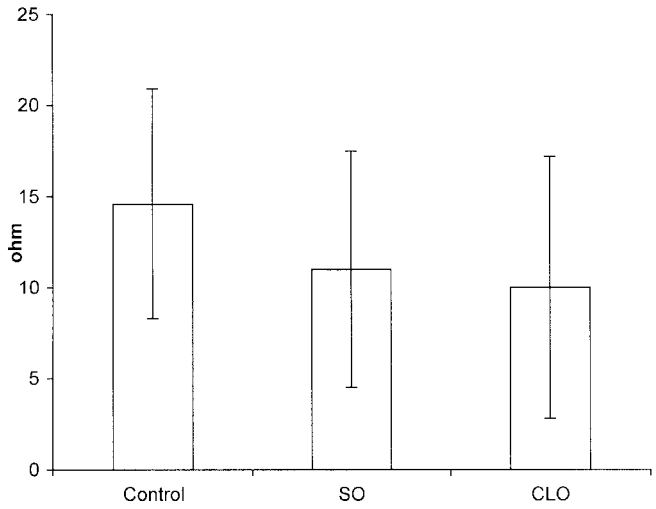


**FIG. 1.** Collagen (1 μg/mL)-induced platelet aggregation (ohm) in whole blood in subjects taking daily supplementation of 15 mL seal oil (SO; $P = 0.07$) and cod-liver oil (CLO; $P = 0.03$) for 14 mon, vs. control (no dietary addition) (mean and SD).

The n-3 fatty acid content of both CLO and SO represented a considerable daily addition, although well below the intake through original Eskimo diet. The serum level of EPA after intake of SO was about 30% higher as compared to CLO (Table 4), in spite of the fact that the EPA content of SO is 30% lower than in CLO (Table 1). It is an open question whether this implies that SO may be more efficient than CLO in preventing cardiovascular events. The mechanism behind the present observation is not clear, but may be related to the fact that EPA is located in position 1,3 in glycerol in SO, and in position 2 in CLO.

The n-3/n-6 ratio was moderately increased after the intake of both oils (Table 4). Still, this is far from the ratio in plasma of Greenland Inuit ($R = 0.7–1.2$) (4) and implies a different biological setting. During the study, the level of n-3 fatty acids in serum increased with *ca.* 100% in the CLO group and with about 140% in the SO group (Table 4). Also the level of n-6 fatty acids was elevated, although not to a similar degree. Intake of n-3 fatty acids may increase the enteral absorption of n-6 fatty acids (21).

The wash-out period of 4 mon hardly affected the serum levels of n-3 fatty acids. The level of DHA was quite similar at the end of the oil supplementation period and after 4 mon washout. Similar findings were reported in normal individuals (22). This probably reflects that the fatty acids were incorporated into the fat tissues (depot fat) and only slowly released.

One implication of this observation is that all crossover studies on PUFA should be interpreted carefully. This would also have significance for studies in subjects with traditionally high and low fish intake, and the finding also indicates that once the body depot fat is saturated with fatty acids, the need of supplementation is reduced, probably to one or two times a week.

Daily dietary addition of 15 mL of oil (about 180 kcal) might be expected to give a slight increase in the body weight. This did not occur (Table 2). The test subjects did not report increased physical activity, nor any significant change in their dietary habits. The n-3 fatty acids may increase β-oxidation of fatty acids and thereby counteract weight increase (23).

Neither SO nor CLO affected the levels of serum total cholesterol, HDL-cholesterol, and apo [Apo A1, Apo B100, Lp(a)]. This is in accordance with findings in normocholesterolemic subjects (13,24,25). By dietary addition of CLO and SO, SFA as well as PUFA are ingested. The level of SFA in serum is also raised after 14 mon of oil supplementation (Table 4). It might be that the intake of SFA is masking a possible lipid-lowering effect of PUFA. The more beneficial levels of total cholesterol in the Eskimos may be due to lower intake of SFA and possibly the fact that they consume a larger amount of unprocessed foods (higher amounts of biologically active compound, i.e., antioxidants) in their diet compared to a Western diet.

Intake of n-3 fatty acids is known to reduce the level of triacylglycerols in the blood (6). This was not confirmed in the present study, in which nonfasting serum was analyzed. This implies that triaclylglycerol in chylomicrons is included, which results in great intra- and interindividual variation in the level of serum triacylglycerol.

CLO supplementation is known to reduce platelet aggregation (18,26,27). In the present study aggregation was done in whole blood, rather than in platelet-rich plasma, which is a more common method. We found that platelet aggregation was statistically significantly reduced in the CLO group, as compared to the controls. Aggregation in the SO group was also lower than in the controls, although this was not statistically significant. The difference between the oil groups was small. Intra- and interindividual variation is substantial with respect to platelet aggregation, which influences the statistical calculations.

The mechanism of reduction in platelet aggregation is probably the same in both of the oil groups. EPA (20:5n-3) substitutes arachidonic acid (20:4n-6) in the platelet membrane phospholipids, and less potent prostanoids (tromboxane) are synthesized. We did not observe any effect of the oil supplementation on monocyte TF activity or TNF in blood. The level of both variables decreased during the study, in all groups. This may be related to biological and seasonal variations.

**TABLE 5**
**The Monocyte-derived Tissue Factor (TF) Activity in Whole Blood and Tumor Necrosis Factor (TNF, ng/mL) Before, During and After Dietary Addition of 15 mL of CLO and SO[a]**

| | | 6 wk before | 10 mon of oil supplementation | Wash out, 4 mon | *n* |
|---|---|---|---|---|---|
| TF | CLO | 16.8 (12.3) | 12.2 (7.8) | 9.9 (9.4) | 38 |
| | SO | 16.5 (8.9) | 16.2 (11.9) | 9.1 (5.8) | 38 |
| | Control | 18.9 (14.6) | 13.3 (10.9) | 9.7 (7.8) | 37 |
| TNF | CLO | 4.4 (1.9) | 1.0 (0.7) | 1.0 (0.6) | 38 |
| | SO | 4.5 (1.3) | 1.3 (1.1) | 1.5 (1.0) | 38 |
| | Control | 4.9 (1.8) | 1.2 (0.9) | 1.5 (1.2) | 37 |

[a]The TF activity is expressed as mU/$10^6$ cells of a standard. Values given as mean and SD. For other abbreviations see Table 2.

The present study mainly focused on certain parameters relevant to the development of cardiovascular disease (n-3 fatty acids in serum, other blood lipids, apo, blood platelets, monocyte activity, inflammation), leaving out aspects of oxidation, although the intake of vitamine E was increased. The oils utilized had been heated to 150°C, which may destroy natural antioxidants. It may well be that the positive influence of the Eskimo diet, and possibly also SO, is acting through such biological systems. This aspect of the Eskimo diet remains to be further explored, as unprocessed food items contain biological active components (antioxidants other than tocopherol), and the relationship between the natural fat and natural antioxidants of Eskimo diet is probably important.

In conclusion we found that dietary addition of SO increased the EPA level considerably more than CLO; after 10 mon of oil supplementation there was no further rise in serum fatty acid levels, and a wash-out period of 4 mon had only a modest effect on these levels.

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# The Composition of Saturated Fatty Acids in Plasma Phospholipids Changes in a Way to Counteract Changes in the Mean Melting Point During Pregnancy

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**ABSTRACT:** It has been demonstrated that in pathological conditions with an increase in the calculated mean melting point (MMP) of phospholipid (PL) fatty acids (FA) there are changes in the composition of the saturated FA (SFA), which partially counteract this effect: shorter-chain SFA with lower melting points are increased, while longer-chain less fluid SFA are suppressed. The aim of this study was to determine whether there are differences in MMP during pregnancy and in the newborn and, if so, whether similar adaptive changes occur in the composition of the SFA. The FA composition of plasma PL was determined in healthy women ($n$ = 16) twice during pregnancy (15–24 wk and 29–36 wk) and at delivery and in umbilical venous blood obtained at birth. The MMP of maternal PL was significantly higher at delivery compared to mid-gestation, due to a loss of highly unsaturated FA (HUFA) which were replaced by SFA. In addition, changes in the SFA occurred: 16:0 with lower melting point was higher while 18:0 with higher melting point was lower at delivery. MMP of PL FA in umbilical plasma was lower than in maternal plasma at delivery, which was due to higher HUFA content. In contrast to maternal plasma, 16:0 was lower while 18:0, 20:0, and 24:0 were higher in umbilical plasma resulting in a higher MMP of SFA, tending to raise the overall MMP. It can be concluded that, during pregnancy and in the newborn, the FA composition of SFA changes in a way to counteract changes in MMP induced by reduced and increased HUFA, respectively.

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Holman *et al.* (1–4) introduced the concept of the calculated mean melting point (MMP) and calculated mean chain length (MCL) of fatty acids (FA) from plasma phospholipids (PL) as surrogate parameters of membrane fluidity. PL are the major structural components of membranes, and the FA pattern of plasma PL reflects that of tissue PL. Membrane fluidity depends among others on the amounts of PL, the FA composition of the PL, and the amount of cholesterol. Membrane fluidity plays an important role in the efficiency of ligand binding, the activity of membrane enzymes, membrane transport, and cell deformability (5). Changes in membrane lipid composition and, consequently, changes in membrane fluidity may result in differences in the function of membrane receptors. In preeclamptic women (6), membrane fluidity of platelets was significantly higher compared to that of normotensive women and was accompanied by higher levels of unsaturated FA in the membrane of platelets. When long-chain polyunsaturated FA (PUFA) are replaced by saturated (SFA) or monounsaturated FA (MUFA), the MMP increases and membrane fluidity decreases (1–4). It has been demonstrated that the MMP of FA of plasma PL of pregnant (36 wk of gestation), lactating, and nonlactating women (6 wk postpartum) is higher compared to that from nonpregnant women (3). Furthermore, it has been demonstrated that the MMP of plasma PL is significantly increased in patients with multiple sclerosis. The latter patients have reduced concentrations of PUFA due to impaired chain elongation, and PUFA are replaced with SFA (1). This overall increase in MMP is accompanied by changes in the composition of the SFA, which partially counteract this effect: shorter-chain SFA with lower melting points are increased while longer-chain, less fluid SFA are suppressed.

The objective of the present study was to determine whether similar adaptive changes in MMP occur during the course of pregnancy and in the newborn. Therefore, we determined the FA composition of PL, isolated from maternal venous plasma during the course of pregnancy and from umbilical venous plasma.

## MATERIALS AND METHODS

*Study population.* Healthy pregnant women at the Department of Gynecology of Ghent University Hospital, Belgium, were asked to participate in this study. All pregnant women signed a written informed consent form, approved by the ethics committee of Ghent University Hospital. Only singleton pregnancies were included. Inclusion criteria were: normotensive (DBP <90 mm Hg), not diabetic, no proteinuria, and not suffering from renal or cardiovascular disease. Twenty pregnant women entered the study. Two pregnant women delivered preterm and were excluded from the study. From one pregnancy the umbili-

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Abbreviations: CI, confidence interval; FA, fatty acid; HUFA, highly unsaturated fatty acid; MCL, mean chain length; MMP, mean melting point; MUFA, monounsaturated fatty acid; OPI, oxidative potential index; PL, phospholipid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

cal blood sample was lost and from another pregnancy we only obtained one antenatal sample. These pregnancies were also excluded. The study population thus consisted of 16 healthy pregnant women and their neonates (seven girls and nine boys).

Maternal venous blood was collected in EDTA-tubes twice during the course of pregnancy, between 15 and 24 wk of gestation (median 20 wk) and between 29 and 36 wk (median 32 wk) and at delivery (median 39.7 wk; range 38.0–41.4 wk). After delivery, a sample of umbilical venous blood was collected. Blood samples were temporarily stored at 6°C. Within 24 h of collection, plasma was isolated by centrifugation (600 × *g* during 5 min at 4°C) and stored in plastic tubes under nitrogen at −80°C until transportation in dry ice to Maastricht for analysis.

*FA analysis*. All samples of a given mother-infant pair were analyzed simultaneously. The FA analysis was performed as described previously (7). Previous to the FA analysis an internal standard [dinonadecanoyl lecithin, phosphatidylcholine 19:0] was added to every sample. Total lipid extracts of plasma were prepared using a modified Folch extraction (8). The PL fraction was isolated by solid-phase extraction on an aminopropyl silica column (9). The PL were saponified and the FA converted to the corresponding methyl esters by reaction with $BF_3$ in methanol (140 g/L) at 100°C during 1 h. The methyl esters were analyzed using a capillary gas–liquid chromatograph equipped with a 50 m BP1 nonpolar column, 0.22 mm i.d. × 0.10 μm film thickness, and a 50 m BP × 70 mm polar column, 0.22 mm i.d. × 0.25 μm film thickness (SGE, Bester BV, Amstelveen, The Netherlands). The injection temperature was set at 250°C and the detector temperature at 300°C. The starting temperature of the columns was 160°C, which after 4 min was increased to 200°C with a rate of 6°C/min. Subsequently, after a stabilization period of 3 min, the temperature was further increased to 270°C at a rate of 7°C/min. The carrier gas was helium, head pressure 370 kPa.

The results are expressed as mole percentage of total FA, and the absolute FA amount in the PL fraction is also reported (mg/L plasma). Thirty-one different FA with chain lengths between 14 and 24 carbon atoms were identified. The sum of all the SFA, the MUFA, the PUFA, the highly unsaturated fatty acids (HUFA: fatty acids with 20 or more carbon atoms and with at least three double bonds), Σn-7, Σn-9, Σn-3, and Σn-6 were calculated and are reported together with the individual FA.

The fluidity of lipids was assessed through the MMP, °C (sum of the mole fraction multiplied by the melting point for each fatty acid) and the MCL (number of carbon atoms, sum of the mole fraction multiplied by the number of carbon atoms in the FA). The oxidative potential index (OPI) of FA in plasma PL was estimated by summing the mole fraction of FA with 1, 2, 3, 4, 5, and 6 double bonds, multiplied by 1, 30, 70, 120, 180, and 240, respectively (10).

*Statistical analysis.* Normality of distribution was ascertained with the Kolmogorov-Smirnov test. The calculated parameters such as MMP, MCL, and OPI had a normal distribution. FA fractions were arcsin transformed to reach normality of distribution. Values are reported as mean [95% confidence interval of the mean (CI)]. Paired Student *t*-test was performed for FA comparisons between maternal samples of the first and second antenatal visit with maternal samples obtained at delivery and for maternal-umbilical FA comparisons at delivery and birth. In order to avoid type 2 errors, due to multiple comparisons, a value of $P < 0.005$ was taken as the criterion of significance. For maternal plasma, the correlations between the fraction of the PUFA and HUFA in the PL on the one hand and the MMP of SFA and the MMP of MUFA on the other hand, were calculated. For these calculations the results of the two antenatal visits and of delivery were used. The degree of association was calculated using Pearson correlation. The data were analyzed using the MedCalc statistical program, version 6 (MedCalc Software, Mariakerke, Belgium) (11).

## RESULTS

*Clinical characteristics.* The mean age of the mothers ($n = 16$) at delivery was 30 yr (range 20–38 yr). The mean body mass index of the women before pregnancy was 21.2 (range 16.5–24.2). All mothers were nullipara, all pregnancies were uncomplicated, and the infants were born healthy with a mean birth weight of 3169.4 g (range 2570–3860 g) and a mean birth length of 50.3 cm (range 47–53 cm). The median Apgar Score 1 min after birth was 9 (range 4–9) and 5 min after birth 9 (range 9–10).

The FA patterns of plasma PL (mol%) from maternal plasma during the course of pregnancy and at delivery and from umbilical plasma shortly after birth are given in Table 1. Calculated values derived from this FA composition are summarized in Table 2.

*Maternal FA composition*. In maternal plasma PL, a few significant differences between gestation and delivery occurred. The total amount of maternal plasma PL-associated FA did not differ significantly between gestation and delivery.

Neither PUFA nor MUFA changed significantly between mid-gestation and delivery. HUFA were lower, while SFA were higher at delivery compared to mid-gestation ($P < 0.005$).

Palmitic acid (16:0) and stearic acid (18:0), the two major SFA, changed significantly, but in opposite directions: 16:0 increased while 18:0 dropped during the last 20 wk of pregnancy ($P < 0.001$).

Few of the individual n-6 or n-3 FA differed significantly between mid-pregnancy and delivery; arachidonic acid (20:4n-6) and its elongation product 22:4n-6 declined ($P < 0.005$), but linoleic acid remained stable. In the series of n-3 FA, only 22:5n-3 was lower at delivery ($P < 0.005$).

The sum of the Δ5 desaturation products (20:5n-3, 20:4n-6, and 20:3n-9) was calculated as a parameter for the essential FA status of the mother because in some disorders in which Δ5 desaturation is affected, the conversion of 20:4n-3 to 20:5n-3, of 20:3n-6 to 20:4n-6, and of 20:2n-9 to 20:3n-9 may be equally altered. This would result in an unchanged triene/tetraene ratio. The sum of the Δ5 desaturase products was lower at delivery as compared to mid-pregnancy: 8.2 (7.6 to 8.8%) vs. 9.3 (8.5 to 10.0%); $P < 0.0005$.

*Umbilical plasma FA composition.* The amount of umbilical

**TABLE 1**
**Amount (mg/L plasma) and Composition (mol% of total fatty acids) of Fatty Acids in Phospholipids Isolated from Maternal Venous Plasma During the Course of Pregnancy and at Delivery and from Umbilical Venous Plasma at Birth: Mean (95% CI of the mean) ($n$ = 16)**

| Fatty acid | Maternal plasma 1st antenatal visit: 15–24 wk | Maternal plasma 2nd antenatal visit: 29–36 wk | Maternal plasma delivery: 38–41 wk | Umbilical plasma birth: 38–41 wk | Paired $t$-test[b] |
|---|---|---|---|---|---|
| Total (mg/L) | 1682.5 | 1810.1 | 1845.5 | 521.4 | |
| | (1532.3 to 1832.7) | (1656.2 to 1964.0) | (1679.1 to 2011.9) | (559.1 to 683.7) | c |
| 14:0 | 0.5 (0.4 to 0.6) | 0.4 (0.4 to 0.5) | 0.45 (0.4 to 0.5) | 0.4 (0.3 to 0.4) | |
| 15:0 | 0.2 (0.2 to 0.3) | 0.2 (0.2 to 0.25) | 0.2 (0.2 to 0.23) | 0.1 (0.1 to 0.2) | b,c |
| 16:0 | 33.5 (32.8 to 34.2) | 34.5 (33.8 to 35.3) | 35.8 (34.9 to 36.6) | 31.9 (30.6 to 33.2) | a,b,c |
| 17:0 | 0.4 (0.4 to 0.5) | 0.4 (0.3 to 0.4) | 0.3 (0.3 to 0.4) | 0.35 (0.3 to 0.4) | a,b |
| 18:0 | 11.0 (10.6 to 11.3) | 10.5 (10.0 to 11.0) | 9.75 (9.4 to 10.1) | 14.7 (14.3 to 15.2) | a,b,c |
| 20:0 | 0.5 (0.5 to 0.5) | 0.5 (0.5 to 0.6) | 0.5 (0.4 to 0.5) | 0.9 (0.8 to 1.0) | c |
| 22:0 | 1.3 (1.2 to 1.4) | 1.3 (1.2 to 1.50) | 1.2 (1.1 to 1.4) | 1.45 (1.3 to 1.6) | |
| 23:0 | 0.5 (0.5 to 0.6) | 0.5 (0.5 to 0.6) | 0.5 (0.45 to 0.6) | 0.2 (0.15 to 0.2) | c |
| 24:0 | 0.9 (0.8 to 1.0) | 0.9 (0.8 to 1.0) | 0.85 (0.75 to 0.95) | 1.4 (1.1 to 1.7) | c |
| 18:3n-3 | 0.1 (0.1 to 0.2) | 0.1 (0.1 to 0.2) | 0.2 (0.1 to 0.2) | ND | |
| 20:4n-3 | 0.1 (0.1 to 0.1) | 0.1 (0.1 to 0.2) | 0.1 (0.1 to 0.1) | 0.1 (0.0 to 0.1) | |
| 20:5n-3 | 0.5 (0.4 to 0.6) | 0.6 (0.4 to 0.7) | 0.4 (0.3 to 0.5) | 0.3 (0.2 to 0.4) | c |
| 22:5n-3 | 0.7 (0.6 to 0.8) | 0.7 (0.6 to 0.8) | 0.6 (0.5 to 0.7) | 0.8 (0.5 to 1.1) | b |
| 22:6n-3 | 4.1 (3.7 to 4.5) | 4.2 (3.6 to 4.7) | 3.7 (3.2 to 4.3) | 6.2 (5.1 to 7.3) | c |
| 18:2n-6 | 20.4 (19.6 to 22.2) | 20.8 (19.2 to 22.4) | 20.8 (19.3 to 22.2) | 7.7 (6.9 to 8.5) | c |
| 20:2n-6 | 0.45 (0.4 to 0.5) | 0.4 (0.4 to 0.5) | 0.4 (0.3 to 0.4) | 0.3 (0.3 to 0.3) | |
| 20:3n-6 | 2.8 (2.5 to 3.0) | 2.8 (2.5 to 3.1) | 3.0 (2.7 to 3.3) | 4.5 (4.1 to 4.95) | c |
| 20:4n-6 | 8.6 (7.9 to 9.3) | 7.9 (7.2 to 8.6) | 7.6 (7.0 to 8.2) | 15.2 (14.4 to 15.9) | b,c |
| 22:4n-6 | 0.3 (0.3 to 0.4) | 0.3 (0.3 to 0.3) | 0.3 (0.3 to 0.3) | 0.7 (0.6 to 0.9) | b,c |
| 22:5n-6 | 0.3 (0.3 to 0.4) | 0.3 (0.2 to 0.3) | 0.3 (0.3 to 0.4) | 0.5 (0.5 to 0.6) | c |
| 24:2n-6 | 0.2 (0.15 to 0.2) | 0.2 (0.2 to 0.3) | 0.2 (0.15 to 0.2) | 0.6 (0.5 to 0.7) | c |
| 16:1n-7 | 0.4 (0.3 to 0.5) | 0.4 (0.3 to 0.6) | 0.7 (0.4 to 0.9) | 0.6 (0.5 to 0.7) | |
| 18:1n-7 | 1.4 (1.2 to 1.5) | 1.3 (1.2 to 1.4) | 1.3 (1.1 to 1.4) | 2.3 (2.1 to 2.5) | c |
| 18:1n-9 | 8.1 (7.7 to 8.6) | 8.3 (7.8 to 8.7) | 8.7 (7.9 to 9.6) | 6.5 (6.3 to 6.7) | c |
| 20:1n-9 | 0.1 (0.1 to 0.1) | 0.1 (0.1 to 0.1) | 0.1 (0.10 to 0.12) | 0.1 (0.05 to 0.1) | a,b,c |
| 20:3n-9 | 0.2 (0.1 to 0.2) | 0.1 (0.1 to 0.2) | 0.2 (0.14 to 0.2) | 0.3 (0.2 to 0.3) | |
| 24:1n-9 | 1.6 (1.5 to 1.8) | 1.7 (1.5 to 1.8) | 1.6 (1.4 to 1.7) | 1.9 (1.7 to 2.1) | |
| Σn-3 | 5.6 (5.0 to 6.2 | 5.7 (4.8 to 6.5) | 5.1 (4.24 to 5.9) | 7.4 (6.0 to 8.7) | c |
| Σn-6 | 33.5 (32.5 to 34.4) | 32.8 (31.6 to 33.9) | 32.6 (31.0 to 34.2) | 29.6 (28.3 to 30.9) | c |
| Σn-7 | 1.7 (1.5 to 2.0) | 1.7 (1.5 to 1.9) | 1.9 (1.6 to 2.3) | 2.9 (2.6 to 3.1) | c |
| Σn-9 | 10.05 (9.6 to 10.5) | 10.2 (9.7 to 10.7) | 10.6 (9.7 to 11.5) | 8.7 (8.4 to 9.0) | c |
| SFA | 48.9 (48.2 to 49.5) | 49.4 (49.0 to 49.9) | 49.6 (48.9 to 50.3) | 51.4 (50.4 to 52.5) | b,c |
| MUFA | 11.6 (11.0 to 12.2) | 11.76 (11.13 to 12.40) | 12.4 (11.2 to 13.5) | 11.29 (10.9 to 11.7) | |
| PUFA | 39.2 (38.4 to 40.1) | 38.6 (37.9 to 39.2) | 37.8 (36.6 to 39.1) | 37.2 (36.4 to 38.1) | |
| HUFA | 17.6 (16.7 to 18.5) | 17.0 (15.8 to 18.3) | 16.3 (15.3 to 17.3) | 28.6 (27.4 to 29.8) | b,c |

[a]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids; ND, not detectable.
[b]Paired $t$ test performed after arcsin transformation of all the fatty acid fractions. a, significant difference ($P < 0.005$) between maternal values of second antenatal visit and delivery; b, significant difference ($P < 0.005$) between maternal values of first antenatal visit and delivery; c, significant difference ($P < 0.005$) between maternal values at delivery and umbilical venous plasma at birth.

plasma PL-associated FA was only 34% [95% confidence interval (CI) 30.2 to 38.7] of maternal plasma PL-associated FA at delivery. The FA composition of umbilical venous plasma PL was very different from that of maternal plasma PL at delivery.

Both SFA and HUFA were significantly higher in umbilical plasma PL compared to maternal values. No significant differences were observed in MUFA and PUFA between mother and neonate.

The SFA in umbilical plasma PL showed significant differences from the SFA content of maternal plasma PL; 16:0, the major SFA, was lower in umbilical plasma compared to maternal values. In contrast, the 18:0 content was significantly higher in umbilical plasma. The other long-chain SFA were also higher in the neonate compared to the mother, 20:0 ($P < 0.0001$) and 24:0 ($P < 0.001$). The odd-chain FA 15:0 ($P < 0.001$) and 23:0 ($P < 0.0001$) were lower in the neonate.

Umbilical plasma PL were significantly enriched in all the individual n-6 HUFA compared to maternal plasma. In contrast, maternal plasma contained more 18:2n-6. α-Linolenic acid (18:3n-3) was not detected in umbilical plasma. Eicosapentaenoic acid (20:5n-3) was significantly lower in umbilical plasma, while its elongation and desaturation product docosahexaenoic acid (22:6n-3) was significantly higher in umbilical plasma PL compared to maternal plasma PL.

*Calculated parameters. (i) Maternal plasma.* The MMP of the FA in plasma PL at delivery is significantly elevated compared to mid-pregnancy (increase of 1.13°C, 95% CI, 0.64 to 1.61, $P < 0.001$). The MCL was significantly lower at delivery compared to gestation. These parameters have an effect on

**TABLE 2**
**Calculated Values Derived from the Fatty Acid Composition of Maternal Plasma Phospholipids During the Course of Pregnancy and at Delivery and of Umbilical Venous Plasma at Birth: mean (95% CI of the mean) ($n$ = 16)**

| Calculated values[a] | Maternal plasma 1st antenatal visit: 15–24 wk | Maternal plasma 2nd antenatal visit: 29–36 wk | Maternal plasma delivery: 38–41 wk | Umbilical plasma birth: 38–41 wk | Paired $t$-test[b] |
|---|---|---|---|---|---|
| OPI | 32.15 (30.87 to 33.44) | 31.50 (29.78 to 33.23) | 29.94 (28.34 to 31.54) | 43.10 (40.54 to 45.66) | a,b,c |
| MCL (overall) | 18.02 (17.98 to 18.06) | 18.00 (17.95 to 18.05) | 17.92 (17.87 to 17.97) | 18.39 (18.30 to 18.47) | a,b,c |
| MCL SFA | 16.86 (16.81 to 16.90) | 16.84 (16.79 to 16.90) | 16.78 (16.72 to 16.83) | 17.04 (16.96 to 17.13) | a,b,c |
| MCL MUFA | 18.80 (18.71 to 18.90) | 18.80 (18.72 to 18.89) | 18.70 (18.59 to 18.81) | 18.90 (18.82 to 18.98) | b,c |
| MCL PUFA | 19.23 (19.15 to 19.30) | 19.22 (19.12 to 19.33) | 19.18 (19.08 to 19.27) | 20.09 (19.99 to 20.19) | c |
| MCL HUFA | 20.62 (20.58 to 20.66) | 20.64 (20.59 to 20.68) | 20.61 (20.55 to 20.66) | 20.57 (20.49 to 20.64) | |
| MMP (overall) | 26.17 (25.49 to 26.84) | 26.84 (26.20 to 27.47) | 27.30 (26.59 to 28.70) | 24.49 (23.48 to 25.50) | b,c |
| MMP SFA | 65.65 (65.52 to 65.79) | 65.61 (65.45 to 65.78) | 65.41 (65.25 to 65.58) | 66.27 (66.02 to 66.53) | a,b,c |
| MMP MUFA | 17.24 (16.78 to 17.69) | 17.22 (16.76 to 17.68) | 16.64 (16.08 to 17.20) | 17.67 (17.27 to 18.07) | b,c |
| MMP PUFA | –20.49 (–21.58 to –19.40) | –20.04 (–21.40 to –18.67) | –19.27 (–20.42 to –18.13) | –31.12 (–32.38 to –29.86) | c |
| MMP HUFA | –39.56 (–40.21 to –38.90) | –39.15 (–39.84 to –38.45) | –38.14 (–39.04 to –37.23) | –39.52 (–40.32 to –38.72) | b |

[a]OPI, oxidative potential index; MCL, mean chain length, expressed as number of carbon atoms; MMP, mean melting point, expressed as degrees Celsius. For other abbreviations see Table 1.
[b]a, significant difference ($P < 0.005$) between maternal values of second antenatal visit and delivery; b, significant difference ($P < 0.005$) between maternal values of first antenatal visit and delivery; c, significant difference ($P < 0.005$) between maternal values at delivery and umbilical venous plasma at birth.

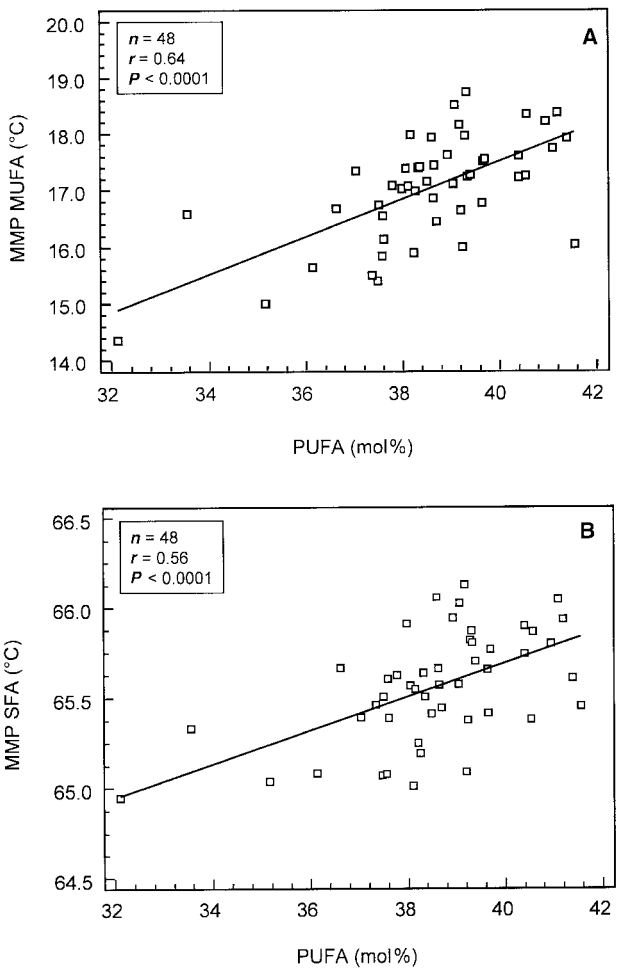


**FIG. 1.** Correlation between the mean melting point (MMP) of the monounsaturated fatty acids (MUFA) and of the saturated fatty acids (SFA) in plasma phospholipids and the fraction of polyunsaturated fatty acids (PUFA) in maternal plasma phospholipids (mol%).

membrane fluidity. The OPI of plasma PL, an index for susceptibility toward peroxidation, was significantly lower at delivery compared to gestation. The change in composition of the SFA results in a shorter MCL and consequently a lower MMP (decrease of 0.24°C, 95% CI, 0.13 to 0.34, $P < 0.001$) of the SFA. The same phenomenon was observed in MUFA, but it was less pronounced, the MCL of the MUFA is shorter at delivery compared to mid-pregnancy ($P < 0.005$) and their MMP is lower (0.60°C, 95% CI, 0.25 to 0.95, $P < 0.005$).

The mole fraction of the PUFA, but not of HUFA, in the PL correlated positively with both the MMP of SFA ($r = 0.56$, $n = 48$, $P < 0.0001$) and with the MMP of MUFA ($r = 0.64$, $n = 48$, $P < 0.0001$) (Fig. 1). Even without the two values with a low PUFA fraction (<34 mol%), the same association still exists.

*(ii) Umbilical plasma*. The high concentration of HUFA in umbilical plasma caused a significantly higher OPI, a longer MCL, and a lower MMP compared to maternal values. The lower global MMP in umbilical plasma PL compared to maternal plasma PL is associated with a higher MMP of the SFA (0.86°C, 95% CI, 0.61 to 1.11, $P < 0.001$) and of the MUFA (1.03°C, 95% CI, 0.46 to 1.60, $P = 0.002$).

Higher concentrations of HUFA in the newborn than in the mother are associated with higher concentrations of SFA, and a shift in the composition of SFA toward longer chain lengths. The MCL of the SFA is significantly higher in umbilical plasma compared to maternal plasma at delivery. These changes partially counteract the reduction in MMP induced by higher levels of HUFA.

## DISCUSSION

The loss of HUFA (mean 1.30% pts, 95% CI, 0.61–1.99) at delivery compared to mid-pregnancy was about equal to the increase in SFA and MUFA (mean 1.44% pts, 95% CI, 0.48–2.41). The lower content of HUFA in maternal plasma PL resulted in a higher MMP of the FA mixture in plasma PL.

HUFA were preferentially replaced by shorter-chain SFA, especially palmitic acid as was also demonstrated in anorexia nervosa (4). These shorter-chain FA are more fluid than their longer-chain homologs because they have lower melting points. The MMP of SFA was significantly lower at delivery compared to pregnancy. Although the shorter chain length of the SFA compensates only partially for the higher overall MMP, it is possible that FA metabolism in pregnancy attempts to maintain MMP homeostasis as was shown previously in multiple sclerosis (1). An additional indication for this hypothesis is the positive correlation between the fraction of PUFA in PL with the MMP of SFA and of MUFA. This indicates that a loss in PUFA, which would result in an increased MMP, is accompanied by an increase in shorter-chain SFA and MUFA, which have an opposing effect on the MMP. A difference as large as 10.8°C was observed by Holman *et al.* (3) between the MMP of plasma PL FA of nonpregnant healthy controls (15.3°C) and the MMP of plasma PL FA of women at parturition (26.1°C). Taking into account the differences in MMP found in different populations , the latter value compares favorably with ours at delivery (27.3°C). In our study population, the MMP increases only 1.13°C from mid-pregnancy until delivery. The MMP of the FA mixture of PL in umbilical plasma (24.49°C) is comparable with a previously calculated value of 20.07°C of PL in normal cord serum (2), but is much lower than maternal values due to higher HUFA status. A higher HUFA fraction in umbilical plasma PL is accompanied with more longer-chain, less fluid SFA. The observed changes in the FA composition of maternal plasma PL during the course of pregnancy are probably not due to changes in dietary intake. Indeed, analysis of food frequency questionnaires (surveyed at the beginning of pregnancy and in the third trimester) of Belgian pregnant women attended by the same obstetrician revealed no significant differences in the FA composition of the diet nor in fat intake during the course of pregnancy (DeVriese, S.R., Matthys, C., De Henauw, S., Christophe, A.B., and Dhont, M., unpublished results). Others confirm these findings: the maternal dietary fat composition of pregnant Dutch women was consistent during pregnancy (12).

Holman *et al.* (3) found that pregnant women in their 36th wk of gestation have significantly suppressed concentrations of all the products of Δ5 desaturation compared to nonpregnant women. Our data reveal a similar pattern; the concentration of Δ5 products in mid-pregnancy is significantly higher than at delivery.

The essential fatty acid composition of maternal PL slightly changed from mid-pregnancy to delivery; 20:4n-6 and its elongation product 22:4n-6 declined, but 18:2n-6 remained stable. No changes were observed in 18:3n-3, 20:5n-3, or 22:6n-3, while 22:5n-3 decreased. The sum of the Δ5 desaturation products was lower at delivery indicating a lower essential FA status of the mother; however, the differences were small. Similar deviations in maternal essential FA status, but of greater magnitude, were reported by Al *et al.* (10th wk of gestation vs. delivery) (13) and by Otto *et al.* (14th wk of gestation vs. delivery) (14). The FA pattern of umbilical plasma presented here is very different from maternal values as was noted in previous studies (2,11–13,15–17). HUFA, especially 20:4n-6 and 22:6n-3, are higher in umbilical than in maternal plasma and both FA are lower in maternal plasma PL after delivery compared to mid-pregnancy. As FA desaturation and elongation by fetal tissues cannot meet neonatal needs (18–20), this could indicate a preferential placental transfer of these long-chain FA to the fetus (21). The total amount of FA in PL (mg/L plasma), and of each individual FA, is much lower in umbilical plasma compared to maternal values (Table 1). This is in concurrence with literature findings (13).

In summary, small but significant deviations were found in maternal plasma PL essential FA between mid-gestation and delivery. This is in concurrence with previous reports on the essential FA status of the mother during pregnancy.

This study extends the concept that changes in overall MMP are counteracted by changes in the MMP of individual FA classes. We demonstrated that in maternal plasma, the loss of HUFA during gestation is accompanied by a shorter MCL and a decrease in the MMP of the SFA. The high content of HUFA in umbilical plasma is associated with a significantly longer MCL and a higher MMP of the SFA. Thus, the FA composition of the SFA changes in a way to counteract changes in the MMP induced by changed HUFA composition. Similar adaptations in the FA composition of SFA, to maintain homeostasis in the overall MMP, were found in multiple sclerosis (2), cystic fibrosis, and anorexia nervosa (4).

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# Influence of Fatty Alcohol and Other Fatty Acid Derivatives on Fatty Acid Uptake into Rat Intestinal Epithelial Cells

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**ABSTRACT**: We investigated the influence of various substrates on the uptake of long-chain fatty acid into IEC-6, rat intestinal epithelial cell line. The uptake of [$^3$H]oleic acid into IEC-6 cells was a saturable function of the oleic acid concentration. Long-chain fatty acids significantly inhibited the oleic acid uptake into IEC-6 cells and shorter-chain fatty acids had little or no effect. Various fatty acid esters suppressed the oleic acid uptake into IEC-6. Fatty alcohols also inhibited oleic acid uptake into IEC-6 and the length of the carbon chain played an important role. These results suggest that long-chain fatty acid uptake was inhibited by the substrates which had a structure similar to long-chain fatty acids, especially those with a long carbon chain. At least two molecules, fatty acid translocase and fatty acid transport protein type 4, which are considered to be involved in the long-chain fatty acid transport into the cell, were expressed on IEC-6 cells, supporting the existence of the carrier-mediated system of long-chain fatty acid transport on IEC-6 cells.

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Fatty acids are important nutrients, liberated from dietary lipids, mainly in the form of triacylglycerol. Today, many people avoid a high-fat diet because excessive intake of dietary fats causes obesity, the most common nutritional disorder in humans. Intestinal absorptive cells can easily absorb free fatty acids. Recently, the mechanism of fatty acid absorption has become an important topic.

Cellular uptake of fatty acids was formerly believed to occur by a purely passive process. However, kinetic properties of facilitated transport of long-chain fatty acids in the small intestine (1,2) and other tissues (3–5) have been exhibited. Now, many molecules that help the absorption of long-chain fatty acids are known to exist in the intestine (6–9). Plasma membrane fatty acid-binding protein (FABPpm) is expressed in several tissues including the small intestine (6), and antibodies against FABPpm decrease oleate uptake (6). However, FABPpm was subsequently identified as the mitochondrial aspartate aminotransferase (10), and the transport mechanism of this protein remains unclear. Fatty acid translocase (FAT), a homolog of human CD36, is localized in the adipocytes and other tissues, and is also expressed in the small intestine (7). The expression of mRNA of FAT, and cytosolic fatty acid-binding protein, was increased in the small intestine of rats fed a high-fat diet (8). Fatty acid transport protein (FATP) has been identified from adipocytes (11). FATP type 4 molecule (FATP4) is expressed in the small intestine and has been suggested to be a novel fatty acid carrier (9).

In this study, we investigated the effects of various components on oleic acid uptake into IEC-6, rat normal intestinal cell line, and the expression of two fatty acid-carrier membrane proteins, FAT and FATP4. The results show that long-chain alcohol and other fatty acid derivatives, especially those that have a long carbon chain, lead to the inhibition of oleic acid uptake by IEC-6 cells and that long-chain fatty acid uptake is possibly mediated by some carrier proteins.

## EXPERIMENTAL PROCEDURES

*Materials*. Bovine serum albumin (BSA) essentially fatty acid free and sodium taurocholate (TC) were obtained from Sigma Chemical Co. (St. Louis, MO). Oleic acid, oleyl alcohol, methyl oleate, linoleic acid, glutamine, glutamic acid, and D-glucose were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Oleyl oleate, oleamide and fatty alcohols were from Tokyo Chemical Industry Co. (Tokyo, Japan). [$^3$H]Oleic acid (1.85 TBq/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA).

*Cell culture conditions*. The intestinal epithelial cell line IEC-6 (from American Type Cell Culture Collection, Rockville, MD), which was established from rat small intestine (12), was maintained in Dulbecco's modified Eagle's medium (GIBCO-BRL, Grand Island, NY) supplemented with 50 mL/L of fetal bovine serum (Bio Whittaker, Walkersville, MD), insulin (4 mg/L), and antibiotics (50,000 units/L of penicillin G sodium and 50,000 μg/L of streptomycin sulfate; GIBCO-BRL) at 37°C in 5% $CO_2$/95% atmosphere. The medium was changed every second or third day. Cells were subcultured when they were 70–80% confluent. For experiments, cells were seeded at a density of $5 \times 10^4$

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Abbreviations: BSA, bovine serum albumin; FABPpm, plasma membrane fatty acid-binding protein; FAT, fatty acid translocase; FATP4, fatty acid transport protein type 4; HBSS, Hanks' balanced salt solution; PCR, polymerase chain reaction; RT, reverse transcription; TC, sodium taurocholate.

cells/well in a 24-well multi-plate (tissue culture-treated) and grown during 3–4 d.

*Preparation of test solutions.* Radioactive oleic acid solution was prepared as follows: [$^3$H]oleic acid and nonradiolabeled oleic acid were solubilized and mixed in ethanol and equilibrated overnight at 4°C (final specific activity was 1.48 GBq/mmol). On the day of experiment, the radioactive oleic acid solution was suspended in Hanks' balanced salt solution (HBSS) containing 10 mM TC to the final concentration 50 μM (radioactivity 74 MBq/L), or various concentrations for determination of dose-response, and incubated at 37°C. Nonradiolabeled oleic acid and other fatty acids and fatty acid derivatives were dissolved in ethanol and the aliquots were suspended in TC-HBSS to the desired concentrations (<1% at ethanol) and incubated at 37°C. The radioactive solution was mixed with the same volume of nonradiolabeled lipid solution or HBSS (as control) just before the incubation with the IEC-6 cells.

*Determination of oleic acid uptake.* Oleic acid uptake into IEC-6 cells was determined based on a procedure previously reported (13), after some modifications. The monolayers of IEC-6 cells were incubated at 37 or 4°C and washed with cold HBSS three times. The test solution (preincubated at 37 or 4°C) was added to an IEC-6 monolayer. After a specified incubation time, the test solution was aspirated off, and the monolayer was washed three times with cold HBSS containing 0.1% BSA (fatty acid free) and 0.05% sodium azide. Then the monolayer was washed once more with cold HBSS and lysed with 1% Triton X-100. The radioactivity of lysate was measured as cellular uptake of oleic acid with Aquasol-2 (Packard Bioscience B.V., Groningen, The Netherlands) using a liquid scintillation counter. The protein concentration of the cell lysate was also determined using a commercial protein assay kit (Bio-Rad Laboratories, Hercules, CA) based on Bradford's method (14).

*RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR).* Total cellular RNA was isolated from IEC-6 and rat intestinal epithelial cells by QuickPrep® Total RNA Extraction Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Rat intestinal epithelial cells were isolated as previously reported (15) from 10-wk-old male Wistar rats (Japan SLC Co., Hamamatsu, Japan). Five micrograms of total RNA was reverse-transcribed to obtain cDNA with an Oligo dT primer (TaKaRa, Kyoto, Japan). RT reaction conditions were 70°C for 10 min, 42°C for 50 min, 70°C for 15 min for 1 cycle. PCR was performed with 1 out of 10 μL of cDNA as a template with Ex Taq DNA polymerase (TaKaRa) in a PCR Thermal Cycler SP (TaKaRa).

The oligonucleotide primers used to amplify respective cDNA sequences were 5′-GAGGTCCTTACACATACAG-3′ and 5′-GTGCCATTAATCATGTCGCA-3′ for FAT [corresponding to nucleotide number from 336 to 819 of the published rat sequence (GeneBank accession number L19658)]; 5′-TGGTGTACTATGGATTCCGCATG-3′ and 5′-TATCCTCATTGACACGTACCAA-3′ for FATP4 [corresponding to nucleotide number from 395 to 862 of the published mouse sequence (GeneBank accession number AF072759)]; and 5′-AACACCCCAGCCATGTACGTAG-3′ and 5′-TGTCAAAGAAAGGGTGTAAAACGC-3′ for β-actin [corresponding to nucleotide number from 462 to 1,250 of the published mouse sequence (GeneBank accession number X03672)]. Samples were heated to 94°C for 1 min. Subsequent cycles were incubated in three temperature steps: 0.5 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 30 cycles. The PCR products were separated on a 2% agarose gel (NuSieve3:1 agarose, TaKaRa).

*Statistics*. Data are presented as means ± SEM. Statistical analyses were carried out with InStat Version 2.00 for Macintosh (GraphPad Software, San Diego, CA). The significance of differences in results among more than three groups was analyzed using a nonparametric test for independent variables (16); for comparisons between two groups at a particular time, a nonparametric test (17) for independent variables was used.

Differences with $P < 0.05$ were considered to be significant in all experiments.

## RESULTS

*Intracellular uptake of oleic acid into IEC-6 cells*. Figure 1 shows the uptake of oleic acid as a function of time (Fig. 1A) and oleic acid concentration (Fig. 1B). When 50 μM of [$^3$H]oleic acid in 10 mM TC-HBSS was incubated with IEC-6 cells, cellular oleic acid uptake was a saturable function of the incubation time (Fig. 1A) and the oleic acid concentration of test solutions (Fig. 1B). When the monolayer was incubated at 4°C, the radioactivity detected in the lysate was significantly lower than that of 37°C at 30 s of incubation and thereafter (Fig. 1A) as reported previously (2,18). Radioactivity detected at 15 s was considered to contain a large amount of oleic acid that bound to cellular membrane nonspecifically because detected radioactivities at 37 and 4°C were almost the same. Since the uptake was linear for 30 s (Fig. 1A) at 37°C, the following experiments were done using 30-s time points. The initial rate of uptake was determined with various oleic acid concentrations in the range of 5 to 600 μM. The uptake was a saturable function of total oleic acid concentration (Fig. 1B). The concentration of oleic acid monomer, which can pass through the cell membrane, was not determined, but the monomer concentration was considered to be proportional to the concentration of the test solution, as previously reported on α-linolenic acid (18).

*Effects of various substrates on oleic acid uptake into IEC-6 cells*. In order to determine whether the uptake of oleic acid by IEC-6 cells was modified by the presence of other substrates in the incubation medium, we examined the effects of several fatty acids, various derivatives of oleic acid, amino acids, and D-glucose, each at 500 μM, added to 50 μM of [$^3$H]oleic acid test solution. The addition of nonradiolabeled long-chain fatty acids, oleic acid, or linoleic acid, reduced the uptake of oleic acid significantly (Fig. 2). In the case of octanoic acid with a shorter carbon chain, the inhibition of oleic acid uptake was weaker and butyric acid, C4, had no effect

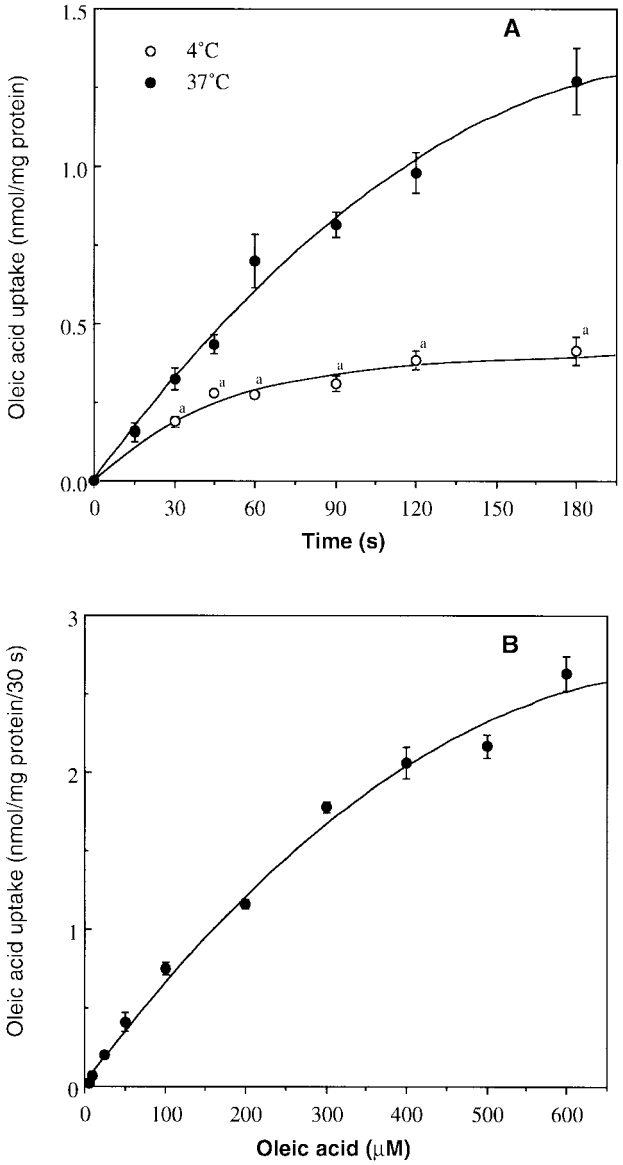


**FIG. 1.** Uptake of oleic acid by IEC-6 cells as a function of time (A) and oleic acid concentration (B). (A) The time course of oleic acid uptake at 37 and 4°C. IEC-6 monolayers (final concentration *ca.* 2.5 g protein/L) were incubated with [$^3$H]oleic acid (50 μM) in 10 mM sodium taurocholate-Hanks' balanced salt solution (TC-HBSS). Results are expressed as means ± SEM of two experiments. Values assigned a letter are significantly different at indicated time points at $P < 0.05$. (B) The uptake of oleic acid for 30 s at 37°C. IEC-6 monolayers (final concentration *ca.* 2.5 g protein/L) were incubated with various concentrations of [$^3$H]oleic acid in 10 mM TC-HBSS. Results are expressed as means ± SEM of two experiments.

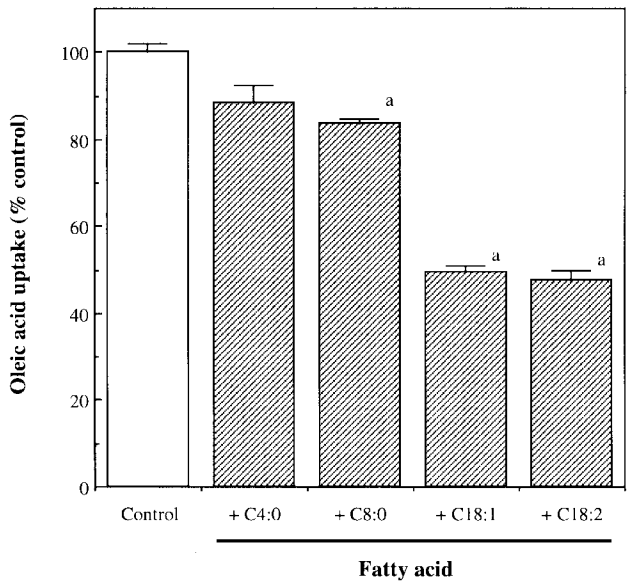


**FIG. 2.** Inhibition of oleic acid uptake with nonradiolabeled fatty acids in IEC-6 cells. IEC-6 monolayers were incubated with the test solutions containing [$^3$H]oleic acid (50 μM) and various fatty acids (500 μM), and mixed as described in the Experimental Procedures section for 30 s. Results are expressed as means ± SEM of four experiments, as % control (no nonradiolabeled fatty acid was contained). Values assigned a letter are significantly different from control at $P < 0.01$.

(Fig. 2). Changes in the cellular uptake of oleic acid induced by oleic acid analogs are shown in Table 1. When the oleic acid derivatives, in which the terminal carboxyl group was blocked (methyl ester, ethyl ester, and oleyl ester), were incubated simultaneously, oleic acid uptake was inhibited significantly. Oleamide and oleyl alcohol also inhibited the cellular uptake of oleic acid significantly. When the incubation medium was supplemented with glutamine or D-glucose that are actively transported by intestinal cells, but are not related to a fatty acid, there was no change in oleic acid uptake. Oleic acid uptake was slightly inhibited by glutamic acid, which has two carboxyl groups, although the differences were not significant. We also measured the uptake of Trypan blue at the same conditions to investigate cell viability. The viability of IEC-6 cells was not influenced by the incubation with these solutions (data not shown).

*Effects of various fatty alcohols on oleic acid uptake into IEC-6 cells*. Long-chain fatty acids inhibited oleic acid uptake into IEC-6 cells, while shorter-chain fatty acids did not.

**TABLE 1**
**Influence of the Various Unlabeled Substrates (500 μM) on Cellular Uptake of 50 μM [$^3$H]Oleic Acid into IEC-6 cells[a]**

| Additives | [$^3$H]Oleic acid uptake |
|---|---|
| | % Control |
| None (control) | 100.0 ± 1.6 |
| Oleic acid methyl ester | 75.1 ± 1.9* |
| Oleic acid ethyl ester | 61.1 ± 3.2* |
| Oleic acid oleyl ester | 56.2 ± 4.6* |
| Oleamide | 55.1 ± 3.6* |
| Oleyl alcohol | 63.2 ± 1.6* |
| Glutamic acid | 79.8 ± 7.8 |
| Glutamine | 94.0 ± 6.2 |
| D-Glucose | 96.0 ± 5.8 |

[a]Values are means ± SEM of four experiments. Oleic acid and additive substrates solutions were prepared separately in the presence of 10 mM of sodium taurocholate and mixed just before the incubation with IEC-6 cells. Uptake of radiolabeled oleic acid into cells was measured for 30 s. *Signficantly different from control, $P < 0.05$.

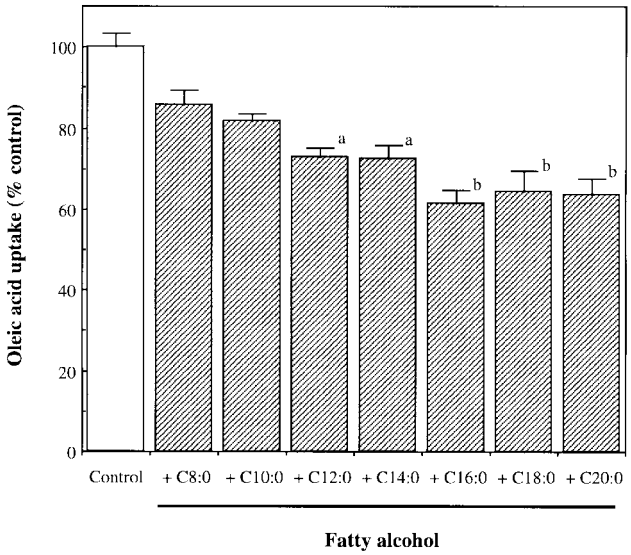


**FIG. 3.** Inhibition of oleic acid uptake with various fatty alcohols in IEC-6 cells. IEC-6 monolayers were incubated with the test solutions containing [$^3$H]oleic acid (50 μM) and saturated fatty alcohols with various carbon chain lengths (500 μM), and mixed as described in the Experimental Procedures section, for 30 s. Results are expressed as means ± SEM of four experiments, as % control (no fatty alcohol was contained). Values assigned letters are significantly different from control, $^aP < 0.01$, $^bP < 0.001$.

The effect of carbon chain lengths on the uptake of oleic acid was tested using various alcohols that had different carbon chain lengths. Oleic acid uptake into IEC-6 was inhibited by

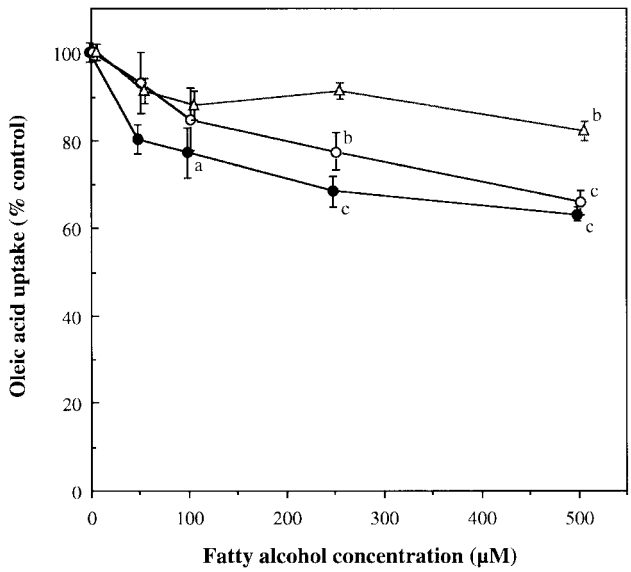


**FIG. 4.** Inhibition of oleic acid uptake with oleyl alcohol (●), stearyl alcohol (○), or octanol (△) in IEC-6 cells. IEC-6 monolayers were incubated with the test solutions containing [$^3$H]oleic acid (50 μM) and various amounts of fatty alcohols (25–500 μM), and mixed as described in the Experimental Procedures section, for 30 s. Results are expressed as means ± SEM of at least two experiments, as % control (no fatty alcohol was contained). Values assigned letters are significantly different from control, $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

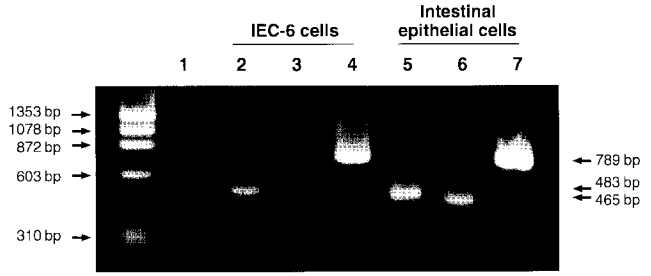


**FIG. 5.** Typical expression of fatty acid translocase (FAT), fatty acid transport protein type 4 (FATP4), and β-actin mRNA in IEC-6 cells and rat small intestinal epithelial cells by reverse transcription polymerase chain reaction (RT-PCR) experiments. Total RNA (5 μg) isolated from IEC-6 cells and intestinal epithelial cells were subjected to PCR (30 cycles), separated on a 2% agarose gel. Lane 1, without reverse transcriptase; lanes 2 and 5, FAT; lanes 3 and 6, FATP4; lanes 4 and 7, β-actin. IEC-6, lanes 2–4; intestinal epithelial cells, lanes 5–7. The size of the mRNA was determined by using a size marker (ϕX174/*Hae* III digest).

simultaneous incubation with fatty alcohol (Fig. 3). Octanol and decanol did not inhibit the uptake significantly, but dodecanol and other longer-chain alcohols inhibited the uptake significantly. Hexadecanol, octadecanol, and eicosanol were much more effective. Octanol also inhibited the oleic acid uptake in a dose-dependent manner, but not as much as oleyl alcohol and stearyl alcohol (octadecanol) (Fig. 4).

*Expression of the candidates of fatty acid transporter on IEC-6 cells.* Various plasma membrane proteins have been suggested to be involved in fatty acid uptake into cells. FAT and FATP4 are candidates of the carriers on intestinal fatty acid uptake. We investigated whether the carrier protein expressed on IEC-6 cells or not. RT-PCR analysis of the total RNA from IEC-6 cells was performed in comparison with rat small intestinal epithelial cells. Figure 5 shows the specific expression of RT-PCR products for FAT (483 bp) and FATP4 (465 bp) in IEC-6 cells and intestinal epithelial cells. FAT and FATP4 mRNA in IEC-6 cells were expressed substantially as in rat small intestine.

## DISCUSSION

Long-chain fatty acids have been believed to be taken into cells by simple diffusion for a long time. However, there are now many reports that various tissue cells express, at least in part, a saturable transport system of long-chain fatty acids (1–5). Here, we investigated whether the cellular uptake of oleic acid into IEC-6 cells was mediated by a kind of transporter or not.

IEC-6 is a normal rat small intestinal epithelial cell line (12). We have utilized IEC-6 cells as an adequate *in vitro* system of intestinal epithelial cells. Previously, we found that fatty acids activate the mobilization of $^{45}Ca^{2+}$, which might be concerned with signaling pathway by dietary fat in IEC-6 cells (19) as well as dispersed rat intestinal cells (15). This effect was inhibited by the presence of a fatty acid methyl ester and glutamic acid. These responses were similar to the effect of these substrates on the secretion of pancreatic juice in rats

(15). Thus, IEC-6 cells had a normal response to dietary components, especially to dietary fat. In this study, we investigated the cellular uptake mechanism of fatty acids using IEC-6 cells as a model and compared the results with the fatty acid recognition on IEC-6 cells.

We showed that oleic acid uptake into IEC-6 cells was saturable. This suggests a saturable transport mechanism and supports the carrier-mediated transport of long-chain fatty acids. Decrease of oleic acid uptake was observed at 4°C. This may be in part due to the change of the state of the cellular membrane and fatty acid micelles. On the other hand, the possibility of the energy-dependent active transport of fatty acids has been reported (18). It is possible that the radioactivity detected at 4°C reflected nonspecific binding and/or uptake into cells because this was almost constant at 30 s (*ca.* 50% of controls) and thereafter. Our results showed that nonradiolabeled long-chain fatty acids inhibited the oleic acid uptake into IEC-6 cells to 50%. In the case of the initial rate of uptake, half of the fatty acid uptake was probably caused by passive diffusion.

Our results showed that nonradiolabeled oleic acid and linoleic acid inhibited the oleic acid uptake into IEC-6 cells, but butyric acid and octanoic acid had little or no inhibitory effect. Similar results were reported previously. Gore *et al.* (18) reported that α-linolenic acid inhibited the linoleic acid uptake by isolated rat enterocytes. On the other hand, short-chain octanoic acid could not inhibit oleic and palmitic acid uptake into Caco-2 cells, a human colon adenocarcinoma cell line, which was generally used as an intestinal cell model (13). These results suggested that the absorption mechanism of long-chain fatty acids was different from that of short-chain fatty acids in the intestine.

Our results also showed that various fatty acid derivatives inhibited the oleic acid uptake into IEC-6 cells. The inhibitory effects varied from 25 to 45%, and the differences were caused by the differences in the structures of these molecules. A fatty acid methyl ester has been reported to inhibit the cellular uptake of linoleic acid and α-linolenic acid (18,20). In this study, we investigated the oleic acid uptake and focused the effect of fatty alcohols. Fatty alcohols exist in the form of wax esters in marine organisms (21) and plants (22) and are indeed contained in human foodstuff. Methyl esters can easily be hydrolyzed in the small intestine and absorbed in a manner similar to fatty acids in rats (unpublished data, Murota, K., Kawada, T., and Fushiki, T.), but fatty alcohols may remain in an intact form in the intestinal lumen and are hardly absorbed. It may be possible that small amounts of fatty alcohols free from wax esters are taken into the gastrointestinal tract and interact with dietary fatty acids. Our results showed that especially the substitutes that had a long carbon chain had a stronger effect. Glutamic acid also inhibited slightly the oleic acid uptake, but glutamine, which does not have a terminal carboxyl group, had no effect. Fatty acid esters and amide had a weaker effect than nonlabeled fatty acids. These results suggest that the carboxyl group also plays a role in fatty acid uptake. However, compared to our previous study on calcium mobilization by lipids (15,19), carboxyl group had a weak effect on fatty acid uptake. The fatty acid recognition mechanism by signal transduction pathways and absorption may not be the same. The test solution used in the calcium mobilization study contained serum albumin, different from taurocholate used in this study. Taurocholate has many hydroxyl groups and may have some interaction similar to fatty alcohol with a fatty acid carrier. Another possible factor is the influence of the condition of the test solution. Previously, we investigated the effect of oleyl alcohol on fatty acid absorpion of rats *in vivo* (23). Dietary fatty acids are solubilized as bile acid micelles in the small intestine. In the *in vivo* study, we used test solutions with a high concentration of oleic acid and oleyl alcohol (100–200 mM) as the emulsion with taurocholate, resulting in oleyl alcohol inhibiting the fatty acid absorption. The emulsions were very turbid, and the micellar condition was not negligible. It is possible that fatty alcohol and other derivatives exert an influence on the micellar condition and inhibit the oleic acid from being free monomers, especially in the high concentration solutions. Although oleic acid was also assembled into micelles with taurocholate to be solubilized in the aqueous phase in this study, the concentrations of oleic acid and other substrates in the test solutions in this study were significantly low (50–500 μM) and the solutions were absolutely clear. In either case, taurocholate has some influence on cellular fatty acid uptake, and fatty acid absorption into enterocytes may be controlled by bile acids *in vivo*.

Tranchant *et al.* (20) reported the inhibition effect of various lipids on the linolenic acid uptake into Caco-2 cells. They showed that the inhibition of initial rate of uptake was not due to a decrease of the monomer concentration of linolenic acid in the incubation medium but due to the inhibition of carrier-mediated transport. Gore *et al.* (18) also reported that the linoleic acid methyl ester inhibited linoleic acid uptake into isolated enterocytes of rats. However, Stahl *et al.* (9) reported that long-chain fatty acid methyl esters and ethyl esters hardly inhibited the incorporation of fluorescently labeled fatty acids into FATP4-expressing cells. It is possible that the characteristics of various molecules, for example fatty acid esters, caused a change in the micellar state and inhibited uptake. However, if fatty acid ester influenced the micelles, the existence of esters should certainly cause the inhibition of uptake in all cases. These reports suggest the existence of several carriers in enterocyte that recognize fatty acid in different way. Previously, both FAT and FATP4 have been considered as possible candidates concerning intestinal fatty acid transport. We showed that FAT and FATP4 were expressed on IEC-6 cells. The expression level of these molecules on IEC-6 cells was substantially same as in rat small intestinal epithelial cells.

In conclusion, oleic acid uptake was inhibited by fatty acid derivatives and the length of the carbon chain played an important role. Thus, our results suggest that long-chain fatty acids are probably taken into IEC-6 cells *via* a kind of transporter, with FAT and FATP4 as possible candidates, and the transporters recognize both the length of carbon chain and carboxyl group of the molecule.

## ACKNOWLEDGMENTS

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# Modulation of Apolipoprotein E-Mediated Plasma Clearance and Cell Uptake of Emulsion Particles by Cholesteryl Ester

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**ABSTRACT:** Cholesteryl ester, along with triglyceride (TG), is the major core component of plasma lipoproteins. We investigated the effect of core composition on the physical state and metabolic behavior of lipid emulsions, as model particles of lipoproteins. Fluorescence studies using 1,6-diphenylhexatriene analogs showed that although cholesteryl oleate (CO) significantly decreased core mobility, the surface rigidity of phosphatidylcholine (PC) monolayers was independent of core composition. When intravenously injected into rats, the increased amount of core CO tended to retard TG emulsion removal from plasma, and the initial clearance rate was correlated with the amount of apolipoprotein E (apoE) bound from plasma. In addition, PC liposomes with a similar emulsion particle size showed negligible binding of apoE and were cleared at a slower rate compared to all emulsions. Furthermore, the effect of CO on the binding behavior of apoE to the emulsion surface and the emulsion uptake by hepatocytes was assessed *in vitro*. Replacing core TG with CO was found to decrease the apoE binding capacity to emulsions markedly without changing the binding affinity and thereby to reduce the cell uptake of emulsion particles by HepG2 cells. These results indicate that the physical state of core lipids, which can be modulated by CO content, plays a role in emulsion metabolism through the alteration in apoE binding.

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Apolipoprotein E (apoE) is a 299-residue plasma apolipoprotein that plays a key role in the hepatic metabolism of triglyceride (TG)-rich lipoproteins, such as chylomicrons, very low density lipoproteins (1–3), and lipid emulsions (4,5). In these particles, the hydrolysis of core TG by lipoprotein lipase leads to the production of relatively apoE-enriched remnant particles, which are rapidly taken up by the liver due to a specific interaction between apoE and cell receptors (6,7). In contrast, apolipoproteins C (C-I, C-II, and C-III-apoCs) inhibit this apoE-mediated hepatic uptake (8–11) probably through the displacement of apoE on these particles (12) or through a direct interaction with apoE (13). These findings indicate that the hepatic uptake of remnant particles is governed by the balance of apoE and apoCs on the particle surface. Although lysophosphatidylcholine (14,15) or cholesterol (16–19) is a key component influencing the balance of both apolipoproteins and consequent remnant metabolism, which factor(s) determine the binding behavior of apolipoproteins in lipoproteins remains obscure.

In addition to surface lipids, several lines of study have shown that the core composition of lipoproteins and lipid emulsions influences their metabolic fates. The increase in core content of cholesteryl ester in low density lipoproteins (LDL) (20) and lipid emulsions (21) facilitates the cellular uptake of these particles, probably through the alteration of apoB or apoE conformation. In fact, physical states of core lipids have been shown to affect the conformations of apoB in LDL (22). We previously demonstrated that cholesteryl oleate (CO) reduces the amount of apolipoprotein A-I (apoA-I) (23) and apoE (18) bound to emulsions and causes an alteration of emulsion metabolism in rats (24). However, the precise mechanism by which core composition affects apolipoprotein binding and metabolism of lipid emulsions is still unclear.

In this study, we investigated the relation of the binding behavior of apoE to plasma clearance in rats and cell uptake by human hepatoma cells (HepG2) of emulsion particles prepared by varying the core content of CO. The data presented here show that the decrease in core mobility caused by CO significantly reduces hepatic uptake of emulsions because of the inhibition of apoE binding.

## EXPERIMENTAL PROCEDURES

*Materials.* Recombinant human apoE (isoform E3) was provided by Pepro Tech EC Ltd. (London, United Kingdom). This apoE was previously shown to have similar physical and biological properties to native human plasma apoE (25). Sodium dodecylsulfate-gel electrophoresis of the current sample showed one major band (34 kDa) and two minor bands at 30–32 kDa (about 10% each). No bands indicating

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Abbreviations: apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; CO, cholesteryl oleate; DPH, 1,6-diphenylhexatriene; FBS, fetal bovine serum; FCR, fractional clearance rate; LDL, low density lipoprotein; PC, egg yolk phosphatidylcholine; py-CE, cholesteryl 1-pyrenedecanoate; TG, triglyceride; TMA-DPH, 1-[4-(trimethylamino)phenyl]phenylhexatriene; TO, triolein.

protein aggregation were observed. Egg yolk phosphatidylcholine (PC) was generously provided by Asahi Kasei Co. (Tokyo, Japan). Triolein (TO) and CO were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Cholesteryl 1-pyrenedecanoate (py-CE), 1,6-diphenylhexatriene (DPH), and 1-[4-(trimethylamino)-phenyl]phenylhexatriene (TMA-DPH) were purchased from Molecular Probes Inc. (Eugene, OR). All other chemicals were of special grade from Wako Pure Chemicals (Osaka, Japan).

*Preparation of emulsions and vesicles.* Emulsion particles were prepared using a high-pressure emulsifier as previously described (18,26). Briefly, mixtures of TO, CO, and egg PC were suspended in 10 mM Tris-HCl buffer (pH 7.4), containing 150 mM NaCl, 1 mM EDTA, and 0.01% $NaN_3$, and successively emulsified at 60–70°C. After removing contaminating vesicles by ultracentrifugation, homogeneous emulsion particles were obtained. Quasi-elastic light scattering measurements (Photal LPA-3000/3100; Otsuka Electronic Co., Osaka, Japan) demonstrated weight-averaged diameters of 107 ± 26, 113 ± 27, and 119 ± 22 nm for TO-PC, TO/CO (1:1)-PC, and CO-PC emulsions, respectively. Egg PC vesicles with a diameter of 108 ± 12 nm were prepared by an extrusion technique (26).

*Cell cultures.* HepG2 cells were grown in a humidified incubator (5% $CO_2$) at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin (27). The FBS was changed to 10% human lipoprotein-deficient serum 24 h before each experiment.

*Fluorescence anisotropy measurements.* Emulsion samples were labeled with DPH or TMA-DPH by adding stock solutions of the probes to yield a probe/core lipids molar ratio of 1:200 for DPH and probe/PC ratio of 1:100 for TMA-DPH. Steady-state fluorescence anisotropy was measured with a Hitachi F-4500 spectrofluorometer (Tokyo, Japan) (26).

*Serum incubation studies.* Binding of apolipoproteins from human serum to emulsions and vesicles was examined as reported previously (18). Emulsions or vesicles were incubated with chylomicron-free serum for 20 min at 37°C, and then the mixtures were centrifuged to separate bound from free apolipoproteins. We did not observe transformation of large unilamellar vesicles into small disks in the present work. In vesicles, 3% sucrose was added to adjust serum density in which vesicles can be separated from lipoproteins by ultracentrifugation. ApoE concentration in the resulting emulsion-free serum was determined in single radial immunodiffusion assay plates, purchased from Daiichi Pure Chemicals (Tokyo, Japan). Intense scattering from lipid particles and ultraviolet absorption of PC and TO interfered with exact spectroscopic determination of the bound protein content. It was also difficult to provide appropriate references for the bound protein in the immunodiffusion assay. Accordingly, the amount of bound apolipoproteins was calculated as the difference between apolipoprotein concentrations in serum before and after incubation. Since the amount of apolipoprotein binding obtained per particle of phospholipid was almost constant, ranging in total lipid concentration of emulsions or vesicles from 1 to 5 mM, saturation of apolipoprotein binding to the particle surface appeared to be achieved in this condition.

*Injection studies in rats.* Fasted male Wistar rats weighing 200 ± 20 g were anesthetized with sodium pentobarbital (Nembutal). py-CE-labeled emulsion or vesicle fractions, containing 4–5 μmol of surface PC that corresponded to 30 μmol of total lipids for emulsions and 10 μmol of total PC for vesicles, were injected into the exposed femoral vein. At the indicated times, blood samples of 250 μL were taken from the carotid vein using a heparinized syringe and immediately centrifuged to separate plasma. To 100 μL of each plasma sample, 600 μL of chloroform/methanol (1:1, vol/vol) was added and then centrifuged to remove proteins from plasma. Fluorescence intensity (excitation 342 nm, emission 377 nm) in each sample was measured with a Hitachi F-4500 spectrofluorometer. Initial plasma clearance rates were calculated as fractional clearance rates from exponential curves fitted by least-squares statistical analysis. Animal work has been reviewed by the Faculty Board.

*ApoE binding assays.* ApoE binding assays were performed in Tris-HCl buffer (pH 7.4) in the presence of 0.25 M sucrose according to the method previously described (23). Briefly, after the incubation of emulsions with recombinant apoE for 30 min at 37°C, the mixtures were subjected to ultracentrifugation to separate both emulsions and lipid-bound apoE from free apoE. The free apoE concentration was determined by measuring the tryptophan fluorescence at 335 nm (excited at 280 nm). The lipid-bound apoE amount was calculated by subtracting the background of free apoE concentration in the top fraction obtained from the results of centrifugation of lipid-free apoE solution (28). Binding data were analyzed using Equation 1 (29),

$$P_f = [\mathrm{PL}]\,(P_f/P_b)\,N - K_d \qquad [1]$$

where $P_f$ and $P_b$ are free and bound protein concentrations, respectively, [PL] is the concentration of phospholipid, $N$ is the binding maximum, and $K_d$ is the dissociation constant.

*Cellular uptake assays.* py-CE-labeled emulsions or vesicles were preincubated with recombinant apoE (16 μg of apoE/μmol of neutral lipid for emulsions and 40 μg of apoE/μmol of PC for vesicles) at 37°C for 30 min to allow apoE equilibrium binding. This apoE-particle complex was diluted and added to the cells. After incubation at 37°C for 2 h, the cells were chilled on ice and washed three times in Hepes buffer, and then dissolved in 0.2% Triton X-100. Fluorescence intensity of py-CE and protein concentration of each sample were determined to calculate particle uptake.

*Lipid and protein analysis.* Phospholipid concentration was determined by phosphorus assay according to the method of Bartlett (30). The concentrations of other lipids were determined using enzymatic assay kits purchased from Wako Pure Chemicals. The protein concentration was determined by the method of Lowry (31), using bovine serum albumin (Bio-Rad, Richmond, CA) as a standard.

## RESULTS

*Fluorescence anisotropy in emulsions.* Figure 1A shows fluorescence anisotropy of DPH incorporated into TO-PC and CO-PC emulsions as a function of temperature. TO-PC emulsions showed a monotonical decrease in DPH anisotropy with increasing temperature, whereas CO-PC emulsions exhibited a temperature break at 41°C, which corresponds to an order–disorder transition of the core CO in emulsions (32). Over the temperature range studied, the anisotropy value of DPH in CO-PC emulsions was much higher than that of TO-PC emulsions, indicating that CO in the emulsion core has a more ordered structure than TO below and above the transition temperature.

As shown in Figure 1B, incorporation of CO into TO core caused an increase in DPH anisotropy with increasing CO. However, change in core mobility had no effect on TMA-DPH anisotropy, indicating that the physical states of lipids on the emulsion surface and in the core seem to be independent (18,33).

*Plasma clearance of emulsions in rats.* Previous work in our laboratory showed that replacing core TG with CO significantly reduces plasma clearance and liver uptake of emulsions in rats (24). To assess the effect of composition and physical states of core lipids on plasma clearance of emulsions more precisely, we evaluated the clearance rates of emulsions varying the core content of CO and of PC vesicles, which have no core lipids and are thought to be extreme particles, showing no effect of core lipids. Since these particles have a similar particle size (about 100 nm in diameter) and the same surface composition, i.e., only PC, the difference in plasma clearance is considered to be due to the core effect.

As shown in Figure 2, *in vivo* clearances of these particles (traced by py-CE label) depended strongly on core compositions. At 1 h after the injection, about 60% of the injected TO-PC emulsions was cleared from plasma. In contrast, increasing CO content in the emulsion core tended to retard the plasma clearance of emulsions. PC vesicles injected at similar numbers particle to emulsions were cleared from plasma at a slower rate compared to all emulsions studied. Table 1 lists the initial fractional clearance rates (FCR) of emulsions and vesicles *in vivo*. The table also summarizes DPH anisotropy values, representing the core mobility in emulsions except PC vesicles, and the binding amount of apoE in human serum, indicating the binding capacity for apoE of each particle. As seen in Table 1, the initial clearance rate of emulsions from plasma appeared to be correlated with apoE binding and inversely correlated with DPH anisotropy, suggesting that the core composition of emulsions regulates the initial plasma clearance *in vivo* through the decrease in apoE bound. In addition, PC vesicles exhibited a negligible binding capacity for apoE and a smaller clearance rate from plasma than all emulsions, providing further evidence for the importance of core lipids for apoE binding and therefore plasma clearance.

*ApoE binding to emulsions.* To further examine the effect of core CO on the binding behavior of apoE to the emulsion surface, we performed binding analysis of recombinant apoE using a centrifugation assay. Figure 3 shows the binding isotherms of apoE to emulsions and vesicles. Although apoE appeared to bind to all particles in a saturable manner, replacing core TO with CO markedly decreased the binding amount of apoE. According to the linearized plot of binding data (23,28), binding parameters, the dissociation constant $K_d$, and the binding maximum $N$ were obtained (Table 2). Increasing CO content in the TO core gradually reduced the binding capacity of apoE without changing the affinity. This was consistent with the results partly showing the effect of CO on apolipoprotein binding in cholesterol-containing emulsions. The binding capacity of apoE to PC vesicles was negligibly small compared to emulsions, as observed for apoA-I (23).

*Cell uptake of emulsion particles.* To determine the contribution of apoE to the cellular uptake of these particles, we compared the particle uptake by HepG2 cells. As shown in Figure 4, apoE enhanced the cellular uptake of all particles

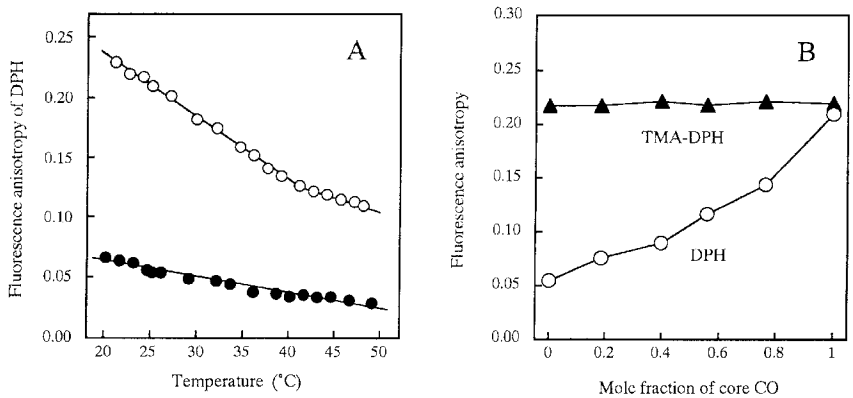


**FIG. 1.** (A) Fluorescence anisotropy of 1,6-diphenylhexatriene (DPH) in triolein-phosphatidylcholine (TO-PC) (●) and cholesteryloleate-phosphatidylcholine (CO-PC) (○) emulsions as a function of temperature. (B) Fluorescence anisotropy of 1-[4-(trimethylamino)phenyl]phenylhexatriene (TMA-DPH) and DPH as a function of CO content in the emulsion core at 25°C.

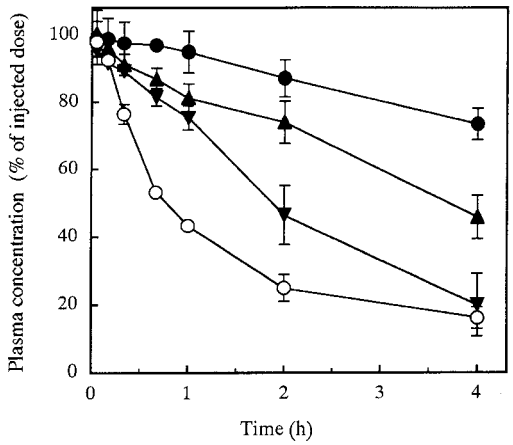


**FIG. 2.** Plasma clearance of cholesteryl 1-pyrenedecanoate (py-CE) after intravenous injections of lipid emulsions and vesicles labeled with py-CE. TO-PC (○), TO/CO (1:1)-PC (▼), CO-PC (▲) emulsions, and PC vesicles (●). Results are mean ± SD of six rats for each. For other abbreviations see Figure 1.

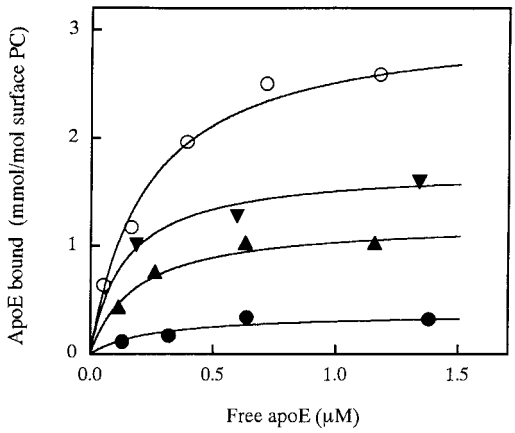


**FIG. 3.** Binding isotherms of apolipoprotein E (apoE) to TO-PC (○), TO/CO (1:1)-PC (▼), CO-PC emulsions (▲), and PC vesicles (●). For other abbreviations see Figure 1.

(35,36). Uptake of TO-PC emulsions increased up to 7–8-fold by the addition of apoE, but the effect of apoE decreased with increasing CO content and was almost negligible on the uptake of PC vesicles. From $K_d$ and $N$ values in Table 2, we estimated the number of apoE molecules bound to each particle at the cell experiment condition: approximately 36, 30, 19, and 5 apoE molecules per particle for TO-PC, TO/CO (1:1)-PC, CO-PC emulsions, and PC vesicles, respectively. The apoE molecules available on particle surface were comparable to the magnitude of particle uptake in the presence of apoE, suggesting that the cell uptake of these particles was modulated by the amount of bound apoE. In addition, the close correlation between the percentage cell association of each particle and the amount of apoE bound per particle, including results of TO-PC emulsions with varying added apoE content in which over 90% of the added apoE bound to the emulsion surface (Fig. 4, inset), clearly indicated that the reduced uptake of CO-containing emulsions in the presence of apoE was due to a decrease in apoE bound on the particle surface, not to the effect of unbound apoE.

**TABLE 1**
**Fluorescence Anisotropy of DPH ($r_s$), Binding Amount of ApoE in Human Serum, and Initial FCR of Emulsions and Vesicles in Rats**

| | $r_s$ (37°C) | ApoE bound (mmol/mol PC)[a] | FCR ($h^{-1}$) |
|---|---|---|---|
| Emulsions | | | |
| TO-PC | 0.038 ± 0.002 | 0.68 | 0.76 ± 0.04 |
| TO/CO(1:1)-PC | 0.086 ± 0.001 | 0.42 | 0.40 ± 0.08 |
| CO-PC | 0.149 ± 0.001 | 0.25 | 0.17 ± 0.04 |
| PC vesicles | — | 0 | 0.13 ± 0.02 |

[a]Values were reproducible within ± 10%. DPH, 1,6-diphenylhexattriene; apoE, apolipoprotein E; FCR, fractional clearance rate; TO, triolein; PC, phosphatidylcholine; CO, cholesterylolate.

**TABLE 2**
**Binding Parameters of ApoE to Emulsions and Vesicles[a]**

| | $K_d$ (μM) | $N$ (mmol/mol surface PC) |
|---|---|---|
| Emulsions | | |
| TO-PC | 0.23 ± 0.06 | 3.08 ± 0.20 |
| TO/CO(1:1)-PC | 0.14 ± 0.04 | 1.71 ± 0.08 |
| CO-PC | 0.18 ± 0.17 | 1.22 ± 0.24 |
| PC vesicles | 0.25 ± 0.16 | 0.38 ± 0.06 |

[a]$K_d$, dissociation constant, $N$, binding maximum; for other abbreviations see Table 1.

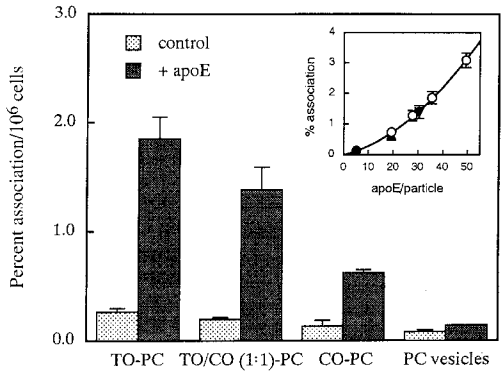


**FIG. 4.** Cell association of emulsion and liposome particles by HepG2 cells in the absence and presence of apoE. HepG2 cells were incubated for 2 h at 37°C with lipid particle alone or together with apoE. Inset, relation between cell association percentage and the amount of bound apoE per particle. Error bars represent standard deviation. Emulsions: TO-PC emulsions with 4, 8, 16, and 24 μg (○), TO/CO (1:1)-PC emulsions with 16 μg (▼), and CO-PC emulsions with 16 μg of apoE/μmol of neutral lipid (▲), respectively. PC vesicles with 40 μg of apoE/μmol of PC (●). For abbreviations see Figures 1 and 3.

## DISCUSSION

During the metabolism of plasma TG-rich lipoproteins, lipoprotein particles become smaller in size and vary in lipid composition, specifically, in their CO content in the core. Particle size is shown to be an important factor in regulating the distribution of plasma apolipoproteins (29) and the hepatic uptake of lipoproteins (37,38). Our previous studies partially indicated the importance of lipid compositions in apolipoprotein binding and plasma clearance of lipid emulsions (18,24). In this study, we systematically compared physical states of core lipids, binding of apoE, and metabolic behavior of emulsions with various core compositions.

As shown in Figure 1, although the increase in core CO content significantly reduced the mobility of core lipids of emulsions, there was no change in surface rigidity as determined from fluorescence anisotropy of TMA-DPH, indicating that the physical state of core lipids had a negligible influence on the acyl chain mobility of the emulsion surface. However, we showed previously (23) and in the present study that the replacement of core TG with CO reduced the binding capacity of apoE and to a lesser extent, binding of apoA-I. This clearly indicates that apolipoprotein binding to the emulsion surface can be regulated by the physical states of not only surface but also core lipids, despite the fact that binding of apolipoproteins appears to occur at the emulsion surface.

Both apoA-I and E have several amphipathic helical segments that are thought to be responsible for lipid binding (39). Based on the binding model of apolipoproteins proposed by Segrest *et al.* (40,41), the apolipoprotein helices are predicted to insert deeply into the surface layers such that the amphipathic helices are buried within the hydrophobic interior of phospholipid monolayers. Taken together with the finding that a significantly small amount of apolipoproteins can bind to the PC vesicle surface compared to TO-PC emulsions, we suggested that the penetration of core lipids into the emulsion surface layers could release the packing stress created by the binding of apolipoproteins, allowing more apolipoproteins to bind to the emulsion surface (18,23). The reduced core mobility in CO-PC emulsions would therefore result in the decrease in the apolipoprotein binding observed in this study. In addition, recent studies have shown that lipid binding of apoE induces the conformational opening in the N-terminal domain, which exposes the hydrophobic faces of four helices, permitting their direct contact with the lipid surface (42,43). Such a conformation of apoE would need large packing defects on the lipid surface layers, which may be unfavorable for particles having a lesser ability to reduce the packing stress.

The decreased binding of apoE caused by increasing CO content in the core is predicted to alter emulsion metabolism significantly. In plasma clearance studies in rats, the increase in core CO content was shown to retard emulsion clearance from plasma, and the calculated initial clearance rate, FCR, was positively correlated with the amount of apoE bound from plasma, including the case of PC vesicles. This suggested that the amount of apoE associated with the particle is crucial for removal of these lipid particles from plasma. Cell experiments, which can exclude the influence of various reactions occurring in plasma such as transfer of apolipoproteins and TG hydrolysis by lipases, further confirmed that core CO significantly affected the liver uptake of emulsion particles. As shown in Figure 4, the enhancement by apoE on cell association of particles diminished with decreasing apoE availability on the particle surface, indicating that the amount of apoE per particle affected directly the liver uptake, namely the uptake by low density lipoprotein (LDL) receptor or heparan sulfate proteoglycans-LDL receptor-related protein pathway (3). These results are consistent with the notion that the absolute number of apoE molecules bound on the particle surface determines the binding affinity of the particle to the receptor (44). Although Quarfordt *et al.* (21) reported that CO enhanced hepatocyte uptake of TO emulsions in the presence of apoE, they did not show the clear relation of the particle content of apoE with the increment in particle uptake by the cells. In addition to the importance of the amount of apoE on the particle, a conformational change of apoE has been suggested to be involved in the emulsion size-dependent receptor affinity (36) or the surface transition-induced change in $K_d$ (45). Since all emulsions used in this study have similar particle sizes and the same surface composition, the conformational change of apoE was unlikely to occur among emulsions, as reflected by the similar values of $K_d$ for all emulsions (Table 2).

When entering plasma, it is known that emulsion particles acquire plasma apolipoproteins, especially apoE and apoCs, and are consequently removed from plasma by the liver (5,46). Several lines of study have indicated that apoE plays a critical role in directing the clearance of emulsions from plasma and that the hepatic uptake of emulsions is modulated by the amount of apoE available (35,36). The present data demonstrate that the increase in core CO exerts its effect on emulsion metabolism by decreasing the binding capacity of apoE. We previously showed that replacing core TG with CO causes the relocation of cholesterol in the surface layers of emulsions, resulting in the alteration in emulsion surface properties (18). In this study, despite a lack of change in the surface rigidity of cholesterol-deleted emulsions, reduced mobility of core lipids caused by increasing CO content was shown to gradually reduce apoE binding and thereby retard cellular uptake of emulsion particles. Enrichment of CO in the metabolism of very low density lipoproteins to LDL is also accompanied by dissociation of apoE in animal plasma (18). Physiologically, the apoE-mediated hepatic uptake of plasma lipoproteins by cell receptors has been argued to be governed by the ratio of apoE to apoCs rather than the absolute apoE content (8,37,47). Further studies of the effects of cholesterol, in addition to those of CO, on the activation of apoE, apoE/apoCs balance and cell uptake of emulsion and lipoprotein particles are in progress.

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# *In Vitro* Behavior of Marine Lipid-Based Liposomes. Influence of pH, Temperature, Bile Salts, and Phospholipase $A_2$

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**ABSTRACT:** To deliver polyunsaturated fatty acids (PUFA) by the oral route, liposomes based on a natural mixture of marine lipids were prepared by filtration and characterized in media that mimic gastrointestinal fluids. First the influence of large pH variations from 1.5–2.5 (stomach) to 7.4 (intestine) at the physiological temperature (37°C) was investigated. Acidification of liposome suspensions induced instantaneous vesicle aggregation, which was partially reversible when the external medium was further neutralized. Simultaneously, complex morphological bilayer rearrangements occurred, leading to the formation of small aggregates. These pH- and temperature-dependent structural changes were interpreted in terms of osmotic shock and lipid chemical alterations, i.e., oxidation and hydrolysis, especially in the first hours of storage. Besides, oxidative stability was closely related to the state of liposome aggregation and the supramolecular organization (vesicles or mixed micelles). The effects of bile salts and phospholipase $A_2$ ($PLA_2$) on the liposome structures were also studied. Membrane solubilization by bile salts was favored by preliminary liposome incubation in acid conditions. $PLA_2$ showed a better activity on liposome structures than on the corresponding mixed lipid–bile salt micelles. As a whole, in spite of slight morphological modifications, vesicle structures were preserved after an acid stress and no lipid oxidation products were detected during the first 5 h of incubation. Thus, marine lipids constituted an attractive material for the development of liposomes as potential oral PUFA supplements.

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Numerous liposome drug administration modes have been envisaged including parenteral, oral, topical, and pulmonary routes (1). Among them, oral intake appears versatile and safe. However, liposome physical stability in digestive tract conditions (low pH in the stomach, degradative enzymes and bile salts in the intestine) and absorption of the encapsulated material through the intestinal barrier remain two major problems to be solved. Whereas hydrosoluble drug delivery with oral liposome formulations may be controversial, lipophile molecule delivery led to more promising results (2). In this context, intake of n-3 polyunsaturated fatty acids (PUFA), mainly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), using liposomes can be envisaged. Indeed, these PUFA are associated with a variety of health benefits such as a reduced risk of coronary heart disease (3) or alleviated symptoms of rheumatoid arthritis (4). However, the benefit of n-3 PUFA intake is likely correlated with the bioavailability of these fatty acids. In humans, the PUFA acylglycerol form showed a better absorption rate than the corresponding ethyl ester form (5). More recently, DHA was shown to be assimilated better by preterm infants when administered in the phospholipid form than in the triglyceride form (6). On this basis, we have focused our attention on liposomes based on a natural mixture of lipids extracted from a marine organism and containing high contents of phospholipids with long-chain n-3 PUFA (7). The resulting liposomes were prepared by a simple method using one filtration step. Indeed, we deliberately chose not to reduce liposome size by sonication or extrusion so taking advantage of the multilamellar character to increase bilayer resistance to digestive fluids and, possibly, to improve PUFA absorption. These liposomes have already shown a good stability upon large pH changes regarding both their physical and chemical bilayer integrities (8). However, in the case of liposome administration by the oral route, the vesicle behavior has to be investigated when the temperature is elevated to a physiological value. Thus, chemical hydrolysis and oxidation as well as phospholipase $A_2$ ($PLA_2$) and bile salt actions were studied. As a whole, for incubation times compatible with the digestion process, we show that the marine lipid-based liposomes used in this study were quite stable as far as oxidation was concerned. Vesicles exhibited a complex morphological behavior as a function of large pH variations between 1.5–2.5 (for the stomach) and 7.4 (for the intestine). Nevertheless, the bilayer structures were mainly preserved so that $PLA_2$ could find the adequate interface for hydrolysis to proceed even in the absence of bile salts.

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Abbreviations: BS, bile salt; cmc, critical micellar concentration; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC; gas chromatography; GEC, gel exclusion chromatography; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; OD, optical density; PC, phosphatidylcholine; PE, phosphatidylethanolamine; $PLA_2$, phospholipase $A_2$; PL, phospholipids; PUFA, polyunsaturated fatty acids; QELS, quasi-elastic light scattering; TG, triglycerides; TLC, thin-layer chromatography.

## EXPERIMENTAL PROCEDURES

*Material.* The natural lipid mixture supplied by IFREMER (Nantes, France) was extracted from a marine organism as previously described (7). The average lipid content was 69

wt% phospholipids (PL), 27 wt% cholesterol, and 4 wt% triglycerides (TG) (8). More precisely, PL were composed of 68 wt% phosphatidylcholine (PC), 23 wt% phosphatidylethanolamine (PE), 2 wt% phosphatidylserine, 2 wt% phosphatidylinositol, and 1 wt% sphingomyelin. In PL, 56% of total fatty acids were PUFA, among which EPA and DHA represented 30 and 59%, respectively. HEPES, bovine bile, and $PLA_2$ extracted from porcine pancreas were obtained from Sigma (St. Louis, MO). Sephacryl S-1000 was purchased from Pharmacia Fine Products (Uppsala, Sweden). All solvents were of analytical grade.

*Bovine bile purification.* Bovine bile was purified from colored pigments on activated charcoal. Dried bile (500 mg) in 10 mL water was added to 4 g of activated charcoal and the mixture was evaporated at 80°C. The powder was dissolved in absolute ethanol and distilled during 2 h. Charcoal was removed by filtration, and the decolorized bile was precipitated using diethyl ether. Precipitate was dried under a stream of nitrogen, and the total mass of purified bile was determined by weight. The different components of initial or decolorized bile were separated by silica gel thin-layer chromatography (TLC) using chloroform/methanol/acid acetic/water (65:25:15:9, by vol) as solvent and visualized by exposing the plates to iodine vapor (9). The same bile salt species were qualitatively identified, in initial and decolorized bile. Total PL amount was assayed by phosphorus analysis (10). Cholesterol and TG contents were analyzed using enzymatic kits (Boehringer Mannheim, Mannheim, Germany). Decolorized bile contained 11 wt% PL, 1 wt% cholesterol, and 1 wt% TG. Accordingly, bile salts represented 87 wt% of decolorized bile.

*Liposome preparation*. Liposomes were prepared by filtration of a lipid suspension according to a method based on Reference 11. Briefly, HEPES buffer (10 mM HEPES, 145 mM NaCl, pH 7.4) was used for hydrating a lyophilized lipid film. The lipid dispersion was successively filtered through polycarbonate membranes of variable pore diameters (Millipore Corp., Bedford, MA), i.e., 5, 1.2, and 0.4 µm. This preparation method led to a mixture of vesicles with a variable number of lamellae (8). Lipid losses by filtration through the membranes were quantified using phosphorus analysis for PL and enzymatic kits for cholesterol and TG. Total lipid concentrations ([lip]tot = [PL] + [cholesterol] + [TG]) given in this work took into account these lipid variations. Liposomes were exposed to acid conditions by dilution in solutions adjusted to pH 1.5 or 2.5 using HCl (10 N). Neutralization was carried out by addition of small volumes of NaOH (10 N) solution.

*Liposome characterization.* Liposome stability as a function of time was followed by turbidity measurements [optical density (OD) at 400 nm] in a 1-mm thick cuvette using a PerkinElmer Lambda Bio 20 spectrophotometer. The samples were maintained at 25 or 37°C in a thermostatted cell support during OD reading.

Gel exclusion chromatography (GEC) was performed using a Sephacryl S-1000 column (30 × 1 cm; Pharmacia) equilibrated at a flow rate of 0.4 mL $min^{-1}$ with HEPES buffer as elution buffer. The column was saturated with lipids by 1-mL repeated injections of a 0.4-µm liposome suspension (15.0 mM) until a constant elution profile was obtained. The liposomes conditioned in acid media (pH 1.5 or 2.5) were diluted in HEPES buffer before injection. Fractions (0.5 mL) were collected, and lipid presence was revealed by absorbance measurements at 280 nm. Fractions of interest were pooled and analyzed for PL content.

Size distribution, i.e., mean diameter and polydispersity index, was determined by quasi-elastic light scattering (QELS) and granulometry measurements. The apparatus used for QELS measurements consisted of a goniometer (BI-200SM; Brookhaven Instrument, Holtsville, NY), a photomultiplier tube (EMI PM-28B), a digital correlator (BI-9000AT; Brookhaven Instrument) and a krypton ion laser (Innova 90; Coherent, Santa Clara, CA) operated at a wavelength of 647.1 nm. The scattering cell was immersed in a refractive index matching fluid whose temperature was controlled at 25 ± 0.1°C. For each sample, data were acquired, typically for a duration of 10 min, for three runs at angles ranging from 30 to 150°C. Size measurements were also performed using a photon correlator spectrophotometer (Malvern Mastersize Ver. 214).

*Liposome micellization by bile salts.* Vesicle bilayer dissolution by bile salts was followed measuring the turbidity at 400 nm. Lipid–bile salt mixtures were prepared by adding defined volumes of a concentrated bile salt solution (650 mM calculated with an average molecular weight of 481 g $mol^{-1}$) to a liposome suspension with [lip]tot ranging from 0.3 to 0.8 mM. After stirring, the mixture was allowed to equilibrate until stable turbidity measurements were obtained. Solubilization was performed at 37°C, (i) on liposomes prepared in HEPES buffer and (ii) on liposomes that were first incubated 3 h in acid medium (pH 1.5) and then neutralized to get a final pH suspension at 7.4.

For each [lip]tot, a solubilization curve was obtained by plotting OD as a function of total bile salt concentration ([BS]tot). The solubilization point, corresponding to the bile salt amount required to completely transform the lipid bilayers into mixed micelles, was determined by the point at which additional detergent only slightly affected the suspension turbidity value, i.e., where OD was quasi-null (12,13). At this solubilization point, the concentration of bile salt molecules that are not associated with the lipids ([BS]bulk) and the bile salt to lipid molecular ratio in the mixed micelles ([BS/lip]mic) are given by the following equation (14):

$$[BS]tot = [BS]bulk + [BS/lip]mic \cdot [lip]tot \quad [1]$$

Using a linear regression analysis, [BS]bulk was found as the extrapolation to zero of the total lipid concentration, and [BS/lip]mic from the slope of the lines.

*Liposome PL hydrolysis by $PLA_2$.* PL hydrolysis in different lipid aggregates, i.e., bilayers and mixed lipid–bile salt micelles, was followed by titration with 0.01 N NaOH solution of the fatty acids liberated at 37°C using a pH-stat (Radiometer). Total lipid concentrations varied from 15.0 to 150.0 mM, which in terms of PL represented concentrations

([PL]tot) ranging from 10.0 to 103.0 mM. Initial liposome preparations were diluted in buffer (10 mM HEPES, 145 mM NaCl, 5 mM $CaCl_2$, pH 8) prior to enzyme addition. Then, liposomes were solubilized by addition of the required amount of bile salts in order to obtain mixed micelles. The hydrolysis reaction was initiated under stirring by $PLA_2$ addition (10 IU) to 2.5 mL of liposome or mixed micelle preparations. The initial velocity ($V_i$), defined as the micromoles of fatty acids liberated per minute, was determined from the initial slope of the titration curves of the hydrolysis experiment.

*Liposome chemical hydrolysis.* The different diacylphospholipid and lysophospholipid classes were assayed after lipid extraction (15) and two-dimensional TLC (8) on liposome dispersions incubated at 25 and 37°C, in HEPES buffer and acid medium (pH 1.5) for 0, 3, and 24 h. The different spots were scraped and analyzed for total phosphorus (10).

*Liposome oxidation.* Aldehydes were analyzed by static headspace gas chromatography (GC) (16). One-milliliter liposome samples ([lip]tot ranging from 0.2 to 7.5 mM) at different pH values (7.4, 2.5, and 1.5) were measured into special headspace 10-mL bottles, sealed under ambient air with Teflon caps, and incubated at two temperatures (25, 37°C) for 24 h. Liposomes and the corresponding mixed lipid–bile salt micelles ([lip]tot = 1.5 mM) were also compared in terms of peroxidation after storage at 37°C up to 30 h in acid medium (pH 1.5 and 2.5). Two-milliliter samples of the headspace were automatically taken and analyzed using a column coated with 1-µm thickness DB1701 (30 m × 32 mm; J&W Scientific, Folsom, CA) in conditions described in Reference 8. Quantification of the different aldehydes formed upon lipid peroxidation was carried out with calibration curves using pure standard solutions (hexanal, pentanal, propanal).

*Statistical analysis.* Differences between two groups were tested for significance using the Student's *t*-test. Data were expressed as mean values ± SD.

## RESULTS

*Liposome behavior under acid conditions.* Initial liposome suspensions were first characterized by granulometry. Vesicle mean diameter was found equal to 6.3 µm. In fact, liposomes prepared by filtration consisted of a polydisperse mixture of vesicles with diameters ranging from 0.5 to 30 µm. In order to mimic the gastrointestinal tract conditions, we examined the influence of medium acidification, as well as temperature increase up to a physiological value, on the vesicle behavior. Figure 1 shows the turbidity evolution, as a function of time, for liposome suspensions diluted in HEPES buffer and acid medium (pH 1.5) and stored at two temperatures (25 or 37°C). At pH 7.4, whatever the temperature, the turbidity remained stable for about 1 d, suggesting that liposome suspension was quite stable over this period of time. When liposomes were placed in an acid medium, turbidity drastically increased, indicating vesicle aggregation as confirmed by optical microscopy observation (8). Granulometry measurements indicated mean diameter of 14 µm for the aggregated

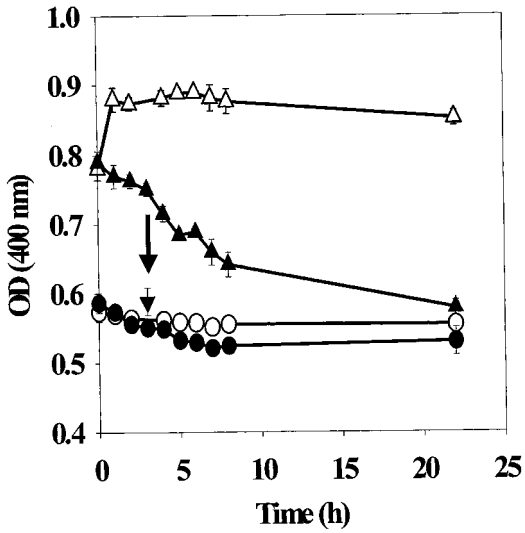


**FIG. 1.** Variations of optical density (OD) at 400 nm as a function of time for liposome suspensions incubated at different pH and 37°C: pH 7.4 (●); pH 1.5 (▲). Liposomes were prepared by the filtration technique using 5-µm pore diameter filters ([lip]tot = 0.3 mM). Acidification was performed using 10 N HCl solution. Sample corresponding to liposomes stored 3 h in acid medium (pH 1.5) was neutralized with 10 N NaOH solution (▼). Data at 25°C, pH 7.4 (○) and pH 1.5 (△), are reproduced from Reference 8. All experiments were performed at least three times with a SD of ±3%.

liposomes. While OD remained at a constant value when liposomes were stored at 25°C, over 24 h (8), a drastic turbidity decrease was observed at 37°C. In the physiological digestion process, liposomes would stay about 2–3 h in acid medium (stomach) before moving to a neutral environment (intestine). To mimic this gap in pH, a liposome sample previously stored under acid conditions during 3 h at 37°C was further neutralized. Medium neutralization induced a decrease in suspension turbidity, the resulting OD being close to that of an untreated sample.

Vesicle elution profile was studied for different incubation times, pH, and temperatures by GEC. The elution pattern of liposomes exposed to pH 7.4 at 25°C during 24 h showed a single peak, the maximum of which corresponded to an elution volume of about 11 mL (Fig. 2). Similar GEC profiles were obtained for liposomes stored, during 0 to 24 h, in the same pH (7.4) and temperature (25°C) conditions (results not shown), confirming the vesicle stability already suggested by the turbidity measurements (Fig. 1). For vesicles stored in an acid medium and/or at 37°C, the chromatograms displayed a second "peak" in the region of small lipid structures, this peak being broader when temperature and pH were combined. QELS measurements were performed on samples corresponding to the second peak. QELS mean diameters ranging from 350 to 500 nm were obtained for the different samples analyzed but their polydispersity in size did not allow discriminating the respective influence of pH and temperature. The relative proportion of the two GEC "peaks" was quantified by total PL analysis. Results are presented in Table 1. Storage of the liposome suspension at room temperature for 24 h led to

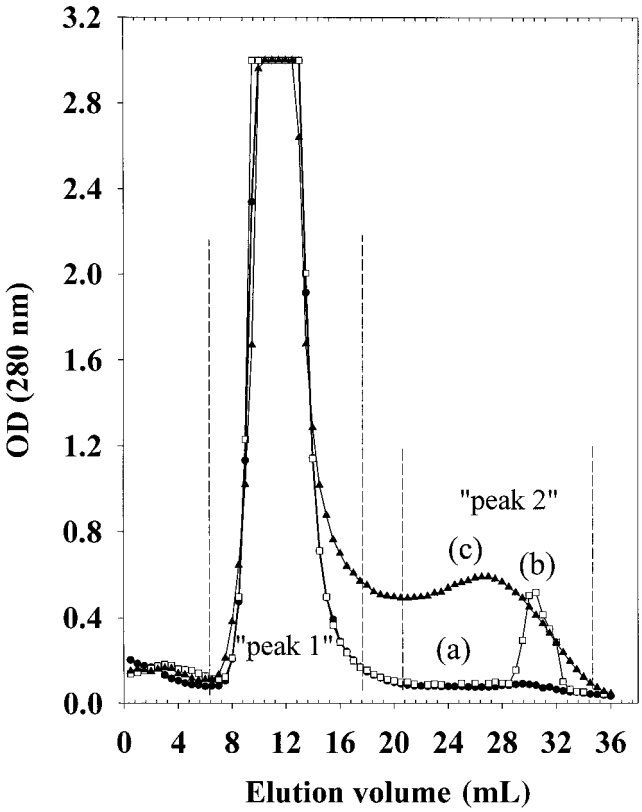


**FIG. 2.** Gel exclusion chromatograms of liposome suspensions after 24-h incubation time (a) in HEPES buffer (pH 7.4) at 25°C (●), (b) in HEPES buffer (pH 7.4) at 37°C (□), (c) after incubation for 3 h in acid solution (pH 1.5) at 37°C before neutralization and then 21 h of storage in buffer (pH 7.4) at 37°C (▲). Liposomes were prepared by the filtration technique using 0.4-μm pore diameter filters ([lip]tot = 15.0 mM, injected volume = 1 mL, flow rate = 0.4 mL $min^{-1}$). The chromatograms represented typical patterns out of four independent experiments.

a slight peak 2 increase (1%), which confirmed, once again, the liposome suspension stability under these storage conditions. When liposomes were incubated at 37°C, the amount of small structures associated with peak 2 slightly increased. However, the combination of pH (1.5 or 2.5) and temperature (37°C) drastically modified the liposome population profile since more than 20% of the initial liposome structures were turned into smaller structures.

*Liposome micellization by bile salts.* The solubilization process was visualized by the turbidity variations measured as a function of bile salt addition. Since detergent was externally added to preformed liposomes, it was essential to work with bile salts completely equilibrated within the lipid structures. Twenty-four hours were required to obtain constant, quasi-null turbidity values for the mixed micelle systems (result not shown), indicating that equilibrium was slowly reached compared to large unilamellar vesicles (14). To mimic the liposome route from the stomach to the intestine, liposomes were incubated 3 h at pH 1.5 then neutralized prior to bile salt addition. Medium acidification combined with temperature favored membrane solubilization by bile salts, and equilibration time was minimized so that 15 min were enough to get the

**TABLE 1**
**Relative Phospholipid Amount of Peak 2 as Defined from GEC Profile[a] of Liposomes Stored at 25 or 37°C at Different pH Values**

| pH | Temperature (°C) | % peak 2[b] |
|---|---|---|
| 7.4[c] | 25 | 9.6 ± 1.4 |
| | 37 | 13.2 ± 1.0* |
| 2.5[d] | 25 | 14.3 ± 0.6* |
| | 37 | 28.6 ± 3.4** |
| 1.5[d] | 25 | 16.2 ± 1.2** |
| | 37 | 29.5 ± 4.4** |

[a]For profile see Figure 3.
[b]The % peak 2 was calculated (phosphorus amount of peak 2)/(phosphorus amounts of peak 1 + peak 2). Peak 1 and peak 2 corresponded to elution volumes ranging from 6 to 18 mL and 21 to 35 mL, respectively. In the initial liposome preparation, peak 2 represented 8.5%.
[c]Liposome incubation time: 24 h in HEPES buffer.
[d]Liposome incubation time: 3 h in acid medium followed by 21 h in neutral medium. *Significantly different from suspensions stored in HEPES buffer at 25°C, during 24 h ($P \leq 0.01$); **significantly different from suspensions stored in HEPES buffer at 25°C, during 24 h ($P \leq 0.001$). GEC, gel exclusion chromatography.

mixed micelles at equilibrium (result not shown). Varying the lipid concentration allowed the calculation of two parameters that describe the complete solubilization of the liposome membranes, i.e., [BS]bulk and [BS/lip]mic. The results are reported in Table 2 for mixed micelles at equilibrium. At neutral pH, increasing the temperature up to 37°C lowered both [BS/lip]mic and [BS]bulk values. However, at pH 7.4, the slight variation of [BS/lip]mic ratio with temperature may be related to the fact that, at 25°C, PL were already in the liquid-crystalline phase. Suspension acidification prior to bile salt action mainly affected the mixed micelle composition since [BS/lip]mic decreased significantly compared to the standard solubilization conditions (pH 7.4, 25°C). Influence of acidification on the amount of detergent molecules in the continuous medium was not so clear. Nevertheless, [BS]bulk values remained above the critical micellar concentration (cmc) of all the detergents (17) suggesting that, at the micellization point and afterward, mixed lipid–bile salt micelles and pure bile salt micelles coexist.

*Chemical stability of liposomes.* When containing PUFA chains, PL may undergo an oxidation process *via* a free-radi-

**TABLE 2**
**Bile Salt/Lipid Molecular Ratio in Mixed Micelles ([BS/lip]mic) at Equilibrium and Corresponding Bile Salt Concentration in the Continuum Medium ([BS]bulk) as a Function of pH and Temperature[a]**

| pH | Temperature (°C) | [BS/lip]mic | [BS]bulk (mM) |
|---|---|---|---|
| 7.4 | 25 | 17.5 | 24.8 |
| 7.4 | 37 | 15.4* | 15.6** |
| 2.5[b] | 37 | 12.0** | 20.2** |
| 1.5[b] | 37 | 9.8** | 17.2** |

[a]All experiments were performed at least four times with a SD of 7%.
[b]Liposome suspensions were incubated 3 h in acid medium and neutralized to pH 7.4 prior to bile salt addition. *Significantly different from dispersions stored in HEPES buffer at 25°C ($P \leq 0.01$); **significantly different from dispersions stored in HEPES buffer at 25°C ($P \leq 0.001$).

**TABLE 3**
**Susceptibility of Liposomes to Lipid Peroxidation Measured by Head Space Capillary Gas Chromatography as a Function of pH, Temperature and Lipid Concentration After 24-h Incubation Time[a]**

| pH | Temperature (°C) | Aldehydes (nmol/µmol total lipids) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Propanal | | | Pentanal | | | Hexanal | | |
| [Lip]tot (mM) | | 0.15 | 1.50 | 7.50 | 0.15 | 1.50 | 7.50 | 0.15 | 1.50 | 7.50 |
| 7.4 | 25 | ND[b] | 4.6 | 0.1 | ND | ND | ND | ND | ND | 0.1 |
| | 37 | ND | 9.5 | 1.6* | ND | 0.4* | ND | ND | 0.7* | 0.5 |
| 2.5 | 25 | 32.9** | 10.9 | 1.7* | ND | 0.5* | 0.2 | 4.2** | 0.5* | ND |
| | 37 | 60.0** | 19.3* | 3.2* | ND | 0.7* | 0.3* | 8.0** | 0.7* | 0.2 |
| 1.5 | 25 | 35.9** | 9.4 | 3.0* | ND | ND | 0.3* | ND | 0.3* | 0.2 |
| | 37 | 65.9** | 22.5* | 11.1** | ND | 1.3* | 0.3* | 5.7** | 1.2* | 0.3 |

[a]All experiments were performed at least twice with a SD of 5%. Data at 25°C and [lip]tot = 1.5 mM are reproduced from Reference 8.
[b]Not detected. For the Student's *t*-test, an arbitrary value of 0.01 corresponding to the minima threshold detection of the head space apparatus was used. *Significantly different from suspensions stored in HEPES buffer at 25°C during 24 h ($P \leq 0.01$); **significantly different from suspensions stored in HEPES buffer at 25°C during 24 h ($P \leq 0.001$).

cal chain mechanism (18). Table 3 presents the analysis of volatile compounds as secondary oxidation products measured by headspace GC for liposomes at different concentrations, stored for 24 h under various pH and temperature conditions. Among the three aldehydes examined, propanal was the more abundant since it resulted from the oxidation of n-3 fatty acids that constituted about 95% of the total PUFA. For a given lipid concentration of 1.5 mM, whatever the pH medium, the rise of temperature from 25 (8) to 37°C increased the oxidized product amounts by a factor 2. For a given temperature, acid storage conditions also doubled oxidation product formation. The influence of the initial liposome concentration was also examined: the more concentrated the suspension, the less oxidation process observed. While at acid pH and high lipid concentration, the lipid oxidative stability may be related to the state of aggregation (19); at neutral pH, the results obtained remained unexplained. The time course of propanal formation in the liposomes and in the corresponding mixed micelles was examined at pH 1.5 and 2.5 up to 24 h (Fig. 3). Liposome suspensions were more stable than mixed micelles as evidenced by (i) the significant formation of propanal that occurred only after a lag period of 5 h instead of 1.5 h for mixed micelles and (ii) the lipid oxidation levels as a function of time that were globally lower in liposomes than in the corresponding mixed micelles. This suggests that the lipid resistance to oxidation is correlated with the different molecular packing conformations of PUFA acyl chains in the two types of lipid structures. Moreover, whatever the structure type, oxidation process was favored by decreasing pH values.

The resistance to chemical hydrolysis upon pH variations and temperature increase of liposomes based on marine lipids was investigated by the relative composition in PC, PE, and the corresponding lysophospholipids, i.e., lysophophatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) (Table 4). At neutral pH and room temperature, 1-d storage slightly affected phospholipid hydrolysis (8). For a given pH, increasing the temperature from 25 to 37°C favored the degradation process as evidenced by significant increases of lysophospholipids. Moreover, hydrolysis was more pronounced in acid medium than in neutral medium, in agreement with ester cleavage being catalyzed in presence of acid (18). Finally, the hydrolysis process was significantly amplified when pH and temperature were combined.

$PLA_2$ catalyzes the hydrolysis of the *sn*-2 fatty acyl ester bonds of PL to produce 2-lysophospholipids and fatty acids. The catalytic behavior was studied on marine lipid-based vesicles exposed beforehand or not to acid conditions. Typical reaction process curves for the hydrolysis by $PLA_2$ of

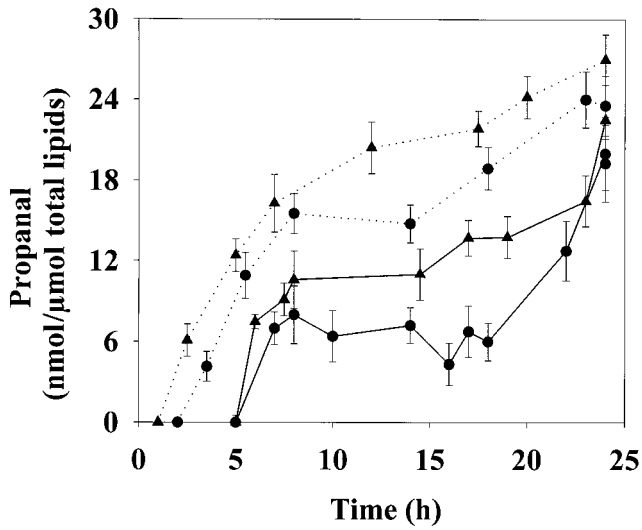


**FIG. 3.** Propanal concentration (nmol/µmol total lipids) determined by headspace capillary gas chromatography as a function of time. Lipid peroxidation was studied on liposomes ([lip]tot = 1.5 mM) incubated at pH 1.5 (—▲—), pH 2.5 (—●—) and on the corresponding lipid-bile salt mixed micelles incubated at pH 1.5 (···▲···) or at pH 2.5 (···●···). All experiments were performed at least three times with a SD of ±9%.

**TABLE 4**
**Relative Composition (wt%) of the Choline and Ethanolamine Species PC, PE, LPC, and LPE for Liposomes Stored Up to 24 h in HEPES Buffer or Acid Medium at 37°C[a]**

| pH | Temperature (°C) | Incubation time (h) | PC | PE | LPC | LPE | Others[b] |
|---|---|---|---|---|---|---|---|
| 7.4 | 25 | 0 | 67.7 | 23.1 | 0.8 | 1.8 | 5.9 |
| | | 24 | 66.9 | 21.5 | 1.4 | 2.5* | 7.6 |
| | 37 | 24 | 65.5 | 20.4 | 2.1** | 5.5** | 6.4 |
| 1.5 | 25 | 3 | 65.7 | 19.0* | 3.3** | 5.2** | 6.7 |
| | 37 | 3 | 60.8* | 15.8** | 8.1** | 9.2** | 6.6 |

[a]All experiments were performed at least four times with a SD of 3%. Data at 25°C are reproduced from Reference 8.
[b]Other phospholipids (degraded or intact species), i.e., phosphatidylinositol, phosphatidylserine, sphingomyelin. *Significantly different from initial suspensions prepared in HEPES buffer at 25°C during 24 h ($P \leq 0.01$); **significantly different from initial suspensions prepared in HEPES buffer at 25°C during 24 h ($P \leq 0.001$). Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine.

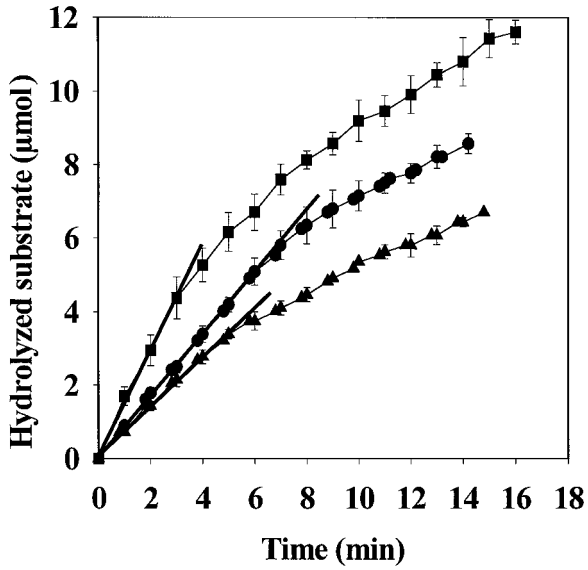


**FIG. 4.** Hydrolysis time course of liposomes (■), liposomes previously incubated 3 h at pH 1.5 and neutralized (●) and mixed lipid-bile salt micelles (▲) by phospholipase $A_2$ ([PL]tot = 27 mM, [$PLA_2$] = 13 µg $mL^{-1}$, [BS]tot = 0.5 M when used). The maximal velocity ($V_{max}$) was determined as the extrapolation of the linear line to 0 abscissa. All the experiments were performed at least four times with a SD of ±8%.

vesicles stored in neutral and acid pH media are shown in Figure 4. In these experiments, the lipid to enzyme ratio was above $10^5$ and ensured a saturating concentration of substrate (result not shown). The shape of the process curve was similar to that obtained by others (21,21), so that it may be assumed that $PLA_2$ hydrolysis obeys Michaelis-Menten kinetics. A similar profile was obtained for $PLA_2$ acting on mixed lipid-bile salt micelles (Fig. 4). The initial velocity per mg of enzyme protein under saturating conditions ($V_{max}$) was calculated from these three curves. Maximum activity value was obtained for the untreated liposome preparation (110 ± 15 µmol $min^{-1}$ $mg^{-1}$ of protein). $V_{max}$ value decreased when enzyme catalysis occurred on mixed micelles containing bile salts (56 ± 2 µmol $min^{-1}$ $mg^{-1}$ of protein). The 40% lower activity on detergent-dispersed micelles may result from a surface dilution of the substrate by the detergent (20). $PLA_2$ activity on vesicles previously incubated under acid conditions led to $V_{max}$ values (74 ± 4 µmol $min^{-1}$ $mg^{-1}$ of protein) intermediate to that obtained for mixed micelles and untreated liposomes. This may be correlated with acid storage conditions that initiated structural and chemical liposome modifications.

## DISCUSSION

Liposomes have been advocated in many applications since the demonstration of the existence of an internal aqueous volume promoted them to carriers of therapeutics and cosmetics agents. However, by virtue of their lipid composition, liposomes could also be effective for lipophilic drug delivery. Marine lipid-based liposomes were prepared with the aim to increase PUFA bioavailability. Nevertheless, liposome characterization with respect to vesicle composition and membrane integrity under various gastrointestinal constraints is needed before considering liposomes as a useful oral dosage form.

Liposome suspension acidification led to an instantaneous vesicle aggregation and to complex morphological bilayer rearrangements (8). Different mechanisms have been advocated to explain the liposome size decrease, including the osmotic shock occurring at acid pH and/or membrane vesiculation attributable to chemical lipid modifications. Increasing the temperature to 37°C seemed to speed these processes as shown by the decrease in turbidity (Fig. 1) and the formation of small lipid particles (Fig. 2 and Table 1). Simultaneously, chemical modifications, in particular oxidation and hydrolysis, occurred which may also proceed to structural changes. First, the greater amount of lysophospholipids formed upon temperature and pH could induce the beginning of a solubilization process or at least alter the membrane permeability. The effect of lysophospholipids and free fatty acids on liposome physical stability has been studied through membrane permeability and particle size measurements. For PC-based liposomes, 10% of lipid hydrolysis was required to promote calcein leakage (18). However, only hydrolysis levels higher than 40% modify liposome stability when monitored by turbidity and size measurements (22). Secondly, oxidation products are also known to increase membrane permeability toward encapsulated products (18). On the whole, the turbidity decrease of marine lipid-based liposome suspensions occurring in the first hours of storage at 37°C suggested that liposome destabilization proceeded by several simultaneous physical and chemical mechanisms. Among them, a simple slow dispersion of the aggregated liposomes as a function of time may not be excluded. With the view of using liposomes by the oral route, 3-h incubation in acid medium corresponds to a long residence time in the stomach for the physiological digestion process. At that time, the lysophospholipid production was not negligible (Table 4), but the oxidative stability was ensured (Fig. 3). Although after 3-h incubation under

acid conditions at 37°C, liposome turbidity had begun to decrease, the liposome membrane organization was globally preserved in simulated gastric conditions as suggested by restored turbidity values following suspension neutralization (Fig. 1). Moreover, optical microscopy observations indicated that part of the liposome aggregates were disrupted so that the suspension mainly contained isolated vesicles (not shown).

In physiological conditions, when in the intestinal tract, dietary lipids are first mixed with bile compounds, i.e., bile salts and phospholipids, so that an adequate lipid-water interface is created allowing pancreatic lipase and phospholipase adsorption and catalysis. Moreover, bile salt solubilization plays an important role in the intestinal absorption of lipids (23). By knowing that bile salt concentration in the intestine of a fasting patient is about 10 mM (24), our results suggested that the solubilization of marine lipid-based liposomes should not be complete in the intestine even after their passage in the stomach. Indeed, [BS]tot required to obtain the bilayer micellization exceeded a critical value of 17 mM for liposomes placed in conditions mimicking the physiological events (3 h at pH 1.5, 37°C, Table 2). The solubilization process is influenced by numerous parameters arising from both experimental conditions and liposome characteristics. Total transformation of PC bilayers into mixed micelles was achieved for a ratio of cholate per PC molecule equal to 8 (25). It is well established that the interactions of detergent with lipid bilayers depend on the lipid composition and especially the amount of cholesterol that stabilizes the membrane against cholate insertion (26). The presence of 27% cholesterol in the natural lipid mixture could explain the higher [BS/lip]mic values obtained in this work (Table 2). Only an acid treatment (pH 1.5) at 37°C prior to liposome solubilization decreased [BS/lip]mic values to that found in the literature for PC membranes. The temperature dependence for solubilization of various lipid–detergent systems has been pointed out for both ionic (27) and nonionic (28) detergents. In the case of ionic detergents, like bile salts, the temperature effect depended, at least partly, on the bile salt species and the lipid to bile salt ratio (27). For example, with taurodeoxycholate, at low lipid to bile salt ratio (corresponding to the presumed coexistence regime of simple and mixed micelles), temperature dependence was similar to the temperature dependence in pure bile salt solutions. The opposite phenomenon was observed at high lipid to bile salt ratio (where only mixed micelles were in presence), i.e., the micellar phase limit decreased with increasing temperature. On the whole, temperature may influence (i) the detergent solubility properties, i.e., the equilibrium between the surfactant dissolved in the aqueous medium and that associated with the lipid to form pure or mixed micelles; (ii) the meeting probability of detergent molecules and lipid membranes through increased Brownian motion. In our case, this temperature effect may explain the sample equilibration times required to obtain stable OD for the mixed micelle solutions: increasing the temperature from 25 to 37°C decreased the equilibration times from 24 h to 15 min (results not shown). Concerning pH influence, the higher [BS/lip]mic values observed at pH 7.4 compared to pH 1.5 may be related to increased amounts of degradation products (especially lysophospholipids) rather than to modifications of lipid packing. Indeed, if PC molecules in the liquid-crystalline phase experience a reduction in the disorder and motion after acidification (29), one must keep in mind that, in our experiments, a medium neutralization was performed prior to vesicle solubilization that should, at least partly, restore the original lipid organization. On the whole, liposome acid treatment seemed to favor bilayer solubilization by bile salts and incubation at 37°C may accelerate the kinetics of liposome micellization. Moreover, high amounts of bile salts required to completely solubilize the liposomes may not be prohibitory for liposome use and PUFA delivery. Indeed, in physiological conditions, $PLA_2$ acts simultaneously with bile salts and generates lysophospholipids that improve the solubilization process.

To mimic the entire lipid digestion process in the intestine, $PLA_2$ action toward liposomes was also addressed. Modulation of enzyme activity toward both physical and chemical membrane properties has frequently been invoked. Thus, phase separation (21,30), membrane curvature (21), or dynamic fluctuations of lipid packing (31) play a prominent role on the catalytic rate. In the same way, lipid composition (32,33), exogenous addition of lysophospholipids (34,35), and changing membrane composition during hydrolysis (36) also affect $PLA_2$ activity. In the present work, the size and lamellarity heterogeneities of the initial liposome dispersion and the membrane evolution as a function of time, pH, and temperature (i.e., small particle generation, lysophospholipid formation) prevented the accurate comparison of $PLA_2$ catalysis on liposomes (acidified or not) and micelles. However, it is clear that $PLA_2$ could act at the interface of liposomes previously incubated under acid conditions that mimic the gastric fluid. This result may be of particular importance for patients with bile secretion deficiency since the detergent would only be required for lipid intestinal absorption and not for the lipid-water interface creation.

In conclusion, we showed that marine lipid-based vesicles exhibited relative membrane physical and chemical stability in the gastric digestion conditions. Acidified liposomes were still an adequate binding surface for $PLA_2$ so that the enzyme was able to hydrolyze the PL present in such structures. On the whole, these results allowed envisaging these liposomes as an oral dosage form.

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# α- and γ-Tocotrienols Are Metabolized to Carboxyethyl-Hydroxychroman Derivatives and Excreted in Human Urine

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**ABSTRACT:** Limited information is available regarding metabolism of vitamin E forms, especially the tocotrienols. Carboxyethyl-hydroxychromans (α- and γ-CEHC) are human urinary metabolites of α- and γ-tocopherols, respectively. To evaluate whether tocotrienols are also metabolized and excreted as urinary CEHC, urine was monitored following tocotrienol supplementation. Complete (24 h) urine collections were obtained for 2 d prior to (baseline), the day of, and 2 d after human subjects ($n = 6$) ingested tocotrienol supplements. The subjects consumed 125 mg γ-tocotrienyl acetate the first week, then the next week 500 mg; then 125 mg α-tocotrienyl acetate was administered the third week, followed by 500 mg the fourth week. Urinary α- and γ-CEHC were measured by high-performance liquid chromatography with electrochemical detection. Urinary γ-CEHC levels rose about four- to sixfold in response to the two doses of γ-tocotrienol and then returned to baseline the following day. Significant ($P < 0.0001$) increases in urinary α-CEHC were observed only following ingestion of 500 mg α-tocotrienyl acetate. Typically, 1–2% of α-tocotrienyl acetates or 4–6% of γ-tocotrienyl acetates were recovered as their respective urinary CEHC metabolites. A γ-CEHC excretion time course showed an increase in urinary γ-CEHC at 6 h and a peak at 9 h following ingestion of 125 mg γ-tocotrienyl acetate. In summary, tocotrienols, like tocopherols, are metabolized to CEHC; however, the quantities excreted in human urine are small in relation to dose size.

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Tocotrienols, members of the vitamin E family, have the same chromanol headgroup and are distinguished by an unsaturated phytyl chain. They are abundant in cereal grains, bran, some nuts, and in palm oil (1). *In vitro,* the tocotrienols are more potent antioxidants than the tocopherols (2,3). Their potent antioxidant activities have been suggested to be due to a combination of higher recycling efficiency from chromanoxyl radicals, more uniform distribution in membrane bilayers, and a stronger disordering effect on membrane lipids, which may make interaction of the chromanols with lipid radicals more efficient (2). Of further interest is the ability of tocotrienols, but not tocopherols, to suppress 3-hydroxy-3-methylglutaryl-CoA reductase activity in mammalian cells (4).

In humans, plasma tocotrienol concentrations remain low (less than 1 μM) after tocotrienol supplementation (5,6). Apparently, tocopherols and tocotrienols are absorbed and secreted in chylomicrons, but the tocotrienols disappear from plasma following chylomicron clearance, while α-tocopherol is preferentially secreted into the plasma. Maintenance of plasma α-tocopherol is under the control of the α-tocopherol transfer protein (α-TTP). This protein selects the naturally occurring *RRR*-α-tocopherol for secretion into the circulation. It has been shown, based on competition studies, that α-tocotrienol (α-T3) possesses only a 12% affinity for α-TTP compared with α-tocopherol (100%) (7), and in terms of biological activity based on the rat fetal resorption assay, the tocotrienols possess only between 10 and 30% activity compared to α-tocopherol (8).

Once tocotrienols and tocopherols are absorbed and delivered to the liver, their fates (other than that of *RRR*-α-tocopherol) are unknown; however, metabolism and/or excretion are likely. Relatively little is known regarding the metabolism of tocotrienols. Tocopherols can be metabolized to carboxyethyl-hydroxychroman (CEHC) derivatives and excreted in the urine (9–11). These metabolites are a result of chain shortening of the tocopherol molecule, presumably *via* a p450-mediated process and β-oxidation, although the exact pathways have not been described. Recent investigations revealed that *ca.* 1% of a dose of deuterium-labeled α-tocopherol is found in urine as the metabolite α-CEHC (11). Based on estimates of dietary intakes of γ-tocopherol, *ca.* 50% of γ-tocopherol may be converted to γ-CEHC (10).

Because of the structural similarity between the tocopherols and tocotrienols (Fig. 1), we hypothesized that α-T3 or γ-tocotrienols (γ-T3) would be metabolized to the same products as the corresponding tocopherols, that is, their CEHC derivatives. This hypothesis was tested in human subjects receiving varying doses of either α- or γ-tocotrienyl acetates as a source of tocotrienols; metabolism was monitored by following the urinary excretion of the CEHC derivatives.

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Abbreviations: CEHC, carboxyethyl-hydroxychroman; HPLC, high-performance liquid chromatography; α-T3, α-tocotrienol; γ-T3, γ-tocotrienol; α-TTP, α-tocopherol transfer protein.

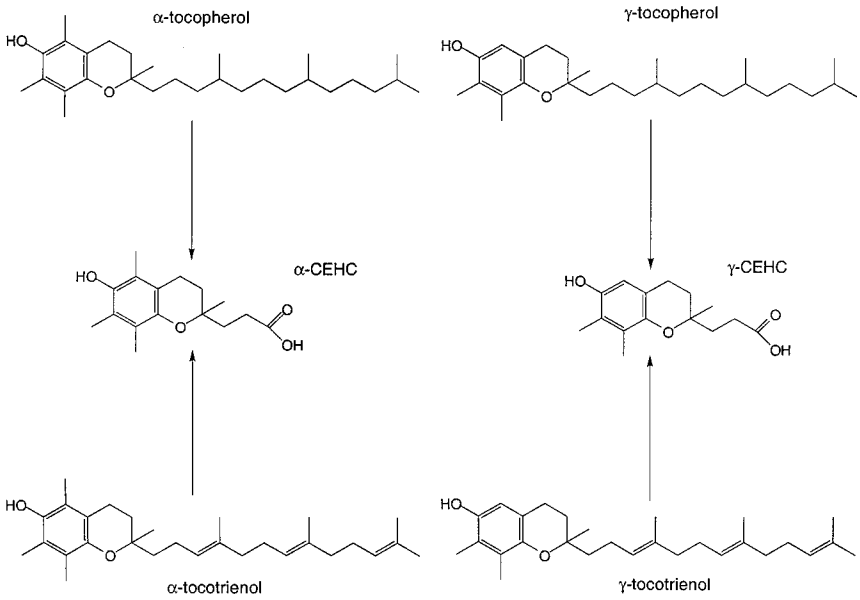


**FIG. 1.** Tocopherols, tocotrienols, and carboxyethyl-hydroxychromans (CEHC). The structures of the vitamin E metabolites α- and γ-CEHC and their respective vitamin E precursors are shown.

## MATERIALS AND METHODS

*Supplements and reagents.* Capsules containing either α- or γ-tocotrienyl acetates (125 mg tocotrienol per capsule) were gifts from Drs. Peter Hoppe and Klaus Krämer of BASF Aktiengesellschaft (Ludwigshafen, Germany). The individual tocotrienols were isolated from food-grade palm oil by preparative high-performance liquid chromatography (HPLC), then acetylated with acetic anhydride, as described (6). The purity of the main component was determined by gas chromatography and verified by $^1$H nuclear magnetic resonance. The content of the other tocols was determined by gas chromatography and verified by HPLC. α-Tocotrienyl acetate contained ≥83% α-tocotrienyl acetate, with minor contamination from α-tocopherol (4.2%), α-tocomonoenol acetate (2.5%), other tocopherols (1.4%), and other tocotrienyls (0.9%). γ-Tocotrienyl acetate contained ≥94% γ-tocotrienyl acetate with minor contamination from β-tocotrienyl (3.4%), α-tocotrienyl (0.7%), and α-tocopherol (0.3%). Residual components were palm oil lipids, none of which exceeded 1%.

Standards of α-CEHC and γ-CEHC were kind gifts from Dr. W.J. Wechter, Loma Linda University (Loma Linda, CA). Trolox and β-glucuronidase were obtained from Sigma-Aldrich (St. Louis, MO). Solvents were all HPLC-grade; all other chemicals and reagents were of the highest purity available.

*Human subjects.* The study protocol was approved by the Institutional Review Board for the use of human subjects at Oregon State University. The subjects ($n$ = 5 males, 1 female) were healthy, nonantioxidant supplement takers and nonsmokers. (One subject dropped out after 2 wk because he found urine collections too time-consuming.) Complete urine collections (24 h) were obtained daily for 2 d before each subject consumed one gelatin capsule containing 125 mg γ-tocotrienyl acetate with breakfast. Urine collections were then obtained for the supplement day and the subsequent 2 d. This protocol was then repeated the following week, except the subjects consumed four capsules (500 mg) γ-tocotrienyl acetate with breakfast. This 2-wk protocol was then repeated with α-tocotrienyl acetate supplement. It should be noted that 250 mg of either of these supplements consumed daily for 8 wk minimally increased plasma tocotrienol concentrations (<1 μM) (6).

Subjects consumed their usual diets; no special restrictions were suggested because the supplements used were large in comparison to usual dietary vitamin E intakes.

*Analysis of vitamin E metabolites.* Urinary CEHC were extracted and analyzed, as described (12). Urine collected the 2 d prior to supplementation represented baseline levels. The total volume of the 24-h urine sample was recorded; then an aliquot was taken, frozen, and kept at −70°C. Prior to extraction, an internal standard was added (10 μg Trolox in ethanol) to 5 mL urine; then conjugated CEHC were hydrolyzed by addition of 400 μL enzyme solution (4 mg β-glucuronidase in 450 μL of 0.1 M sodium acetate buffer, pH 4.5) and incubation for 4 h at 37°C. After cooling on ice, the samples were acidified by addition of 50 μL 6 M HCl and extracted with 15 mL diethylether. An aliquot of the ether layer was dried under nitrogen, and the residue reconstituted in 200 μL ethanol and stored at −20°C until analysis. On the day of analysis (usually within 2 d of extraction), the samples were diluted 1:10 with ethanol to be in the range of the HPLC assay (see below). Unconjugated CEHC were extracted directly from urine, as described above except without hydrolysis.

CEHC were analyzed by HPLC (Shimadzu, Pleasanton, CA) with amperometric electrochemical detection (Bioanalytical systems, West Lafayette, IN), as described (12). Separa-

tion was performed using a 5 μm particle size column (Ultrasep ES100, RP 8 norm; Sepserv, Berlin, Germany) with a gradient system of acetonitrile (solvent A) and McIlvaine buffer (0.01 M citric acid, 0.02 M diammonium hydrogen phosphate, pH 4.15) (solvent B) both containing 1% lithium perchlorate. The gradient was established at a flow rate of 0.4 mL/min, as follows: 80% B for 6.5 min, decrease to 60% B in 1 min, 60% B for 12 min, then decrease to 5% B in 4 min, 5% B for 7 min, increase to 80% B in 1 min, and 80% B for 5 min. The electrochemical detector was operated at an applied voltage of 0.55 V. CEHC were identified by comparing their peak retention times to those of authentic compounds. Usual retention times were 19.5, 23.5, and 26 min for Trolox, γ-, and α-CEHC, respectively. To quantitate CEHC, peak areas of γ- and α-CEHC were compared to that of Trolox (internal standard).

Creatinine was measured using a kit (Sigma-Aldrich Company).

*Statistical analyses.* Data were analyzed using an analysis of variance model with repeated measures (StatView; SAS Institute Inc., Cary, NC). Differences were considered statistically significant if $P < 0.05$. If significant differences were found, then Fisher's least square mean *post-hoc* tests were used for making pairwise comparisons. Here differences between means were considered statistically significant if $P < 0.03$.

## RESULTS

*Daily α- and γ-CEHC excretion.* To test whether α- and γ-CEHC excretion increased in response to tocotrienyl acetate supplementation, urinary CEHC were monitored. The urinary excretion of γ- and α-CEHC during the 1 mon of supplementation with the various amounts and kinds of tocotrienols is shown in Figure 2. In response to the 125 mg γ-tocotrienyl acetate supplement administered during the first week, γ-CEHC excretion increased significantly in the 24-h urine sample collected on the day of supplementation compared with collections on each of the other days ($P < 0.0001$, except the day after supplementation $P = 0.0007$) (Fig. 2A). Supplementation with 500 mg γ-tocotrienyl acetate during the second week also resulted in a significant increase in γ-CEHC excretion on the day of supplementation compared with collections on each of the other days ($P < 0.0001$, except the day after supplementation $P = 0.03$). Urinary α-CEHC excretion was unaffected by either dose of the γ-tocotrienyl acetate supplement (Fig. 2B, weeks 1 and 2).

The smaller dose of the α-tocotrienyl acetate supplement (125 mg) did not appreciably increase α-CEHC excretion. However, supplementation with 500 mg α-tocotrienyl acetate resulted in significant increases in α-CEHC excretion on the day of supplementation compared with collections on each of the other days ($P < 0.0001$, except the day after supplementation $P = 0.0003$). γ-CEHC excretion did not change in response to supplementation with either dose of α-tocotrienyl acetate supplement.

Figure 2 also shows the individual values for each of the six subjects for the study month, except subject 5 who dropped out after 2 wk. The baseline α-CEHC excretion was relatively low and constant for all of the subjects, while γ-CEHC excretion was higher and more variable. Indeed subject 2 was quite responsive to dietary γ-tocopherol and excreted large amounts of γ-CEHC following a meal rich in soybean oil during week three of the study.

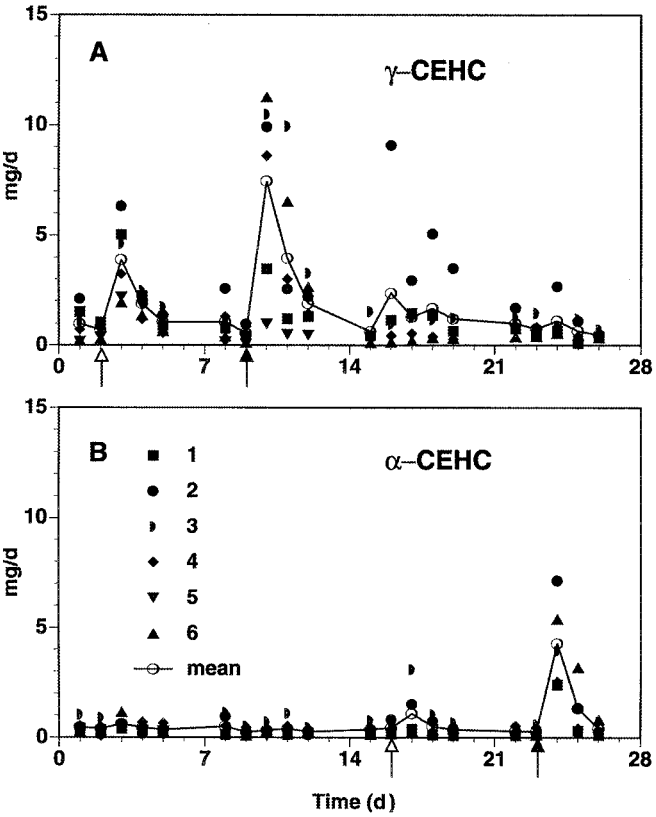


**FIG. 2.** Metabolism of tocotrienols to their CEHC derivatives following supplementation with tocotrienyl acetates. Excretion of γ-CEHC (A) and α-CEHC (B) in daily 24-h urine collections, 2 d prior to and following a 125-mg dose (open arrowhead) or a 500-mg dose (closed arrowhead) of either γ-tocotrienyl acetate (weeks 1 and 2) or α-tocotrienyl acetate (weeks 3 and 4). Shown are the individual responses for the six subjects (except #5 who dropped out after weeks 1 and 2); the open circle represents the mean. Urinary γ-CEHC on the day of 125 mg γ-tocotrienyl acetate supplementation was significantly different from the other days of week 1 ($P < 0.0001$, the day after supplementation $P = 0.0007$). Urinary γ-CEHC on the day of 500 mg γ-tocotrienyl acetate supplementation was significantly different from the other days of week 2 ($P < 0.0001$, except the day after supplementation $P = 0.03$). α-CEHC excretion in the 24-h urine sample following 500 mg α-tocotrienyl acetate supplementation was significantly different from collections the other days of week 4 ($P < 0.0001$, except the day after supplementation $P = 0.0003$). α-CEHC excretion was not affected by γ-tocotrienyl acetate supplementation nor was γ-CEHC excretion affected by α-tocotrienyl acetate supplementation. See Figure 1 for abbreviation.

*Total α- and γ-CEHC excretion in response to tocotrienol supplements.* The total amount of each respective CEHC excreted in urine on the 3 d following each tocotrienol supplement was calculated by summing the daily CEHC excreted minus the average CEHC excreted on days 1 and 2 of each week (Table 1). Overall, the increase in γ-CEHC excretion

**TABLE 1**
**Urinary Excretion of α- or γ-CEHC in Response to α- or γ-Tocotrienyl Acetate Supplementation**

| | γ-Tocotrienyl acetate | | α-Tocotrienyl acetate | |
|---|---|---|---|---|
| | γ-CEHC (mg above baseline)[a] | | α-CEHC (mg above baseline)[a] | |
| Subject | 125 mg | 500 mg | 125 mg | 500 mg |
| 1 | 4.70 | 4.18 | 0.23 | 2.51 |
| 2 | 5.58 | 9.38 | 0.69 | 8.35 |
| 3 | 5.87 | 20.70 | 2.74 | 4.50 |
| 4 | 2.95 | 9.80 | 0.21 | 2.04 |
| 5 | 4.32 | 1.59 | ND[b] | ND |
| 6 | 3.15 | 19.67 | 0.56 | 8.88 |
| Mean ± SD[c] | 4.4 ± 1.2 | 10.9 ± 7.8 | 0.9 ± 1.1 | 5.3 ± 3.2 |
| Percentage of dose excreted as metabolite[d] (mean ± SD) | 6.1 ± 1.7% | 3.7 ± 2.7% | 1.2 ± 1.4% | 1.8 ± 1.1% |

[a]The mg above baseline = average carboxyethyl-hydroxychroman (CEHC) excreted on days 1 and 2 was subtracted from values obtained on days 3, 4, and 5 of each week; the remainder was summed and is an estimate of the CEHC excreted in response to the tocotrienyl acetate supplement.
[b]ND, not determined; subject 5 did not complete this trial.
[c]γ-CEHC excretion was higher than α-CEHC (main effect, $P < 0.01$). The dose size of γ-tocotrienyl acetate did not affect the amount γ-CEHC excreted, while α-CEHC excretion increased with α-tocotrienyl acetate dose ($P < 0.02$).
[d]Percentage of dose excreted as metabolite was calculated using the molecular weights of the CEHC and their respective tocotrienyl acetates for each subject. The percentage of γ-tocotrienol dose excreted as γ-CEHC was higher than that of percentage of α-tocotrienol dose excreted as α-CEHC (main effect, $P < 0.0003$); there was no effect of dose size.

was higher than that of the α-CEHC increase in response to the respective supplements (main effect, $P < 0.01$). However, there was no effect of γ-tocotrienol dose on the amount of γ-CEHC excreted largely because of the intrasubject variability, while the increase from 125 to 500 mg α-tocotrienyl acetate administered resulted in a significant increase in α-CEHC excretion ($P < 0.02$).

The percentage of the dose administered, which was recovered as excreted CEHC, is also shown in Table 1. Typically the percentages of the doses recovered as the metabolites were low and never exceeded 8% for γ-CEHC or 3% for α-CEHC. The percentage γ-tocotrienol dose excreted as γ-CEHC was higher than that of percentage α-tocotrienol dose excreted as α-CEHC (main effect, $P < 0.0003$); the percentage of the dose excreted was not statistically significantly altered by dose size.

*γ-CEHC excretion time course*. Since the majority of γ-CEHC excretion appeared to occur on the day of the supplement ingestion, it was of interest to monitor the excretion of CEHC at intervals throughout the day following ingestion of 125 mg of γ-tocotrienyl acetate. A sample of urine was taken at each time of urination from two of the volunteers (subjects 4 and 6) and the time recorded. The excretion of CEHC was normalized to urinary creatinine, which was constant throughout the day. Figure 3 shows the result as a representative study. Increased γ-CEHC excretion was detected between 5 and 6 h following the supplement and reached a maximum observed at 9 h. In contrast, α-CEHC excretion remained relatively constant throughout the day. The excretion of the unconjugated form of γ-CEHC was also measured since this form has been discovered as a natriuretic factor in human urine (13). Excretion of unconjugated γ-CEHC also followed a similar pattern with a maximum after 9 h (Fig. 3B), and typically represented 10% of the total γ-CEHC, as previously shown (12).

## DISCUSSION

In response to tocotrienol supplements, an increase in urinary CEHC was observed, suggesting that humans metabolize tocotrienols to the same products as the corresponding tocopherols. Labeled tocotrienols were not used in this study, and therefore the conversion to CEHC cannot be claimed with certainty. However, the temporal relationship between the increase in urinary CEHC and the administration of tocotrienol supplements (which was observed with two different tocotrienols at two dif-

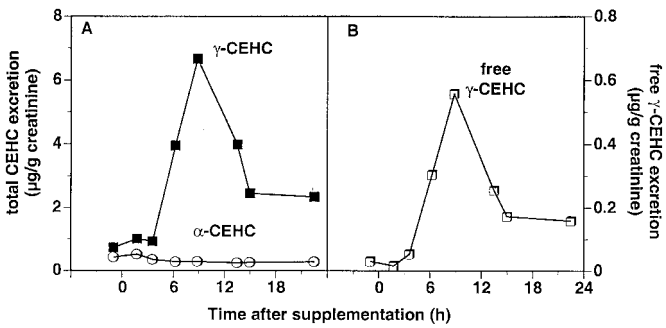


**FIG. 3.** Excretion of total and unconjugated γ-CEHC during a 24-h period. Urinary excretion of (A) total α- and γ-CEHC and (B) unconjugated γ-CEHC, throughout a 24-h period following a 125-mg dose of γ-tocotrienyl acetate in a representative subject of two. See Figure 1 for abbreviation. ■, Total γ-CEHC; ○, total α-CEHC; □, free γ-CEHC.

ferent doses, Fig. 2) provides strong evidence that tocotrienols are metabolized to their respective CEHC.

Only a small percentage of the ingested tocotrienol dose was recovered in the urine: about ~1–2% of α-T3 was recovered as α-CEHC, while ~4–6% of γ-T3 was recovered as γ-CEHC (Table 1). The fractional excretion as CEHC was not dose-dependent (at least between 125 and 500 mg). The fate of the remainder of the dose is unknown; however, there are a number of plausible explanations. The relatively low urinary excretion may be a consequence of either (i) poor absorption and bioavailability, (ii) poor selectivity of the metabolizing system for tocotrienols as compared to tocopherols, or (iii) an alternative pathway for metabolism and/or excretion.

Limited information is available regarding tocotrienol absorption and bioavailability; it is known that plasma tocotrienol levels increase only marginally after supplementation (5,6). Plasma concentrations could be low as a result of low absorption or poor re-incorporation of tocotrienols into plasma. With respect to intestinal absorption in humans, experiments with α-tocopherol have shown *ca.* 70% of the ingested dose is absorbed, as measured by fecal radioactivity (14); however, values as low as 21% have also been reported (15). Although there is apparently no intestinal discrimination of vitamin E in humans, it has been reported that α-T3 absorption in rats is significantly higher than that for α-tocopherol and the other tocotrienols (16). Thus, it is likely in humans that fractional absorption of tocotrienols and α-tocopherol is similar. Despite similar absorption, plasma tocotrienol concentrations are typically extremely low. Therefore, it is likely that the hepatic α-TTP discriminates against tocotrienols along with forms of tocopherol other than 2*R*-α-tocopherols. This is demonstrated in the relatively low biological activity of α-T3 (10–30%) compared to α-tocopherol (100%) (8), a value which correlates with the affinity of the vitamin E analogs for α-TTP (7).

With respect to excretion, studies in humans demonstrated that plasma α-tocopherol reached a maximum *ca.* 8 h after vitamin E administration (17–19), while biliary vitamin E was maximal 12 h after dosing (17). Urinary excretion of γ-CEHC following a dose of γ-tocotrienyl acetate was maximal *ca.* 9 h after ingestion (Fig. 3), thus it appears that vitamin E metabolism is rapid. Although relatively little of the tocotrienol doses were excreted as urinary CEHC, what percentage of the administered vitamin E is actually metabolized to CEHC is in fact unknown. Possibly CEHC are excreted in bile and then reabsorbed into the circulation for ultimate excretion in the urine. This enterohepatic circulation has been proposed to be important for the re-uptake of α-tocopherol; indeed α-tocopherol entering the bile does re-enter the circulation and is distributed to tissues (20).

γ-CEHC was discovered and named LLU-α by Wechter *et al.* (13) because it was found to be a potent natriuretic factor in human urine. Since the metabolite is biologically active, it is likely that specific pathways for the conversion of γ-tocopherol to γ-CEHC exist. Such a specific pathway would explain the relatively higher efficiency for conversion of γ-tocopherol to γ-CEHC. Based on dietary intakes of γ-tocopherol and baseline values of γ-CEHC (10), ≥50% of γ-tocopherol consumed may be converted to γ-CEHC. However, in the present study only ~4–6% of the large dose of γ-tocotrienol was recovered as γ-CEHC.

The fractional conversion of α-tocotrienol to α-CEHC observed in this study is similar to that reported previously for the conversion of α-tocopherol to α-CEHC (9,11), and the baseline amounts found in urine are similar to those of normal humans (12,21). In the present study, large interindividual differences were seen in the quantity of γ-CEHC excreted. These variations are presumably due to differences in absorption and metabolism within individuals. However, both α- and γ-CEHC baseline values fall into the same range as we reported previously (11,12) but are in some cases one-tenth of those reported for γ-CEHC by Swanson *et al.* (10).

Since forms of vitamin E other than naturally occurring *RRR*-α-tocopherol have been shown to be preferentially metabolized to CEHC (11), it was hypothesized that increased CEHC excretion would result from tocotrienol supplementation. However, relatively small amounts of the tocotrienol doses were recovered as urinary CEHC. Previously we observed that after 8 wk of tocotrienyl acetate supplementation, plasma concentrations remained less than 5% of plasma α-tocopherol concentrations (6). Taken together, these data suggest that alternative pathways for metabolism and/or excretion of the tocotrienols exist.

γ-T3 has received interest as a plasma cholesterol-lowering agent (22–24); however our rigorous controlled study in hypercholesterolemic subjects found no effect on plasma cholesterol levels (6). Nevertheless, it is interesting that its metabolite, γ-CEHC, may have an additional beneficial effect as a natriuretic factor, suggesting that specificity for γ-tocopherol exists, even though the actual metabolic pathways for vitamin E metabolism are unknown. For this reason the analysis of unconjugated forms of the metabolite is of importance. It will be of interest to investigate possible ways of increasing γ-CEHC excretion, especially as a possible treatment for hypertension, a risk factor for cardiovascular disease.

In summary, α-T3 and γ-T3 are metabolized to their CEHC derivatives and excreted in the urine. The percentage of dose recovered as the metabolites in the urine is low (only up to 8%). Further studies are required to investigate the biological fate of the remainder of the absorbed dose.

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# Mechanisms of Dimer and Trimer Formation from Ultraviolet-Irradiated α-Tocopherol

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**ABSTRACT:** α-Tocopherol (α-TH) undergoes ultraviolet (UV)-induced photooxidation on the surface of mouse skin to produce a dihydroxydimer, a spirodimer, and trimers as the major products. To study the photochemistry involved, we UV-irradiated α-TH in a thin film on a glass petri dish. Photooxidation yielded a mixture of dihydroxydimer, spirodimer, and trimers. In the time-course studies, the dihydroxydimer accumulated and then was further oxidized, whereas the spirodimer and trimers accumulated more gradually. Reaction of two tocopheroxyl radicals forms the dihydroxydimer, whereas the spirodimer may be formed either by photooxidation of α-TH to an orthoquinone methide (*o*-QM) followed by a Diels-Alder reaction or by photooxidation of α-TH to the dihydroxydimer, followed by two-electron oxidation. Irradiation of a mixture of $d_{10}$-labeled and unlabeled ($d_0$) dihydroxydimer produced a mixture of labeled and unlabeled spirodimers as detected by positive atmospheric pressure chemical ionization–mass spectrometry. The absence of mixed label spirodimers among products indicated that direct oxidation of the dihydroxydimer is a facile route to the spirodimer and is probably the major spirodimer-forming reaction in α-TH photooxidations. Trimer formation from the dihydroxydimer and the spirodimer was observed, however, and requires an *o*-QM intermediate. Photooxidation of $d_{10}$-labeled and unlabeled ($d_0$) dihydroxydimers yielded mixed isotopomers of the trimer products, thus demonstrating that the dihydroxydimer and spirodimers underwent conversion to *o*-QM intermediates. Photochemical conversion of α-TH to UV-absorbing dimer and trimer products may contribute to photoprotection by topically applied α-TH.

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Increasing concern regarding ultraviolet (UV)-induced photodamage to skin has resulted in an increased effort to prevent the deleterious effects of UV radiation on humans. This has been accomplished mainly through the topical application of compounds capable of eliciting photoprotective effects. Several studies have shown that α-tocopherol (α-TH) provides protection from UV-induced photodamage. α-TH inhibits both UV-induced liposomal lipid peroxidation *in vitro* (1) and UV-induced pyrimidine dimer formation, a marker of direct UV-induced DNA damage *in vivo* (2) and *in vitro* (3). However, α-TH depletion occurs in a time-dependent manner when α-TH is exposed to UV-B radiation both in liposomes *in vitro* and in mouse skin *in vivo* (1,4).

The major photoproducts derived from topical application of α-TH to mouse skin followed by UV-irradiation have been identified as a mixture of α-TH dimers (4). Dimers previously have been observed as products in numerous oxidations of α-TH (5–13). In addition, trimers also have been described as α-TH oxidation products (9). The two most commonly observed dimers derived from α-TH oxidation are the 5a,5a′-dihydroxydimer **4** and the 5a,5a′-spirodimer **5** (5,13,14). The formation of the 5a,7a′-, 7a,5a′-, and 7a,7a′-dihydroxydimers seems to be less favorable. This preference for reactivity at the 5a position vs. the 7a position of α-TH has been observed in other α-TH oxidation products (15–17). This difference in reactivity may reflect steric and/or stereoelectronic factors at C7 vs. C5.

Several mechanisms have been postulated to explain the formation of α-TH dimers and trimers (5,10,18). The proposed mechanistic routes are summarized in Scheme 1. The initial step in tocopherol photooxidation is a one-electron oxidation of α-TH, which produces the tocopheroxyl radical **1** (Scheme 1). In pathway A, **1** undergoes a second oxidation (removal of one electron and a proton at the 5a-methyl position) to form an orthoquinone methide (*o*-QM), **2**. The spirodimer **5** is then formed as the result of a Diels-Alder cyclization of two *o*-QM (pathway D) (10,19). A second pathway to the spirodimer involves the initial formation of the dihydroxydimer **4**. Dihydroxydimer formation results from the combination of two carbon-centered, 6-hydroxy-7,8-dimethyltoc-5-ylmethyl radicals (orthomethyl tocopherol radicals) 3. Formation of **3** itself may occur through two possible pathways. The first (pathway C) involves a disproportionation between α-TH and an *o*-QM yielding a phenoxyl radical and an orthomethyl tocopherol radical **3** (18). Alternatively, it has been postulated that an intramolecular hydride transfer may occur between the oxygen and the 5-methyl group, producing the orthomethyl tocopherol radical (pathway B) (5). Finally, the spirodimer is formed by an overall two-electron

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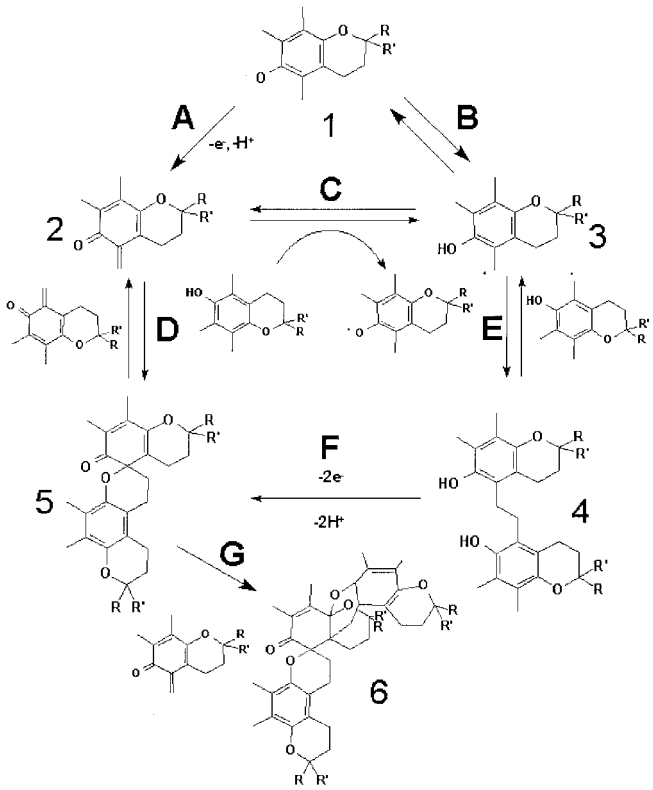
Abbreviations: APCI-MS, atmospheric pressure chemical ionization mass spectrometry; ESI–MS, electrospray ionization–mass spectrometry; MS–MS, tandem mass spectrometry; *o*-QM, orthoquinone methide; RP-HPLC, reversed-phase high-performance liquid chromatography; α-TAc, α-tocopherol acetate; α-TH, α-tocopherol; UV-vis, ultraviolet-visible.




**SCHEME 1**

oxidation of the dihydroxydimer, followed by intramolecular cyclization, which likely occurs in a stepwise fashion (pathway F). The trimers (**6**) are believed to result from a Diels-Alder addition of **2** to **5** (6,19).

We have studied the photochemical formation of tocopherol dimers and trimers in an effort to elucidate their mechanisms of formation. We have compared the products generated by UVB (290–320 nm) photooxidation of α-TH with those generated by silver oxide oxidation. In these studies, we focused on the roles of quinone methide intermediates vs. the dihydroxydimer **4** as precursors to the spirodimer and trimers. Our data suggest that the dihydroxydimer **4** is a key intermediate in α-TH photooxidation and that *o*-QM intermediates are contributors to trimer formation.

## EXPERIMENTAL PROCEDURES

*Materials.* α-TH [(*R*,*R*,*R*)-α-tocopherol] was a gift from Henkel Fine Chemicals (La Grange, IL). The [5a,7a-$(C^2H_3)_2$]-α-tocopherol ($d_6$-α-TH) was synthesized from [5a,7a-$(C^2H_3)_2$]-α-tocopherol acetate ($d_6$-α-TAc) (15). Other reagents were obtained commercially from standard sources and were used without purification. Westinghouse FS-20 lamps were purchased from National Biological Corp. (Twinsburg, OH), and a UVX digital radiometer with a UVX-31 sensor was purchased from Ultraviolet Products, Inc. (San Gabriel, CA). Of the lamp output *ca.* 80% was in the UVB (290–320 nm), whereas the remainder was in the UVA (320–400 nm). UV-visible (vis) spectra were recorded on a Beckman DU-640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

*Synthesis of α-TH dimers and trimers.* Tocopherol dimer standards were purified by reversed-phase, gradient high-performance liquid chromatography (HPLC) on a Spherisorb ODS-2, 5 μm, 250 × 4.6 mm column (Alltech, Deerfield, IL) with the UV detector set at 300 nm. A flow rate of 1.5 mL/min was used, with the initial mobile-phase composition consisting of methanol/ethyl acetate (75:25, vol/vol) for 18 min, increased to 30:70 over 10 min, and then back to 75:25 over 5 min. The identities of both the spirodimer and the dihydroxydimer were confirmed by atmospheric pressure chemical ionization–mass spectrometry (APCI-MS), tandem mass spectrometry (MS–MS), and by comparison of the UV-vis spectra with literature values (5). α-TH spirodimer **5** was prepared by $K_3FeCN_6$-catalyzed oxidation of α-TH using the method of Nelan and Robeson (13). Briefly, 50 mM α-TH in hexane was treated with 50 mM $K_3FeCN_6$ in NaOH (0.2 M) at 25°C for 5 min. The hexane layer was removed, washed with $H_2O$, dried over sodium sulfate, and the bright yellow liquid dried to leave a yellow residue. The spirodimer product eluted at 25 min under the above HPLC conditions. The dihydroxydimer **4** was prepared by the reduction of **5** with $LiAlH_4$/diethyl ether, using the method of Skinner and Alaupovic (5). Briefly, $LiAlH_4$ (600 mmol) was added slowly to spirodimer (10 μM) dissolved in diethyl ether. The reaction mixture was refluxed for 30 min, cooled to room temperature, and the reaction quenched by the addition of $H_2O$. The ether layer was removed, filtered, dried with sodium sulfate, and evaporated leaving a yellow residue. The residue was dissolved in mobile phase and purified by HPLC, with **4** eluting at 17 min.

The trimers could be further resolved into at least four separate species by an isocratic HPLC protocol, with a flow rate of 1.5 mL/min, utilizing a mobile phase consisting of methanol/ethyl acetate (65:35, vol/vol). The trimer peaks eluted at 27, 29, 38, and 40 min. Analysis by APCI-MS and MS–MS yielded values consistent with those expected for tocopherol trimers. The deuterium-labeled dimers and trimers were prepared in the same manner as the unlabeled compounds, except that $d_6$-α-TH was used in place of α-TH, and $LiAlD_4$ was used in the reduction of **2** to **1**.

*UV irradiation.* A solution of α-TH, dihydroxydimer, or spirodimer [100 nmol in ethyl acetate (200 μL)] was transferred to a 9.5-cm diameter glass petri dish. The solvent was allowed to evaporate at room temperature, leaving a thin film on the surface of the dish. The sample was irradiated from directly above for 60 or 240 min with a UV lamp at a dose rate of 10.0 J/m$^2$s (total dose 36,000 J/m$^2$). The sample was dissolved in ethyl acetate (2 × 1 mL), transferred to a test tube, and evaporated. The residue then was dissolved in mobile phase (ethyl acetate/methanol, 1:3 vol/vol). The photooxidation products were separated by reversed-phase HPLC on a Spherisorb ODS-2, 5 μm, 4.6 × 250 mm column. A flow rate of 1.5 mL/min was used, with the UV detector set at 300 nm. The initial conditions consisted of a mobile phase consisting of methanol/ethyl acetate (75:25, vol/vol) (0–18 min), ramped

to 30:70 (18–28 min), and then back to 75:25 (28–33 min).

*UV irradiation of* $d_0$ *and* $d_{10}$ *dihydroxydimer or spirodimer.* A solution of $d_0$- and $d_{10}$-dihydroxydimer ([5,5′-$(C^2H_2)_2$-7,7′-$(C^2H_3)_2$]-tocopherol dimer) or spirodimer [250 nmol, 1:1 (mol/mol)] in ethyl acetate (400 μL) was added by syringe to a 9.5-cm diameter glass petri dish. The sample was irradiated and isolated as above for APCI-MS analysis. The trimers from the initial HPLC collection were subjected to further purification as indicated above. The trimers from the spirodimer irradiation were analyzed by ESI–MS as Ag complexes (see below).

*MS analysis.* Product identities were confirmed by APCI-MS or ESI–MS with a Finnigan TSQ 7000 (Finnigan MAT, San Jose, CA) instrument in the Southwest Environmental Health Sciences Center Analytical Core laboratory at The University of Arizona. APCI-MS samples were analyzed by flow injection using acetonitrile as the mobile phase. Flow injection MS–MS analysis was also performed on α-TH dimer oxidation products and compared to MS–MS analyses of authentic standards prepared by $K_3FeCN_6$-catalyzed oxidation of $d_0$- or $d_6$-α-TH. The Ag-complexed species were prepared in methanol/ethyl acetate (65:35, vol/vol) containing 35 μM $AgNO_3$ and analyzed immediately by flow injection using methanol/ethyl acetate (65:35, vol/vol) containing 35 μM $AgNO_3$ in the mobile phase. Theoretical MS isotope distribution calculations were performed using the software package XMASS/TOF (Bruker, Billerica, MA).

## RESULTS AND DISCUSSION

The fate of UV-irradiated α-TH may involve several possible photooxidative pathways, which are summarized in Scheme 1. In accordance with Scheme 1, we first examined different ways in which the spirodimer **5** could be formed by nonphotooxidative reactions. $K_3FeCN_6$-catalyzed oxidation of α-TH is known to produce a mixture of both the dihydroxydimer **4** and the spirodimer **5** (13). In contrast, oxidation of α-TH with silver oxide in acetonitrile results in the formation of an *o*-QM, which forms the spirodimer exclusively as may be seen in the HPLC chromatogram shown in Figure 1A, suggesting that pathway A–D is a viable route to spirodimer formation. In addition, the spirodimer also may be prepared directly from the dihydroxydimer *via* silver oxide-catalyzed oxidation, as shown in Figure 1B,C.

A time-dependent depletion of α-TH with concomitant formation of dimers **4** and **5** is observed when α-TH undergoes UV irradiation *in vitro* in a thin film. The dimer levels appear to reach a maximum in 20–30 min, and then further

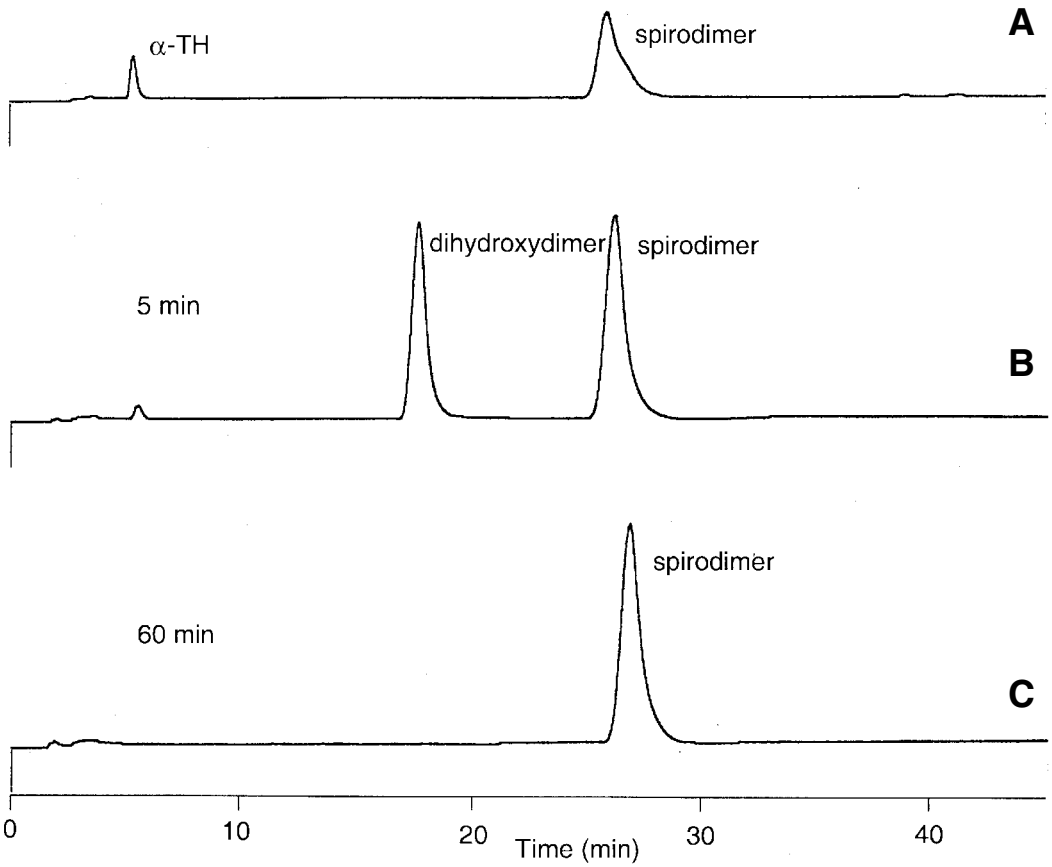



**FIG. 1.** High-performance liquid chromatography (HPLC) chromatogram of products for (A) silver oxide-catalyzed oxidation of α-TH to spirodimer, (B) silver oxide-catalyzed oxidation of dihydroxydimer **4** to spirodimer **5**, 5 min, and (C) silver oxide-catalyzed oxidation of dihydroxydimer to spirodimer, 60 min.

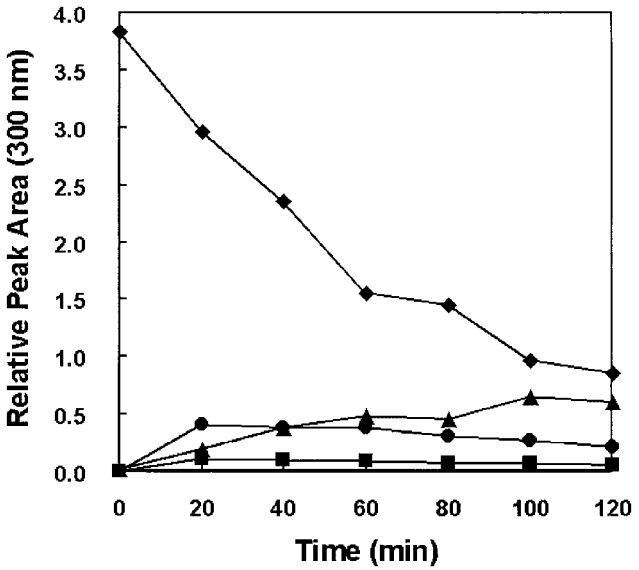



**FIG. 2.** Time course of ultraviolet (UV)-induced α-tocopherol (α-TH) depletion in a thin film. α-TH (◆), dihydroxydimer **4** (●), spirodimer **5** (■), and trimers (▲) were measured by HPLC as described in the Experimental Procedures section.

photooxidation yields trimers and other oxidation products (Fig. 2). Although we have not quantified yields of the dimer and trimer products, it appears that the levels observed probably do not account for α-TH consumption. This can be attributed to the photosensitivity of both dimer and trimer products, which most likely yields polymeric products that are not detected in our analyses. UV irradiation of dihydroxydimer **4** in the absence of α-TH also yields **5** and **6**, in addition to other oxidation products (Fig. 3). UV irradiation of **5** results in slow conversion to a mixture of trimers and unidentified

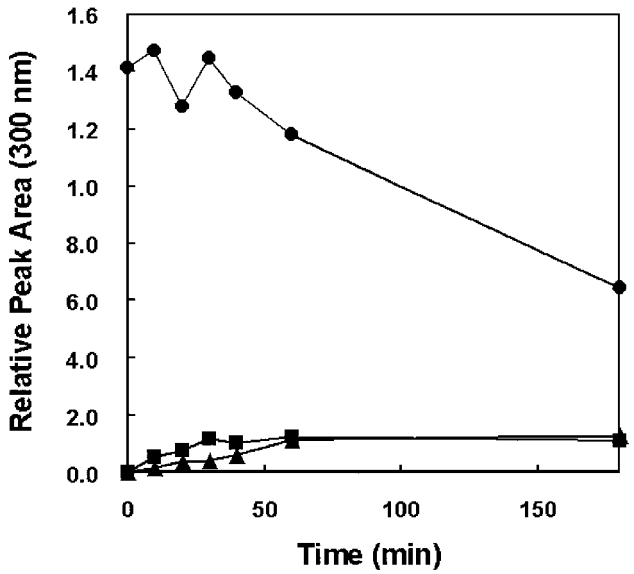



**FIG. 3.** Time course of UV-induced dihydroxydimer 4 depletion in a thin film. α-TH dihydroxydimer 4 (●), spirodimer 5 (■), trimers (▲) were measured by HPLC as described in the Experimental Procedures section. See Figures 1 and 2 for abbreviations.

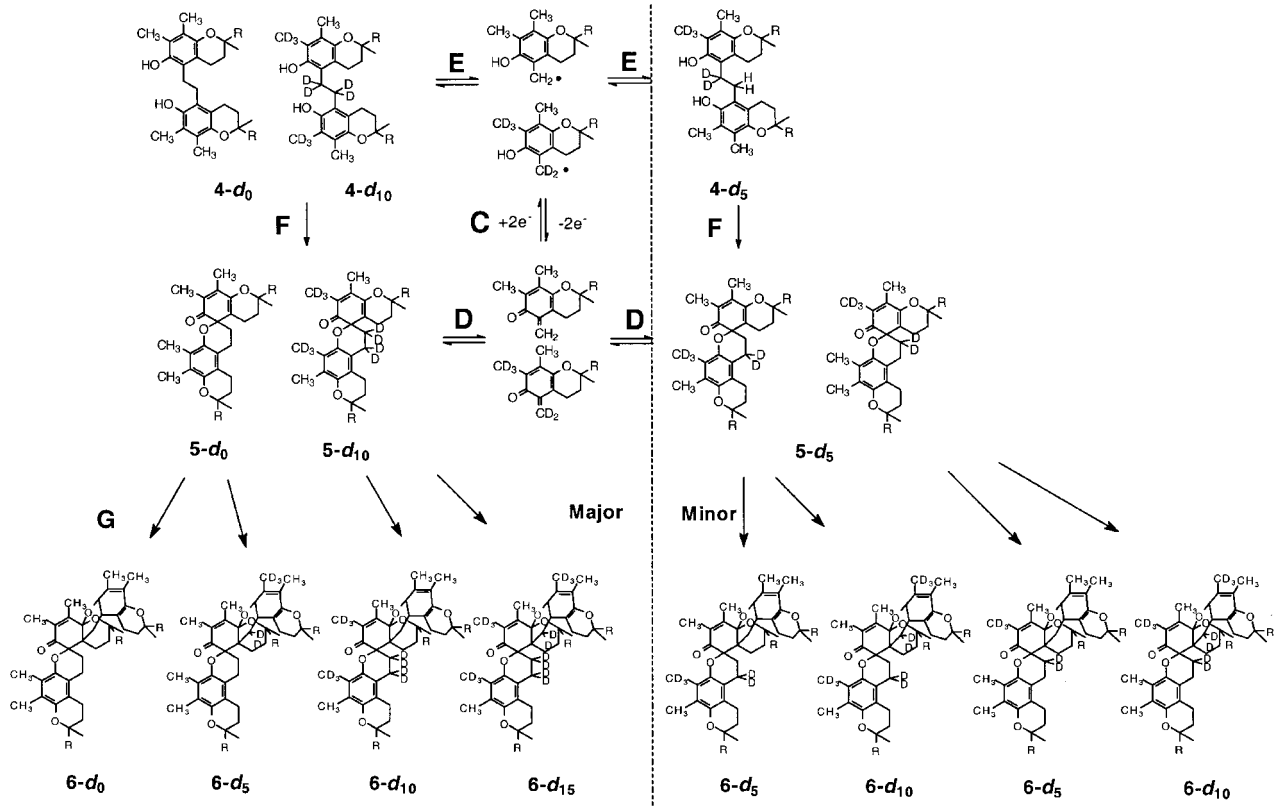
products (not shown). Thus, according to Scheme 1, either reactions D, E, or both must be reversible in order for trimer formation to occur from the dihydroxydimer.

α-TH trimer formation is believed to occur *via* a Diels-Alder reaction of a spirodimer with an *o*-QM (19). For this to occur, however, either the dihydroxydimer or the spirodimer must undergo photolytic cleavage to yield two monomeric units, at least one of which must be an *o*-QM. If a label could be incorporated into the dimers that would remain in each monomeric unit upon dissociation, then the fate of the label could be followed by MS. Accordingly, we prepared both the dihydroxydimer and the spirodimer from $d_6$-α-TH, in which the 5 and 7 methyl groups are fully deuterated. This results in $d_{10}$-dihydroxydimer and $d_{10}$-spirodimer that differ only in their mass from the unlabeled ($d_0$) dimers. Irradiation of a 1:1 mixture of **4-$d_0$** and **4-$d_{10}$** provides a method to follow the fate of the monomeric tocopherol units, which has been summarized in Scheme 2. If the spirodimer were formed *via* reversion and recombination (pathway E-C-D), then a mixed spirodimer, (**5-$d_5$**) in addition to **5-$d_0$** and **5-$d_{10}$**, would be formed. Under this scenario, it also follows that dissociation of **4** would form the mixed dihydroxydimer (**4-$d_5$**) *via* recombination of radicals derived from both **4-$d_0$** and **4-$d_{10}$**. Similarly, *o*-QM formation *via* dissociation of **5** (pathway D) should also produce some mixed spirodimer (**5-$d_5$**) in addition to **5-$d_0$** and **5-$d_{10}$**. Finally, direct oxidation of **4** to **5** without dissociation to monomeric units would produce no mixed (**$d_5$**) spirodimer products.

We irradiated a mixture of **4-$d_0$** and **4-$d_{10}$**, as a thin film, collected the products by HPLC, and then analyzed the products by APCI-MS. Figure 4A,B represents the mass spectra of standards of $d_0$-([M + H]$^+$ at *m/z* 860) and $d_{10}$-dihydroxydimer ([M + H]$^+$ at *m/z* 870), respectively. The mass spectrum of the dihydroxydimer HPLC peak (11 min) is shown in Figure 4C. A mixed dihydroxydimer ($d_5$) would have a mass between the two pure species ([M + H]$^+$ at *m/z* 865). No such mass is evident in the spectrum. Instead, a 1:1 mixture of **4-$d_0$** and **4-$d_{10}$** is observed.

The spirodimer was collected and analyzed in the same manner as for the dihydroxydimer. Figures 5A and 5B are representative spectra of standards of the $d_0$-([M + H]$^+$ at *m/z* 858) and $d_{10}$-spirodimers **5** ([M + H]$^+$ at *m/z* 868), respectively. Figure 5C shows the mass spectrum of the spirodimer product collected from the photooxidation (16 min peak), which does not demonstrate any evidence for the formation of a mixed dimer (**5-$d_5$**) ([M + H]$^+$ at *m/z* 863). Unfortunately, we were unable to obtain useful APCI-MS data for the trimer products of these photooxidations, since excessive fragmentation of the trimer [M + H]$^+$ ions occurred during analysis (figure not shown).

Because mixed isomers were not detected for either the dihydroxydimer or the spirodimer, dissociation of the dihydroxydimer to the tocopherol monomers (pathway E) and subsequent disproportionation to the *o*-QM (pathway C) appear to be a relatively minor pathway compared to direct oxidation of the dihydroxydimer to the spirodimer (pathway F). This may not be surprising since the acidity of excited singlet-state




**SCHEME 2**

phenols can be several orders of magnitude greater than in the ground state (20). This suggests that photodissociation of the phenolic hydroxyl group may be faster than other ground state reactions, so that a photodissociation of the dihydroxydimer followed by cyclization to the spirodimer would occur more rapidly than a Diels-Alder reaction. Although direct oxidation of α-TH to the spirodimer (pathway A–D) may occur, it appears to be a minor product-forming pathway.

Since UV photooxidation of the spirodimer also produces α-TH trimers, we performed an irradiation of a 1:1 mixture of **5-*d*$_0$** and **5-*d*$_{10}$** in a thin film. Once again, the spirodimer peak and the trimer peaks were collected by HPLC for MS analysis. APCI-MS resulted in extensive fragmentation of trimer $[M + H]^+$ ions, which precluded accurate measurements of isotopic distributions. Thus, we used a novel approach, in which compounds are ionized for electrospray by complexation with $Ag^+$ in the HPLC mobile phase (21,22). As was the case in the dihydroxydimer photooxidation, no mixing of label (**5-*d*$_5$**) was observed for the residual spirodimer (not shown). Standards of the $d_0$- and $d_{15}$-Ag-complexed trimers were detected by ESI–MS (Fig. 6A,B). We also were able to successfully detect Ag-complexed trimers from irradiation of a 1:1 mixture of the $d_0$- and $d_{10}$-spirodimers by this method (Fig. 6C). The spectrum indicates that some mixing of label, i.e., the formation of **6-*d*$_5$**, **6-*d*$_{10}$**, occurred.

Since spirodimer consumption was low in this experiment and we observed no mixed label spirodimer, this would seem to indicate that the majority of *o*-QM were trapped as trimers through reaction with the $d_0$- and $d_{10}$-spirodimers. This should result in a product ratio of 1:1:1:1 ($d_0/d_5/d_{10}/d_{15}$) being observed. However, the isotope distribution in Figure 6C does not reflect this ratio. We calculated isotope distributions for the $[M + Ag]^+$ ions for different ratios of the $d_0/d_5/d_{10}/d_{15}$ Ag-complexed trimers (not shown) to compare to our experimental results depicted in Figure 6C. The calculated isotope distribution closest to our experimental result in Figure 6C was a ratio of 1:4:1:4 ($d_0/d_5/d_{10}/d_{15}$).

This result suggests that trimer formation by the $d_5$-*o*-QM (to form $d_5$ and $d_{15}$ trimers) is favored over trimer formation by the $d_0$-*o*-QM. It may be that $d_{10}$ spirodimer dissociates more rapidly than the $d_0$-spirodimer, or that the $d_5$-*o*-QM reacts more rapidly to form trimers than does the $d_0$-*o*-QM. The rate of a Diels-Alder reaction is influenced by an inverse deuterium isotope effect (23), whereas a normal deuterium isotope effect is observed in the retro Diels-Alder (24). The former would favor trimer formation by $d_5$-*o*-QM, whereas the latter would reduce the availability of $d_5$-*o*-QM relative to $d_0$-*o*-QM for trimer formation. Our results are consistent with an inverse deuterium isotope effect, in which the Diels-Alder reaction of the $d_5$-*o*-QM with the spirodimer is more rapid than reaction of the $d_0$-*o*-QM with the spirodimer.

In summary, we have demonstrated that the major route of α-TH photooxidation is *via* tocopheroxyl radical dimerization to form dihydroxydimer **4**, which then undergoes further oxidation to spirodimer **5**. Although *o*-QM intermediates are not major contributors to spirodimer formation, they appar-

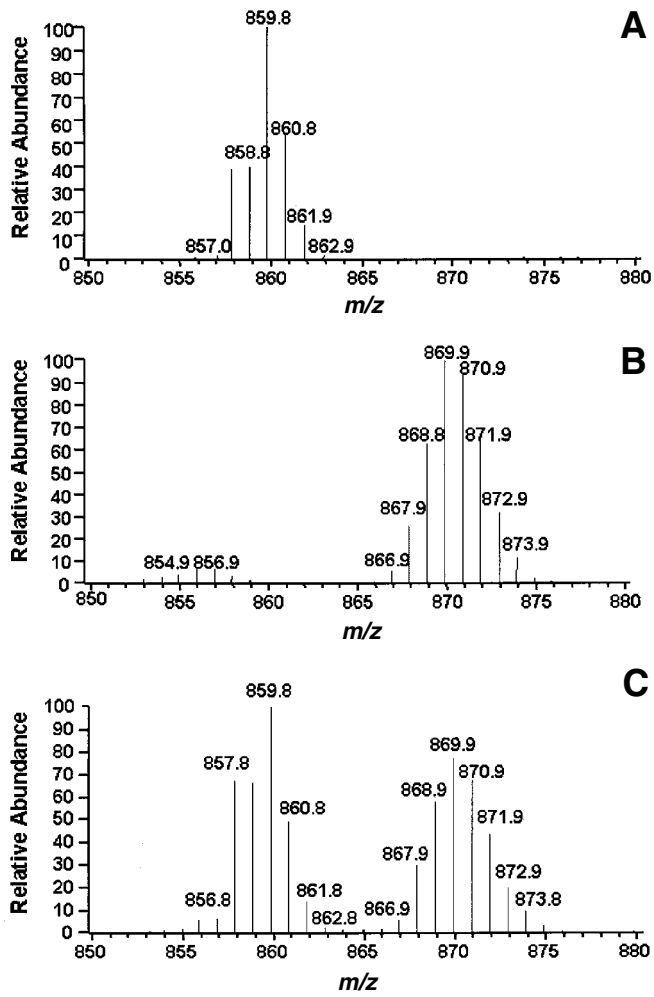



**FIG. 4.** Atmospheric pressure chemical ionization–mass spectrometry (APCI–MS) spectra of (A) $d_0$-dihydroxydimer ($[M + H]^+$ at *m/z* 860), (B) $d_{10}$-dihydroxydimer ($[M + H]^+$ at *m/z* 870), and (C) dihydroxydimer HPLC peak collected from the mixing experiment using $d_0$- and $d_{10}$-5,5′-dihydroxydimer. See Figure 1 for other abbreviation.

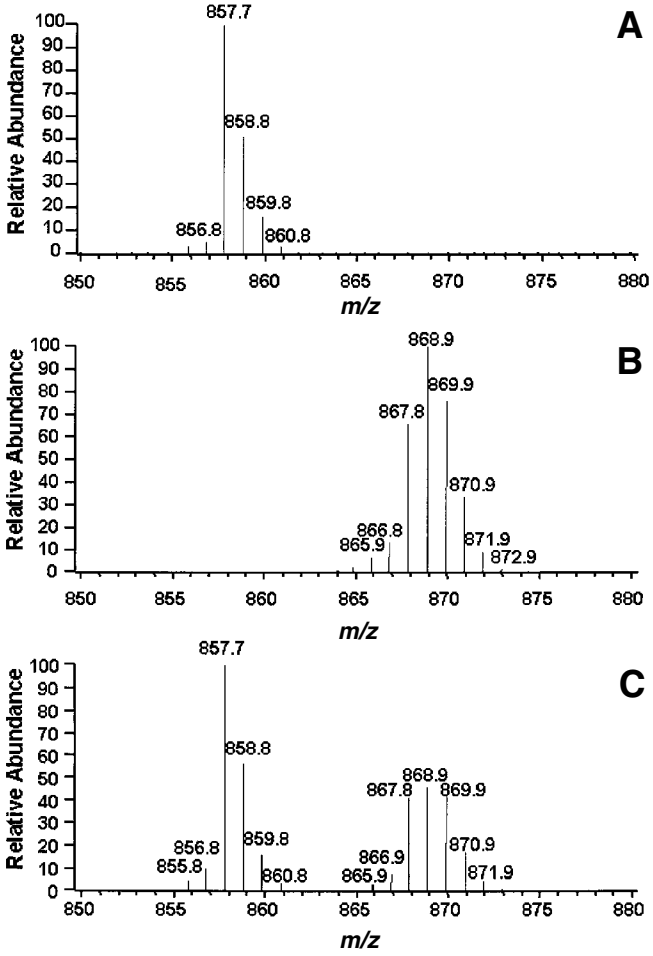



**FIG. 5.** APCI–MS spectra of (A) $d_0$-spirodimer ($[M + H]^+$ at *m/z* 858), (B) $d_{10}$-spirodimer ($[M + H]^+$ at *m/z* 868), and (C) spirodimer HPLC peak collected from the mixing experiment using $d_0$- and $d_{10}$-dihydroxydimer. See Figures 1 and 4 for abbreviations.

ently are formed in photooxidations, as indicated by the formation of trimer products. The UV-induced photooxidation of α-TH may play an important role in the photoprotective effects exerted by topically applied α-TH. DNA photodamage is markedly reduced by topically applied α-TH, although the photoprotective effect evidently occurs concomitantly with significant photochemical degradation of α-TH. The predominant products of α-TH photooxidation observed in this study are those detected on the surface of mouse skin after topical α-TH application and UV-irradiation *in vivo*. This suggests that α-TH dimers and trimers, which display UV spectra similar to α-TH, may contribute to photoprotective effects of topically applied vitamin E.

## ACKNOWLEDGMENTS

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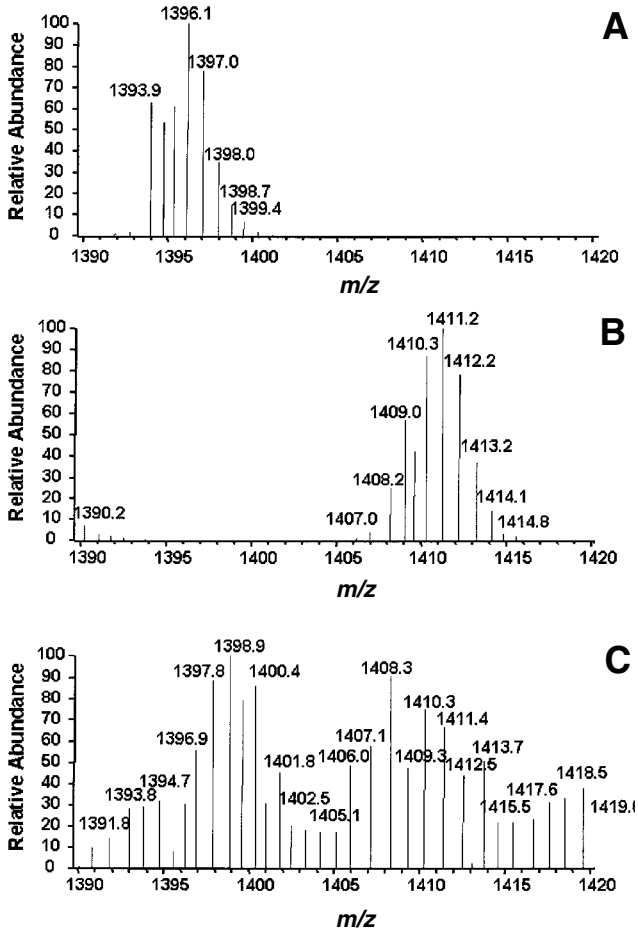


**FIG. 6.** Electrospray ionization–mass spectra of Ag-complexed (A) $d_0$-trimer ($[M]^+$ at *m/z* 1395, 1397), (B) $d_{15}$-trimer ($[M]^+$ at *m/z* 1410, 1412), and (C) trimer HPLC peak collected from the mixing experiment utilizing $d_0$- and $d_{10}$-spirodimer. See Figure 1 for other abbreviation.


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# Effects of Ascorbate on Membrane Phospholipids and Tocopherols of Intact Erythrocytes During Peroxidation by *t*-Butylhydroperoxide: Comparison with Effects of Dithiothreitol

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**ABSTRACT:** Peroxidation of intact human erythrocytes by *t*-butylhydroperoxide (tBHP)) was studied. By incubation of the erythrocytes with 1 mM tBHP, reduced glutathione (GSH) was exhausted within 1 min, and tocopherols (Toc) and phospholipids (PL) decreased to nearly their lowest levels (in this study) within 5 min. The rate of decrease of α-Toc was faster than that of γ-Toc, but α-Toc was never exhausted. The rates of decrease of Toc were faster than that of PL. Malondialdehyde increased slowly to reach a maximal value at 30 min. Methemoglobin (metHB) reached a maximum at 15 min. The maximal levels of these substances were maintained until 90 min incubation, which indicated that the peroxidation by tBHP had stopped spontaneously until at least 90 min. By the incubation with tBHP for 30 min, phosphatidylethanolamine (PE) and α-Toc decreased to about 70 and 30% of control levels, respectively, and γ-Toc and GSH were almost exhausted. Ascorbate (0.1 mM) afforded protection of 92% to PE, 50% to α-Toc, and 65% to γ-Toc against peroxidation, but ascorbate had no preventive effect at all on the formation of metHB and the decrease of GSH. These results may indicate that ascorbate-mediated protection of the membrane PL against the peroxidation depends primarily on Toc. On the other hand, dithiothreitol (DTT) (5 mM) almost completely prevented the formation of metHB, and DTT completely protected the PL and Toc against peroxidation, indicating the importance of sulfhydryl groups in erythrocytes.

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Peroxidation of cellular membrane lipids is implicated in the development of various diseases and in the process of aging (1,2). α-Tocopherol (α-Toc) may be the most important lipid-soluble antioxidant found in cellular membranes, whereas ascorbate (AsA) is the major water-soluble antioxidant in cells (3). Reportedly, AsA reduces tocopheroxyl radicals to α-Toc (3–8). However, whether AsA can regenerate α-Toc in living cells is still unsettled (9–15). Elucidation of the interaction between these two major vitamins in living cells seems to be important.

*tert*-Butylhydroperoxide (tBHP), an organic hydroperoxide, is known to induce oxidative damages to intact human erythrocytes (16–20). The susceptibility of erythrocytes to peroxidation is unique, because they have large amounts of hemoglobin. Hemoglobin may be oxidized to methemoglobin (metHB) by tBHP (16), and metHB itself may lead to peroxidative changes of the cell membranes (21–23). On the other hand, tBHP can cause peroxidative changes in the purified erythrocyte membranes (ghosts) without metHB (24,25). Therefore, molecular mechanisms of the peroxidation of erythrocyte membranes by tBHP may be complicated. Furthermore, it is unclear whether AsA can protect membrane Toc and phospholipids (PL) of intact erythrocytes against peroxidation by tBHP. One purpose of the current study was to investigate the time-dependent changes of tocopherols (Toc), PL, reduced glutathione (GSH), and hemoglobin during the peroxidation of intact human erythrocytes by tBHP. Another purpose was to investigate effects of AsA on membrane Toc and PL during peroxidation. GSH is implicated in antioxidative activities of erythrocytes (26) and is also related to reduction of dehydroascorbate to AsA in erythrocytes (8,27). Because dithiothreitol (DTT) is a protective agent for sulfhydryl groups in cells including GSH, effects of DTT on peroxidation were studied in parallel.

We recently reported a method employing high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection that can detect all of the major PL classes of erythrocyte membranes including phosphatidylserine (PS) by means of a single chromatographic elution (28,29). We applied the HPLC method to investigate changes of membrane PL in intact human erythrocytes; these changes are expressed as the ratio of each PL to sphingomyelin (SM). Furthermore, α-Toc, γ-tocopherol (γ-Toc), and cholesterol of the erythrocytes in the total lipid extraction were simultaneously measured by an

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Abbreviations: AsA, ascorbate; BHT, butylhydroxytoluene; DTNB, 5,5′-dithiobis(2-benzoic acid); DTT, dithiothreitol; ELSD, evaporative light-scattering detector; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; metHB, methemoglobin; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PS, phosphatidylserine; SM, sphingomyelin; tBHP, *tert*-butylhydroperoxide; TEA, triethylamine; α- and γ-Toc, α- and γ-tocopherol; UV, ultraviolet.

HPLC method, and cholesterol was regarded as an internal standard for HPLC (29). Combination of the methods is relatively simple, and they are sensitive to the peroxidative changes of intact erythrocytes.

## MATERIALS AND METHODS

*Materials*. L-Ascorbic acid, tBHP, DTT, and 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB) were obtained from Wako Pure Chemical Co. (Osaka, Japan). α-Toc and γ-Toc were obtained from Sigma Chemical Co. (Tokyo, Japan). Standard PL were purchased from Serdary Research Laboratory (Funakoshi Co., Tokyo, Japan). Other reagent-grade chemicals and HPLC-grade solvents were purchased from Wako Pure Chemical Co.

*Sample preparation.* Human venous blood from healthy adult volunteers who had fasted overnight was drawn into tubes containing EDTA-$Na_2$ and was processed immediately. Plasma and buffy coats were removed after centrifugation at 1000 × *g* for 10 min at 4°C, and erythrocytes were washed and centrifuged three times under the same conditions in cold phosphate-buffered saline (PBS), composed of 138 mM NaCl, 5 mM KCl, 6.1 mM $Na_2HPO_4$, 1.4 mM $NaH_2PO_4$, and 1 mM $MgCl_2$.

*Peroxidation of erythrocytes*. The washed erythrocytes were suspended in PBS to make a 10% hematocrit value. A cell counter (Sysmex F-800; Sysmex, Kobe, Japan) was used for the blood counts. The total volume of the reaction mixture was brought up to 4 mL by the addition of PBS. The final concentration of the erythrocytes was 5% hematocrit value. In some experiments, 1 mM tBHP, 0.1 mM AsA, 5 mM DTT, and 5 mM glucose were included. The incubations of different experimental conditions for the blood from each person were done in parallel. After pre-incubation for 5 min, the incubation at 37°C with shaking was started by addition of tBHP. Reactions were terminated by cooling in an ice bath, and then the erythrocytes were pelleted by centrifugation at 1000 × *g* for 6 min at 4°C.

*Extraction of total lipids from erythrocytes.* Extractions of total lipids including Toc from the packed erythrocytes were done immediately after the incubations. The extraction method was according to Burton *et al.* (30) with some modification. Briefly, the packed erythrocytes were hemolyzed by addition of 1 mL of 5 mM phosphate buffer (pH 7.4) containing 5 mM AsA, which was followed by addition of 1 mL of 80 mM sodium dodecyl sulfate. The solution was then sonicated for 30 s. Next, 2 mL of ethanol containing 1.2 mM butylhydroxytoluene (BHT) was added to the mixture, and the mixture was left at room temperature for 60 min. The 5 mM AsA was used to protect Toc during the extraction procedures (30,31). Next, 2 mL of *n*-hexane containing 1.2 mM BHT was added to the mixture, and it was vigorously mixed by a vortex mixer for 1 min (30 s × 2). After a brief centrifugation at 1000 × *g*, the *n*-hexane layer was transferred to another tube. The remaining aqueous layer was washed again with 2 mL of *n*-hexane. This hexane was combined with the first extract, and the combined hexane layer was dried under a stream of nitrogen gas.

*Determination of Toc and cholesterol*. Toc and cholesterol were measured simultaneously by an HPLC method (29) with minor modification. The dried hexane layer was dissolved in 300 μL of *n*-hexane/isopropanol (3:1, vol/vol), and after filtration through a 0.45 μm filter (Millipore Co., Tokyo, Japan) an aliquot of the solution (usually 5 μL) was injected into an HPLC system consisting of a Waters LC system (Waters Co., Tokyo, Japan) equipped with a four-solvent delivery system, a helium degassing unit, and an automatic injector. The system was connected in series to a fluorescence detector (FS-8020; Tosoh, Tokyo, Japan) and an evaporative light-scattering detector (ELSD) (SEDEX-55; Vitry sur Seine, France). The system was also connected to an interface (HP-35900E) and an HP-Vectra XM computer with HPLC ChemStation software (Hewlett-Packard, Tokyo, Japan). The column was a TSK-GEL ODS-80Ts, 250 × 4.6 mm (Tosoh) and the guard column was a Symmetry C18, 20 × 4 mm (Waters Co.). The mobile phase was methanol, and the flow rate was 1 mL/min at a 45°C column temperature. α-Toc and γ-Toc were detected with the fluorescence detector at 290 nm excitation and 330 nm emission (32), and cholesterol was detected with ELSD, which was set at 50°C for evaporation temperature and 2.4 bars for nebulization gas (compressed air).

*Separation of PL by HPLC*. The HPLC method for separation of the erythrocyte membrane PL was modified somewhat from our previous report (28,29). The column was a Wakosil 5 $NH_2$, 150 × 4.6 mm (Wako Pure Chemical Co.) and the guard column was a μ-Bondpak $NH_2$, 20 × 4 mm (Waters Co.). The mobile phase was composed of acetonitrile/methanol/0.25% triethylamine (TEA), pH 3.5 (68:21:11, by vol). The pH of the TEA solution was adjusted with phosphoric acid. HPLC was done by isocratic elution at 1 mL/min at a column temperature of 40°C. Each PL was detected with UV light at 205 nm. An aliquot (5 μL) of the lipid extract, which was used for the detection of Toc and cholesterol, was injected into the HPLC system.

*Determination of malondialdehyde (MDA) by HPLC*. The MDA concentration of the reaction mixtures was measured by the HPLC method reported previously (28). A fluorescence detector set at 515 nm for excitation and 553 nm for emission was used to detect MDA. 1,1,3,3-Tetramethoxypropane was used as standard for MDA.

*Determination of erythrocyte GSH concentration*. An aliquot of the reaction mixture (1.0 mL) was hemolyzed with 1 mL of water; and then 3 mL of metaphosphoric acid solution, which was composed of 1.67 g of metaphosphoric acid, 0.2 g of EDTA-$Na_2$, and 30 g of NaCl in 100 mL, was added. After filtration through a filter paper (No. 5B; Advantec, Tokyo, Japan), the filtrate (1 mL) was subjected to determination of GSH by using DTNB (33).

*Determination of metHB*. An aliquot (0.4 mL) of the reaction mixture was hemolyzed with 5 mL of a solution containing 100 mM phosphate buffer, pH 6.8, and 1% Triton X-100 (4:6, vol/vol) and then divided into two parts. Absorbances of the first part were read at 630 nm in the absence and then the presence of potassium cyanide. Absorbances of the second part were read at 630 nm in the presence of potassium ferricyanide and then at 630 nm after addition of potassium cyanide (34).

*Statistical analysis*. Statistical analyses of the differences of mean values compared to the control values using the *t*-test were performed with Microsoft Excel 2000 on Windows 98 (Microsoft, Tokyo, Japan). Differences with a $P$ value < 0.05 were considered statistically significant.

## RESULTS

*Changes of the membrane Toc*. α-Toc, γ-Toc, and cholesterol from the erythrocytes were clearly separated by the HPLC and were identified by the retention times for each standard substance (Fig. 1). Calibration curves of the Toc were linear up to at least 20 ng/30 μL, and that of cholesterol was linear up to at least 3 μg/30 μL (data not shown). The detection limit of α-Toc in the present system was less than 1 ng/30 μL, and that of cholesterol was less than 0.1 μg/30 μL. Because peroxidation of the erythrocyte membranes usually spares SM (28,35), the ratio of cholesterol to SM in each reaction mixture was calculated. No significant differences for the ratios were noted between the control incubations and the incubations with tBHP. The results of the present study indicated that no significant oxidation of cholesterol by tBHP occurred. Therefore, cholesterol was regarded as an internal standard for chromatography, and changes in Toc are expressed as the ratios of Toc to cholesterol (Figs. 2,3).

In the presence of 1 mM tBHP, α-Toc decreased rapidly to about 26% of the control levels at 3 min (Fig. 2A), and thereafter α-Toc remained at almost the same level for 90 min (Fig. 2B). γ-Toc decreased to about 10% of the control levels at 4 min (Fig. 2B) and then deceased gradually to almost exhaus-

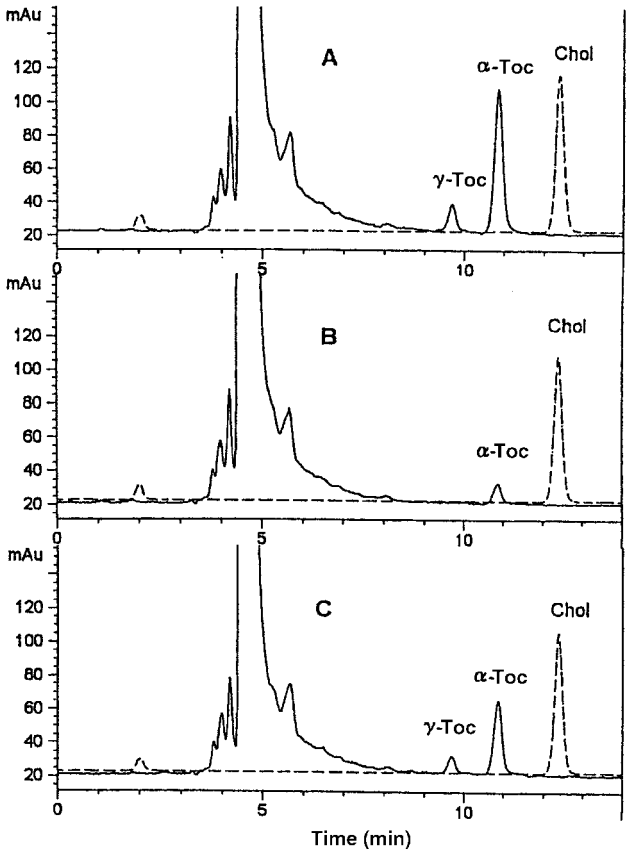


**FIG. 1.** Typical high-performance liquid chromatograms of tocopherols and cholesterol. Tocopherols were detected with a fluorescence detector (—), and cholesterol was detected with an evaporative light scattering detector (----). The column was TSK-GEL 80Ts, 250 × 4.6 mm (Tosoh, Tokyo, Japan). The mobile phase was methanol, and the flow rate was 1 mL/min at a column temperature of 45°C. In each chromatogram, the same amount of erythrocytes was incubated at 37°C for 30 min. (A) Control incubation; (B) incubation with 1 mM *tert*-butylhydroperoxide (tBHP); (C) incubation with both 1 mM tBHP and 0.1 mM ascorbate (AsA). Abbreviations: α-Toc,α-tocopherol; γ-Toc,γ-tocopherol; Chol, cholesterol; mAu, milli-absorbance units.

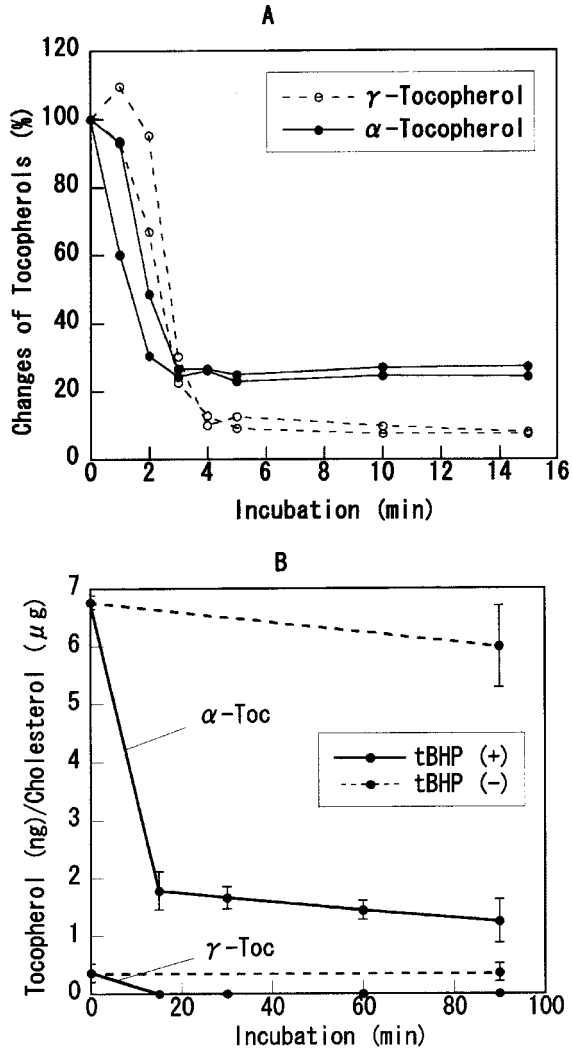


**FIG. 2.** Time courses for the changes of Toc after incubation with tBHP. (A) α-Toc and γ-Toc decreased to almost their lowest levels at 3 and 4 min, respectively, and (B) these minimal levels were maintained at least until 90 min. In (A) each data point represents the mean of two experiments using the same erythrocytes, the results from two different erythrocytes are shown. In (B), the mean ± SD of three experiments using same erythrocytes are shown. Abbreviations: tBHP (+), incubated with tBHP; tBHP (–), incubated without tBHP. For other abbreviations see Figure 1.

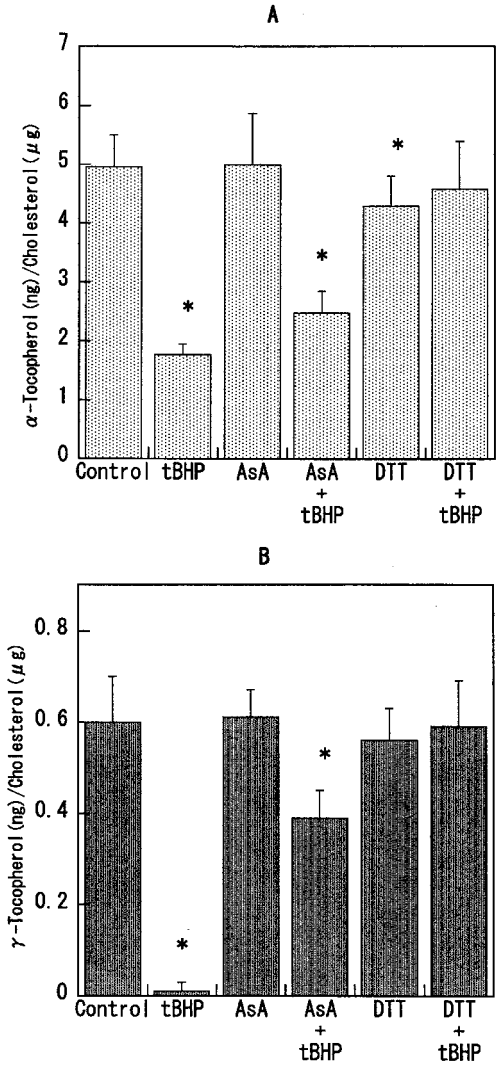


**FIG. 3.** Effects of tBHP, AsA (0.1 mM), and dithiothreitol (DTT) (5 mM) on the contents of α-Toc (A) and on γ-Toc (B) of the erythrocytes after incubation for 30 min with tBHP. The results are the mean ± SD of eight experiments of four different erythrocytes. Abbreviations: tBHP, incubation with 1 mM tBHP; AsA, incubation with 0.1 mM ascorbic acid; AsA + tBHP, incubation with both 0.1 mM AsA and 1 mM tBHP; DTT, incubation with DTT; DTT + tBHP, incubation with both tBHP and DTT; *, difference is statistically significant compared to control ($P < 0.05$).

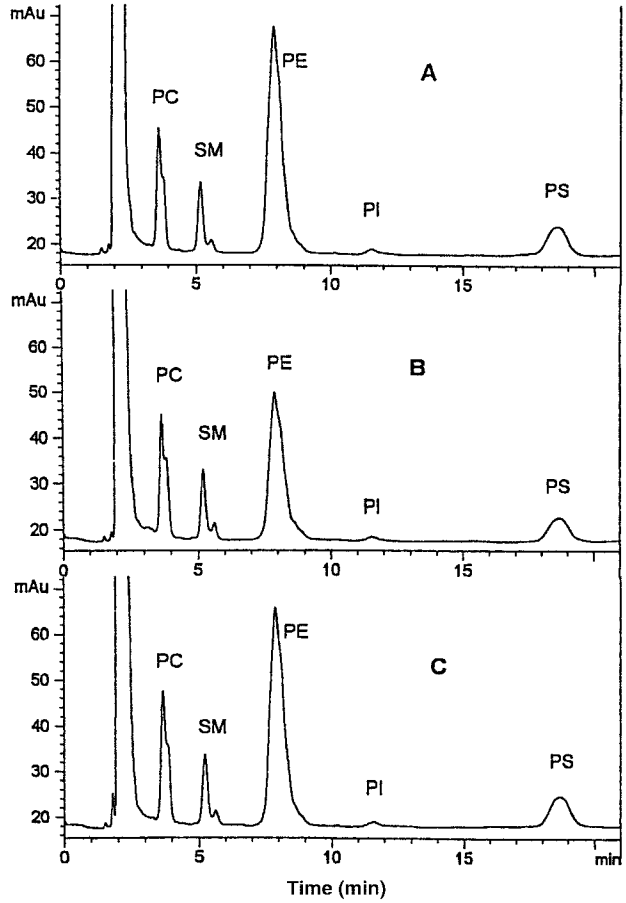


**FIG. 4.** High-performance liquid chromatograms of the erythrocyte membrane phospholipids. The column was a Wakosil 5 $NH_2$, 150 × 4.6 mm (Wako Pure Chemical Co., Osaka, Japan) at 40°C. The mobile phase was acetonitrile/methanol/0.25% triethylamine, pH 3.5 (68:21:11, by vol) delivered isocratically at 1 mL/min. Phospholipids were detected with ultraviolet at 205 nm. An aliquot of the same extraction used in Figure 1 was injected into the high-performance liquid chromatography (HPLC) system. (A) Control incubation; (B) incubation with 1 mM tBHP; (C) incubation with both 1 mM tBHP and 0.1 mM AsA. Abbreviations: PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; for other abbreviations see Figure 1.

tion (Fig. 2B). When 0.1 mM AsA was included together with tBHP in the reaction mixture, α-Toc and γ-Toc were preserved at about 50 and 65 of the control levels respectively, after 30 min incubation, (Fig. 3A,3B). Incubation with DTT alone for 30 min showed a significant decrease of α-Toc ($P < 0.02$), but incubation with both tBHP and DTT showed no significant differences in α-Toc or γ-Toc levels from the control levels (Fig. 3). The addition of 5 mM glucose in the reaction mixtures from the start of the incubation did not show any significant differences from those without the addition of glucose (data not shown).

*Changes of the membrane PL.* Major PL classes of human erythrocytes were separated within 20 min of the initiation of the experiment, and the PL classes were identified by the retention times of each standard PL (Fig. 4). The concentration of each PL is expressed as its ratio to SM (Figs. 5,6). After the incubation of the intact erythrocytes with tBHP, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and PS decreased to almost their lowest levels within the first 5 min, and then decreased more slowly until 15 min (Fig. 5). Thereafter, the maximal change of each PL was maintained until 90 min (Fig. 5). The degrees of decrease of PC, PE, and PS at 30 min incubation were about 90, 68, and 76% of each control level, respectively (Fig. 6). The incubation with AsA or DTT for 30 min showed no difference in PL from the control incubation (Fig. 6). The incubation with both AsA and tBHP for 30 min resulted in about an 8% decrease in PE ($P < 0.04$) without decreases of PC and PS (Fig. 6). The incubations with both DTT

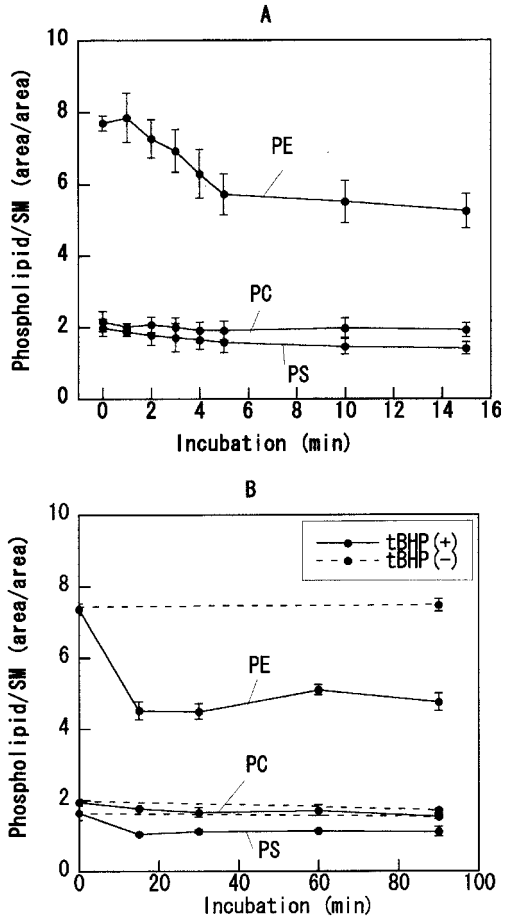


**FIG. 5.** Time courses of the changes in phospholipid levels after incubation with tBHP. (A) Phospholipids decreased rapidly to an almost minimal level at 5 min, and (B) the minimal levels were maintained until 90 min. Data points are the means ± SD of four experiments of two different erythrocytes (A) and of three experiments using the same erythrocytes (B). See Figures 1 and 4 for abbreviations.

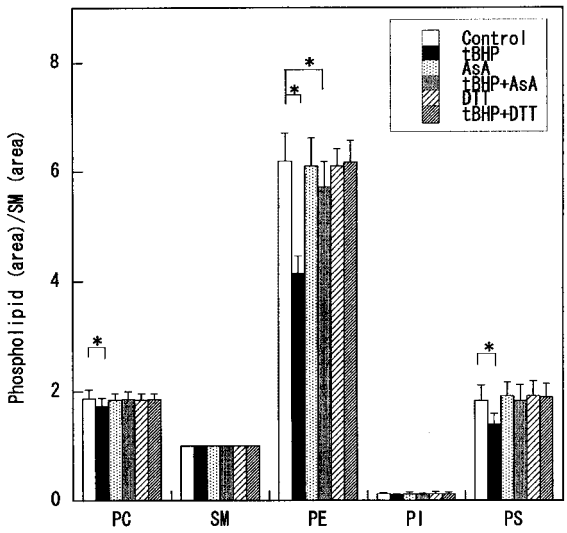


**FIG. 6.** Effects of tBHP, AsA (0.1 mM), and DTT (5 mM) on the phospholipid classes of erythrocyte membranes after incubation with tBHP for 30 min. Data points represent the means ± SD of eight experiments of four different erythrocytes. See Figures 1, 3, and 4 for abbreviations. *, difference is statistically significant compared to each control ($P < 0.05$).

and tBHP for 30 min showed no significant changes of PL (Fig. 6). The addition of 5 mM glucose to the reaction mixtures did not cause any significant differences from controls.

*Increase of MDA*. The MDA concentration in the supernatant from the control incubation was 30 ± 10 nM. Upon incubation with tBHP, MDA did not increase significantly until 3 min and then increased almost exponentially until 15 min (Fig. 7). MDA increased until 30 min upon incubation with tBHP (2,060 ± 330 nM) and thereafter MDA had almost the same value until 90 min (Fig. 7). The incubation with both AsA and tBHP for 30 min showed an increase of MDA (360 ± 80 nM), but the incubation with both DTT and tBHP showed no increase of MDA (40 ± 10 nM) (Fig. 8).

*Changes of GSH concentration*. The incubation of the erythrocytes with tBHP resulted in exhaustion of GSH within 1 min, and thereafter it was slightly recovered and maintained at the low levels until at least 60 min (Fig. 9). The incubation with both tBHP and AsA for 30 min did not show any differences from the incubation with tBHP only (Fig. 10). Because DTT

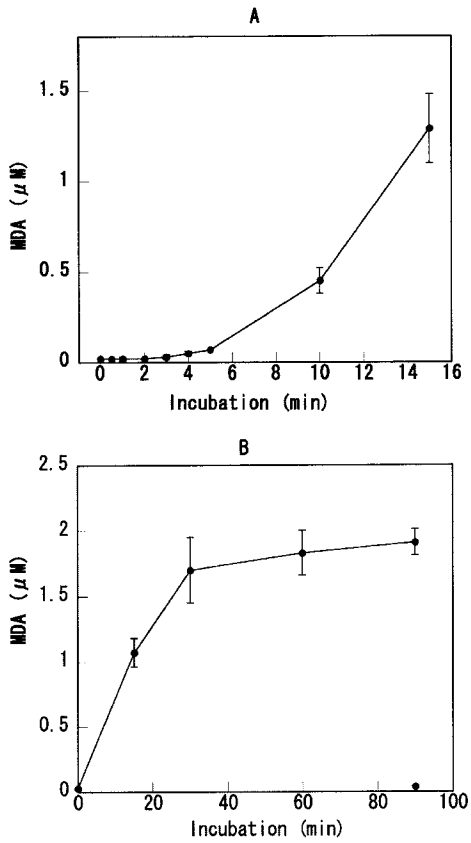


**FIG. 7.** Increase of malondialdehyde (MDA) in the supernatant of the reaction mixture after incubation with tBHP. Data are the means ± SD of four experiments (A) of two different erythrocytes and (B) of three experiments of same erythrocytes. In (B) the lower of the two MDA values at 90 min represents the value for the control incubation. For other abbreviation see Figure 1.

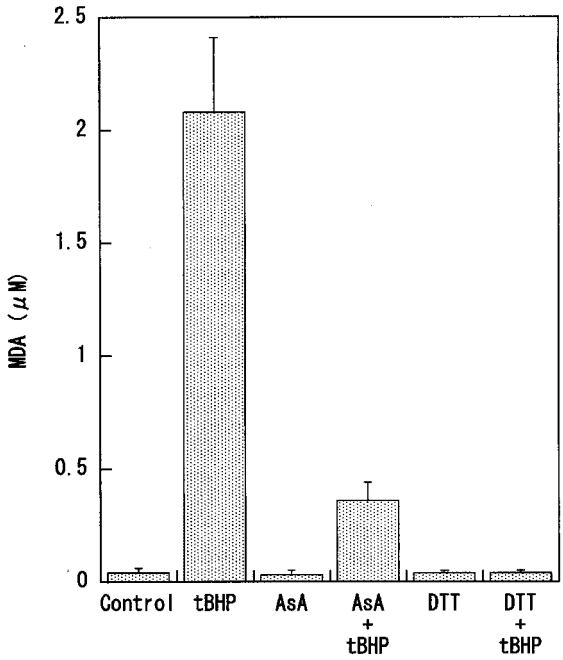


**FIG. 8.** Effects of tBHP, AsA (0.1 mM), and DTT (5 mM) on production of malondialdehyde (MDA) in the supernatant of the reaction mixture after the incubation with tBHP for 30 min. Data are the means ± SD of four experiments with two different erythrocytes. See Figures 1, 3, and 7 for abbreviations.

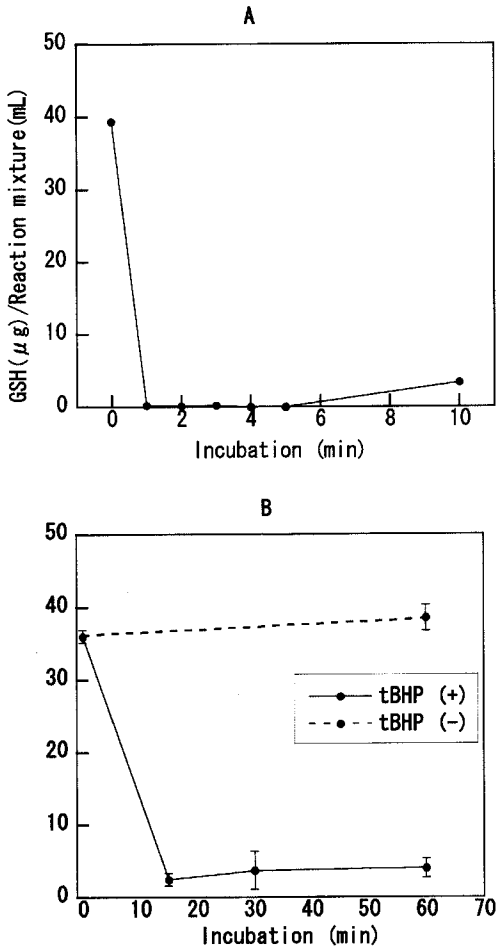


**FIG. 9.** Change of erythrocyte reduced glutathione (GSH) after the incubation with tBHP. Each value is the mean of the two experiments (A) and is the mean ± SD of three experiments (B). See Figure 1 for other abbreviation.

may react with DTNB, GSH level in the presence of DTT was not determined.

*MetHB*. After the incubations with 1 mM tBHP at 37°C, metHB increased almost linearly until 15 min, and then metHB stayed constant until at least 60 min (Fig. 11). AsA did not prevent at all the formation metHB by tBHP (Fig. 12), but the presence of 5 mM DTT together with tBHP prevented almost completely the formation of metHB at least until 30 min (Fig. 12). None of the supernatants of the reaction mixtures after the incubations of the erythrocytes showed coloration by monitoring at 540 nm, which indicated that no hemolysis was occurred in any conditions used in the present study.

## DISCUSSION

In this study, Toc and cholesterol in the total lipid extracts were measured simultaneously by an HPLC method, and the cholesterol was regarded as a kind of internal standard for the HPLC. Actually, the changes in the cholesterol levels on the chromatograms appeared to be negligible when compared to the changes of Toc (Fig. 1). Therefore, the changes of Toc are expressed on the basis of cholesterol (Figs. 2,3). Thus, variability of Toc values on the basis of blood counts after the long process of determination by the HPLC is diminished, and no blood counts of the samples are needed. We recently confirmed, by using human erythrocyte membranes, that the detection of membrane PL with UV depends primarily on unsaturated fatty acids (28,36) and that primary targets of peroxidation of the membrane PL are polyunsaturated fatty acids (28,35). Because SM of the human erythrocytes contains very small amounts of polyunsaturated fatty acids (28,37,38), peroxidation of the membrane PL usually spares SM (28). Therefore, the changes in the ratios of PC, PE, and PS to SM reflect changes in polyunsaturated fatty acid contents of these PL (Figs. 5,6).

After the incubation of the erythrocytes with 1 mM tBHP, GSH, Toc, and PL decreased almost maximally within a few minutes (Figs. 2,5,9). GSH was exhausted within 1 min, α-Toc decreased to about 30% of the control level at 3 min, and γ-Toc decreased to about 10% of the control level at 4 min. PE decreased to about 70% of the control at 5 min. However, MDA in the supernatant of the reaction mixture appeared slowly and increased until 30 min (Fig. 7). MetHB was formed linearly and reached a maximum at 15 min (Fig. 11). These results show that the peroxidative changes of the cell membranes of intact erythrocytes by 1 mM tBHP occur very rapidly and that the speeds and the degrees of decreases by tBHP are different among α-Toc, γ-Toc, and PE. The decrease of α-Toc is faster than that of γ-Toc, and the decreases of the Toc are faster than PE. The large discrepancy of time between the actual decreases

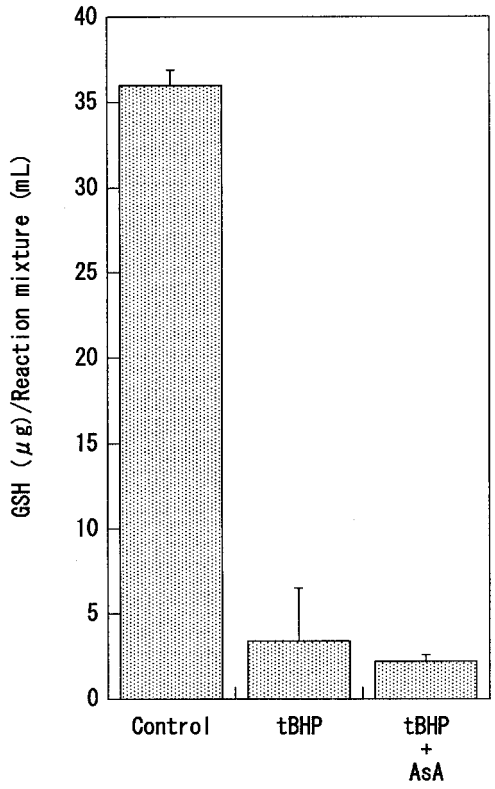


**FIG. 10.** Effects of tBHP and AsA (0.1 mM) on GSH of the intact erythrocytes after incubation for 30 min. GSH contents after the incubation with DTT were not determined because 5,5′-dithiobis(2-benzoic acid) may react with DTT. The data are the means ± SD of three experiments. See Figures 1, 3, and 9 for abbreviations.

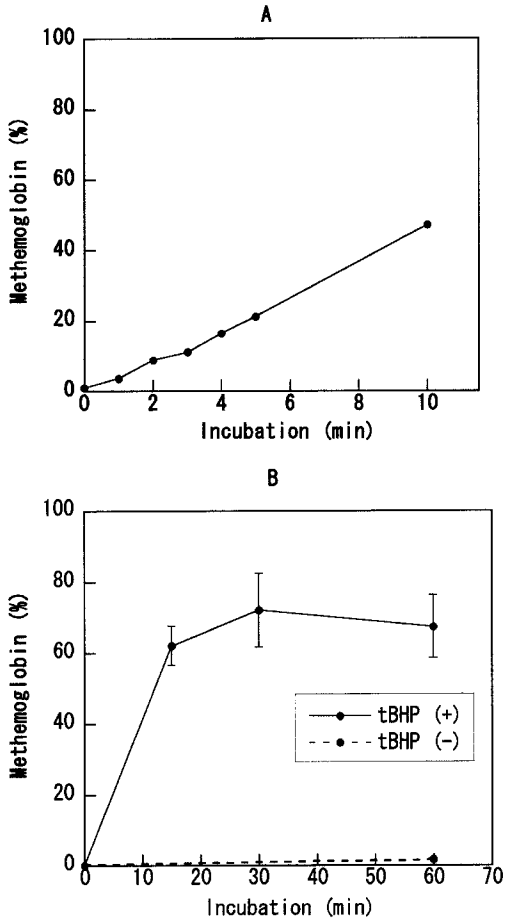


**FIG. 11.** Time course of the formation of methemoglobin (metHB) after the incubation with tBHP at 37°C. Values are mean of the two experiments (A) and are the means ± SD of three experiments (B). The erythrocytes for (A) and (B) were from different persons. See Figure 2 for abbreviation.

of PL and the increase of MDA may also be noteworthy. The results may indicate that the peroxidative changes of the cell membranes precede the formation of metHB.

After rapid initial changes, α-Toc, PL, and MDA showed almost the same levels until at least 90 min (Figs. 2,5,7). metHB was also at a nearly constant level until at least 60 min after reaching the maximal increase at 15 min (Fig. 11). The results indicated that propagation of the peroxidative changes was stopped spontaneously and was prevented spontaneously for a long period. These phenomena seem to be very important for understanding the molecular mechanisms of peroxidation of intact erythrocytes. GSH was exhausted within 1 min (Fig. 9), but α-Toc was never exhausted (Fig. 2). GSH recovered slightly after exhaustion (Fig. 9), but α-Toc had not significantly changed at 90 min (Fig. 2). However, exact mechanisms for the spontaneous cessation of the propagation of peroxidation over a long period remain to be elucidated.

When 0.1 mM AsA was included in the reaction mixture with tBHP, 92% of PE, 50% of α-Toc, and 65% of γ-Toc were preserved at the end of 30 min of incubation (Figs. 3,6). However, AsA did not prevent the formation of metHB and the decrease of GSH (Figs. 10,12). These results show that 0.1 mM AsA can protect the membrane PL and Toc of the intact erythrocytes against peroxidation by tBHP despite the formation of metHB and the decrease of GSH. In the presence of both AsA and tBHP, the decrease of PE was only 8% from the control level at 30 min of incubation, but the decrease of α-Toc was about 50% of the control. The results may support the notion that AsA-mediated protection of the membrane lipids depends primarily on α-Toc even in intact erythrocytes. Reportedly, the rate of entry of AsA into erythrocytes is more than 10 times slower than that of dehydroascorbate (39). The present study clearly shows that exogenously added AsA can protect and spare the membrane Toc of the cell, but it may not necessarily indicate that AsA regenerates tocopheroxyl radicals to Toc. Perhaps AsA merely spared the Toc by reacting directly with tBHP outside the cells.

γ-Toc showed more remarkable changes than α-Toc in both the decrease by peroxidation and the preservation by AsA; γ-Toc was almost exhausted by tBHP and was preserved at 65% of the control level by AsA, whereas α-Toc decreased to about 30% of the control and was preserved at 50% of the control (Fig. 3). In our previous study (29) γ-Toc of the purified membranes was exhausted by the incubation with lipoxygenase for 45 min. After this, further incubation of the mem-

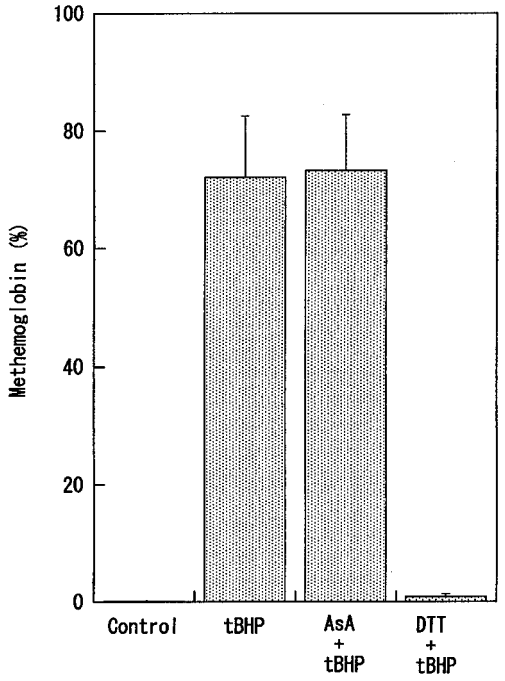


**FIG. 12.** Effects of tBHP, AsA (0.1 mM), and DTT (5 mM) on the formation of metHB after incubation for 30 min at 37°C. The results are the means ± SD of three experiments. See Figures 1, 3, and 11 for abbreviations.

branes with AsA resulted in partial recovery of γ-Toc. Therefore, it is probable that exogenously added AsA can regenerate the membrane Toc of intact erythrocytes within 30 min. The concentration of AsA in human plasma is reported to be 0.03–0.15 mM (3). Therefore, the 0.1 mM AsA used in the present study can be considered as physiological level.

The incubation of erythrocytes with 0.5 mM tBHP caused inhibition of Ca + Mg ATPase, and 20 mM AsA significantly decreased the inhibition of the ATPase (19). However, 20 mM ascorbic acid is far more than physiological concentration of human plasma (3). Dise and Goodman (17) reported that the incorporation of tritium-labeled oleic acid into PE of the erythrocyte membranes was enhanced, whereas incorporation of the oleic acid into PC decreased, in the presence of 1 mM tBHP and that the addition of 0.5 mM AsA in the reaction mixture with tBHP abolished the enhanced incorporation of the oleic acid into PE but not the decreased incorporation of the oleic acid into PC. Our results, in which PE decreased more than PC in the presence of 1 mM tBHP and membrane PL were preserved in the presence of AsA, may be related to the observation of Dise and Goodman (17).

DTT protected Toc and PL completely from peroxidation by tBHP (Figs. 3,6), and DTT also almost completely prevented the formation of metHB by tBHP (Fig. 12). The results show that DTT protects the membrane PL and Toc against peroxidation by tBHP in a different way from AsA. Because normal erythrocytes contain large amounts of GSH, the effects of DTT observed in the present study may largely reflect the effects of GSH (20,39–42). Reportedly, 1 mM DTT was completely effective in preventing tBHP-induced inhibition of the Ca-pump ATPase (20). Furthermore, the ability of DTT to protect the ATPase was abolished when glutathione peroxidase was inhibited by an inhibitor, which indicates that the protection of ATPase by DTT is due to protection and/or regeneration of GSH (20). The effects of DTT on the membrane PL and Toc presented in this study seem to be in agreement with these observations. On the other hand, reduction of metHB in normal erythrocytes depends primarily on NADH-cytochrome $b_5$ reductase, which is not directly related to GSH (43,44). Therefore, no single mechanism likely accounts for the complete prevention of metHB formation by DTT. The present results confirmed the importance of sulfhydryl groups of the erythrocytes on protection against the peroxidation.

After the incubation of the erythrocytes with DTT (without tBHP), a small but significant ($P < 0.02$) decrease of α-Toc and a tendency of γ-Toc to decrease were observed (Fig. 3). The results may indicate that DTT generated a superoxide in the erythrocytes *via* its reaction with GSH (45).

Finally, the procedure for combining the two HPLC methodologies for Toc and PL used in this study is relatively simple and is sensitive to the peroxidative changes of these substances in the cell membranes.

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# Cacao Liquor Polyphenols Reduce Oxidative Stress Without Maintaining α-Tocopherol Levels in Rats Fed a Vitamin E-Deficient Diet

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**ABSTRACT:** The effect of crude polyphenols (CLP) from cacao liquor on vitamin E-deficient rats was examined. The CLP fraction contained 49.8% antioxidative polyphenols such as catechins and their oligomers. Supplementation of the vitamin E-deficient diet with CLP for 7 wk did not prevent the decrease in α-tocopherol levels in the liver, kidney, heart, brain, and plasma. The lipid peroxide levels in these tissues increased in the group fed the vitamin E-deficient diet compared with the control group. However, these changes were inhibited in a dose-dependent manner as a result of supplementation of the vitamin E-deficient diet with 0.25, 0.5, or 1.0% CLP. The lipid peroxide levels in plasma increased in the group fed the vitamin E-deficient diet. This change tended to be suppressed as a result of supplementation of the diet with CLP, but the difference was not significant. There was no evidence of absorption and distribution of CLP to the tissues; however, CLP intake resulted in a decrease in oxidative stress without maintaining vitamin E levels in the plasma and the tissues.

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A protective role of plant polyphenols against atherosclerosis has been suggested by several studies. According to the results of epidemiological studies, plant polyphenol consumption is associated with a reduced risk of coronary heart disease (1–3). These studies suggested that such health benefits of polyphenols are dependent on their antioxidative activity. It has been reported that plant polyphenols reduce oxidative stress *in vivo*, as demonstrated using experimental animal models. Sesame seed (4)-, green tea (5)-, and grape (6)-derived polyphenols have been shown to decrease oxidative stress induced by vitamin E (VE) deficiency in rats.

Cacao beans, the seed of *Theobroma cacao*, are known to contain various polyphenolic substances (7–9). It has been reported that cacao liquor, one of the main ingredients of cocoa and chocolate, prepared by fermentation of raw beans, is rich in polyphenols in spite of the processes involved in its manufacture. Epicatechin (EC), catechin, and its oligomers as procyanidins are confirmed to be the major antioxidative components of cacao liquor (10–12).

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Abbreviations: CLP, cacao liquor crude polyphenols; EC, epicatechin; PMC, 2,2,5,7,8-pentamethyl-6-chromarol; TBARS, thiobarbituric acid-reactive substances; VE, vitamin E.

In the present study, we examined the effect of polyphenolic antioxidants from cacao liquor on VE-deficient rats as an experimental model of oxidative stress.

## EXPERIMENTAL PROCEDURES

*Animals and diets.* Three-week-old male Sprague-Dawley rats were obtained from Clea Japan (Tokyo, Japan). The rats were housed individually and kept in a room maintained at 23–25°C and with controlled lighting in a 12-h light/dark cycle. The animals were fed a basal diet (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) for 4 d. Forty rats were divided into five equal groups and assigned one of five diets shown in Table 1. The five experimental diets were a normal diet (control), a VE-deficient diet (VE-deficient), and VE-deficient diet containing 0.25, 0.5, or 1.0 % cacao liquor crude polyphenol (CLP; 0.25, 0.5, 1.0%). The rats were fed the assigned diet for 7 wk. The normal diet was the reformulated AIN-76 diet, containing 59.9 mg/kg α-tocopherol. The VE-deficient diet was prepared using VE-free corn oil and VE-free vitamin mixture. The VE-free corn oil was made from commercial oil using activated charcoal and silica gel. The VE-free vitamin mixture was the same as AIN-76 vitamin mixture except that it lacked VE acetate. The α-tocopherol concentrations in the corn oil and the VE-free corn oil were analyzed by high-performance liquid chromatography (HPLC) by the method of Ueda and Igarashi (13). The VE-deficient diet contained less than 6.0 μg/kg α-tocopherol. The present study was approved by the Animal Committee of Meiji Seika Functional Foods R&D Laboratories, and the animals received humane care under the guidelines laid down by this committee.

CLP was prepared from cacao liquor by the method described in a previous report (10). Briefly, cacao liquor was defatted with *n*-hexane and extracted with 80% ethanol. The extract was applied to a Diaion HP2MG column (Mitsubishikasei Co., Ltd., Tokyo, Japan), and the column was washed with 20% vol/vol ethanol to remove contaminants including xanthine derivatives. The fraction eluted with 80% vol/vol ethanol was collected as CLP and freeze-dried. The concentration of total polyphenols in the CLP fraction was about 50% as determined by the Prussian blue method using EC as the standard (14). The catechins and procyanidins in CLP measured by the HPLC method of Ricardo da Silva *et al*. (15)

**TABLE 1**
**Composition of the Experimental Diets**

| | Groups | | | | |
|---|---|---|---|---|---|
| (%) | Control | VE-deficient | CLP 0.25% | CLP 0.5% | CLP 1.0% |
| Corn starch | 41.5 | 41.5 | 41.5 | 41.5 | 41.5 |
| Casein | 25.0 | 25.0 | 25.0 | 25.0 | 25.0 |
| α-Potato starch | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| Cellulose powder | 8.0 | 8.0 | 7.75 | 7.5 | 7.0 |
| Corn oil | 6.0 | 0 | 0 | 0 | 0 |
| VE-free corn oil[a] | 0 | 6.0 | 6.0 | 6.0 | 6.0 |
| Mineral mixture[b] | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Sucrose | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Vitamin mixture[c] | 1.0 | 0 | 0 | 0 | 0 |
| VE-free vitamin mixture[d] | 0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CLP | 0 | 0 | 0.25 | 0.5 | 1.0 |

[a]Vitamin E (VE)-free corn oil was prepared from corn oil treated with activated charcoal and silica gel.
[b]Mineral mixture was AIN-76 mixture obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).
[c]Vitamin mixture was AIN-76 mixture including choline bitartrate obtained from Oriental Yeast Co., Ltd.
[d]VE-free vitamin mixture was the same as the AIN-76 mixture except that it lacked VE. Abbreviation: CLP, cacao liquor crude polyphenols.

with slight modification were as follows: total polyphenol, 49.5%; catechin, 0.46%; EC, 1.83%; procyanidin B2, 1.69%; procyanidin C1, 2.37%; and cinnamtannin A2, 2.01%.

*Study design.* The animals were fed the assigned diet for 7 wk. Blood was taken from the tail vein before (0 time) and 2, 4, 6, and 7 wk after start of feeding, and the plasma α-tocopherol level was monitored. Lipid peroxides in plasma, assayed as thiobarbituric acid-reactive substances (TBARS), were measured 4 and 7 wk after the start of feeding. After 7 wk of feeding the assigned diet, the animals were anesthetized with ethyl ether, and blood samples were taken from the abdominal vein using heparinized needles and syringes. The tissues of the animals (liver, kidney, heart, and brain) were removed and homogenized in 1.15% KCl solution. An erythrocyte hemolysis test was conducted immediately after collection of the blood samples. Plasma and tissue homogenates were kept at −80°C until analysis.

*Analysis.* The oxidative hemolysis of erythrocytes was measured using dialuric acid (Tokyo Kasei Co., Ltd., Tokyo, Japan) (16). The α-tocopherol concentrations in the plasma and tissues were measured using a high-performance liquid chromatograph equipped with a fluorometric detector, with 2,2,5,7,8,-pentamethyl-6-chromarol (PMC; for biochemistry and analysis; Eisai Co., Ltd., Tokyo, Japan) as the internal standard (13). Lipid peroxides in plasma were measured as TBARS by a fluorometric method using LPO-test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Liver, kidney, heart, and brain TBARS were measured by the method of Ohkawa *et al.* (17), using tetraethoxypropane (Wako Pure Chemical Industries, Ltd.) as the standard. Protein concentrations in tissue homogenates were determined by the method of Lowry *et al.* (18). Levels of EC and its metabolites in plasma were determined by HPLC (19). Glucuronide, sulfate and glucuronide-sulfate conjugates of EC in plasma were hydrolyzed to nonconjugated EC by treatment with sulfatase type H-5 (Sigma, St. Louis, MO). The sample pretreated with enzymes was analyzed by HPLC.

*Statistical analysis.* The data were expressed as the mean ± SD. Analyses were performed using SPSS statistical software (SPSS Inc., Chicago, IL). When analysis of variance revealed $P < 0.05$, the data were further analyzed using Scheffe's multiple range test. Differences were considered statistically significant at $P < 0.05$.

## RESULTS

In this study, food intake, body weight gain, and food efficiency were equal in all of the experimental groups, as shown in Table 2. The plasma α-tocopherol concentrations in the groups fed the VE-deficient diet or the 0.25, 0.5, and 1.0% CLP-containing VE-deficient diet decreased markedly in

**TABLE 2**
**Total Food Intake, Body Weight Gain, and Food Efficiency[a]**

| Groups | Total food intake (g) | Body weight gain (g) | Food efficiency (%) |
|---|---|---|---|
| Control | 846.3 ± 15.7 | 285.2 ± 12.3 | 0.34 ± 0.02 |
| VE-deficient | 838.8 ± 19.0 | 277.4 ± 13.0 | 0.33 ± 0.01 |
| CLP 0.25% | 840.6 ± 21.1 | 278.1 ± 10.7 | 0.33 ± 0.02 |
| CLP 0.5% | 836.6 ± 24.9 | 281.6 ± 8.3 | 0.34 ± 0.01 |
| CLP 1.0% | 813.4 ± 32.8 | 278.1 ± 18.9 | 0.34 ± 0.02 |

[a]Values are means ± SD. For abbreviations see Table 1.

**TABLE 3**
**Plasma α-Tocopherol Concentrations in Rats Fed the Experimental Diets[a]**

| | α-Tocopherol concentration (μg/mL) | | | | |
|---|---|---|---|---|---|
| Groups | 0 wk | 2 wk | 4 wk | 6 wk | 7 wk |
| Control | 10.38 ± 1.67 | 8.85 ± 3.10 a | 13.38 ± 1.45 a | 11.59 ± 0.73 a | 10.86 ± 2.02 s |
| VE-deficient | | 2.28 ± 0.41 b | 1.40 ± 0.15 b | 0.86 ± 0.28 b | 1.00 ± 0.26 b |
| CLP 0.25% | | 2.58 ± 0.35 b | 1.93 ± 0.84 b | 0.76 ± 0.24 b | 0.93 ± 0.44 b |
| CLP 0.5% | | 2.36 ± 0.59 b | 1.32 ± 0.29 b | 0.96 ± 0.19 b | 0.80 ± 0.22 b |
| CLP 1.0% | | 2.35 ± 0.45 b | 1.61 ± 0.59 b | 0.73 ± 0.17 b | 0.53 ± 0.16 b |

[a]Values are means ± SD. Values in the same column not sharing the same letters are significantly different, $P < 0.05$. See Table 1 for abbreviations.

**TABLE 4**
**α-Tocopherol Concentrations of Various Tissues in Rats Fed Experimental Diets[a]**

| | α-Tocopherol concentration (μg/g) | | | |
|---|---|---|---|---|
| Groups | Liver | Kidney | Heart | Brain |
| Control | 65.56 ± 16.59 a | 58.77 ± 14.98 a | 88.67 ± 13.09 a | 28.48 ± 6.64 a |
| VE-deficient | 9.45 ± 5.16 b | 11.11 ± 4.88 b | 6.53 ± 3.00 b | 13.34 ± 3.05 b |
| CLP 0.25% | 17.29 ± 11.29 b | 13.03 ± 5.55 b | 10.05 ± 4.16 b | 13.19 ± 2.61 b |
| CLP 0.5% | 10.43 ± 3.17 b | 17.18 ± 6.99 b | 11.28 ± 2.37 b | 17.04 ± 2.95 b |
| CLP 1.0% | 8.53 ± 4.21 b | 8.91 ± 3.29 b | 7.73 ± 1.71 b | 17.63 ± 3.58 b |

[a]Values are means ± SD. Values in a row not sharing the same letters are significantly different at $P < 0.05$. See Table 1 for abbreviations.

comparison with the control (Table 3). The results of the test examining erythrocyte hemolysis, which is an index of VE deficiency, are shown as follows (mean ± SD): control, 0.42 ± 0.80%; VE-deficient, 97.07 ± 2.28%; CLP 0.25%, 97.89 ± 2.22%; CLP 0.5%, 92.34 ± 13.53%; CLP 1.0%, 96.46 ± 2.33%. In all of the groups fed a VE-deficient diet, a high degree of hemolysis was evident, and no significant difference was noted comparing these groups.

Lipid peroxide levels in the plasma are shown in Figure 1. After 4 wk, the plasma TBARS level was significantly increased in the group fed the VE-deficient diet without CLP, compared with the control. In the groups fed the CLP-containing VE-deficient diets, the plasma TBARS levels were slightly increased. After 7 wk, the plasma TBARS in the rats fed the VE-deficient diet were elevated to a level twice that in the control. The plasma TBARS levels showed a tendency to decrease in the groups fed the CLP-containing VE-deficient diets, but the changes were not significant.

The α-tocopherol concentrations in various tissues are shown in Table 4. In the liver, kidney, heart, and brain of the rats in the VE-deficient group, the α-tocopherol concentrations were decreased by 86, 81, 93, and 53% compared with the control. In the groups fed the CLP-containing VE-deficient diets, the α-tocopherol levels in the tissues were decreased to a similar extent.

The lipid peroxide levels in various tissues are shown in Figure 2. TBARS levels in various tissues of rats in the VE-deficient diet group were elevated fourfold in the liver,

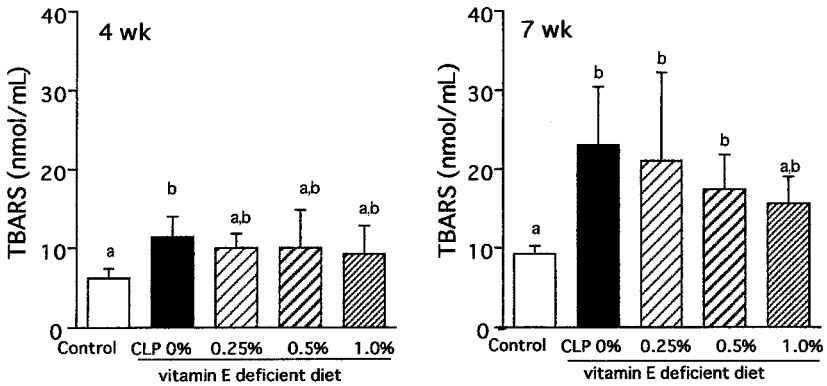


**FIG. 1.** Effect of cacao liquor crude polyphenols (CLP) on plasma lipid peroxide levels in rats fed the experimental diets, as determined after 4 wk and 7 wk. Values are means ± SD. Values not sharing the same letters are significantly different, $P < 0.05$. Abbreviation: TBARS, thiobarbituric acid-reactive substances.

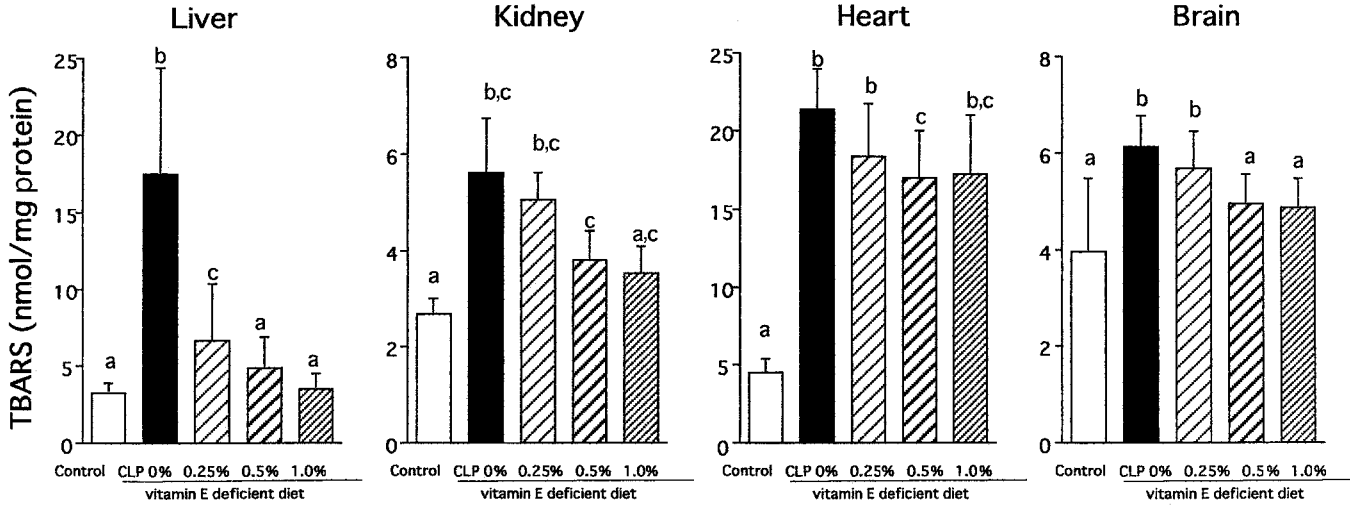


**FIG. 2.** Effect of CLP on lipid peroxide levels in various tissues of rats fed the experimental diets. Values are means ± SD. Values not sharing the same letters are significantly different, $P < 0.05$. For abbreviations see Figure 1.

twofold in the kidney, fourfold in the heart, and 1.5-fold in the brain compared with the control. In the case of rats fed the CLP-containing VE-deficient diets, the liver TBARS levels were decreased markedly in a dose-dependent manner compared with the rats fed the VE-deficient diet. A significant reduction of the kidney TBARS levels was observed in the groups fed the 0.5 and 1.0% CLP-containing VE-deficient diet, but such a decrease was not observed in the 0.25% CLP group. A significant suppression of the increase in heart TBARS levels was evident only in the group fed the 0.5% CLP-containing VE-deficient diet. The groups fed the 0.5 and 1.0% CLP-containing VE-deficient diets showed significant decreases in brain TBARS levels.

In plasma, neither free EC nor its metabolites produced through enzymatic treatment coud be detected by HPLC.

## DISCUSSION

The effect of antioxidants derived from cacao liquor on VE-deficient rats was evaluated. As a result, it was confirmed that the α-tocopherol concentrations in plasma and various tissues were markedly decreased, and lipid peroxide levels were markedly increased in rats fed the CLP-free VE-deficient diet (Tables 3 and 4; Figs. 1 and 2). VE is known to function as a strong antioxidant. Among the experimental oxidative stress models, VE -deficient animals have been well investigated. In these animals, there is a deficiency of VE in the cell membranes, the antioxidative status in the lipid bilayers is decreased, and severe membrane damage occurs (20). Yamashita *et al.* (21,22) evaluated the effects of antioxidative lignans in sesame seeds on VE-deficient rats. These reports indicated that sesame seed lignans reduced oxidative stress in VE-deficient rats. They speculated that the antioxidative mechanism of action of these lignans involved maintenance of VE levels in the organs. According to a previous report, supplementation of a low-selenium and VE-deficient diet with various flavonoids and phenols was not effective to maintain VE levels in plasma and tissues of animals (23,24). However, the increase in lipid peroxide levels in the organs of rats fed the low-selenium and VE deficient diet was suppressed by ingestion of these compounds.

In this study, CLP supplementation did not affect α-tocopherol metabolism in the case of VE deficiency (Tables 3 and 4). However, TBARS production as a consequence of VE deficiency was reduced by intake of CLP in a dose-dependent manner (Fig. 2). CLP inhibited the oxidative damage, especially in the liver. It was considered that the antioxidative component of CLP was absorbed and distributed to the liver more than to other tissues. The mechanisms of absorption and metabolism of polyphenols remain obscure, but recently many studies focusing on these aspects have been reported. Piskula and Terao (19) reported that epicatechin, which is one of the antioxidative components of CLP, was absorbed and could be detected in the blood of rats. In most cases, glucuronide and/or sulfate conjugates were formed. It has been reported also that epicatechin was detected in plasma after intake of black chocolate in man (25). In addtion, Hollman *et al.* (26) reported that orally administered epicatechin was absorbed and distributed in various tissues including liver and kidney. We have reported that cacao liquor polyphenols are absorbed and can be detected in plasma in rats (27). Thirty to 60 min after oral administration of cacao liquor to rats, the concentrations of epicatechin and its metabolites reached a maximum in plasma. At the same time, the level of plasma lipid peroxide production induced by a radical generator was reduced compared with the level before supplementation. In this study, the epicatechin concentration in plasma did not increase following oral administration because the rats were given CLP as a component of the diet. Under these conditions we could not detect epicatechin in plasma nor inhibition of lipid peroxide production induced by a radical generator (data not shown). However, our findings suggested that CLP in the

diet was absorbed and showed antioxidative activity similar to the case of oral administration.

In conclusion, when consumed orally CLP markedly reduce oxidative stress induced by VE deficiency without maintaining VE levels in the plasma and tissues.

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# Removal of Fat from Cow's Milk Decreases the Vitamin E Contents of the Resulting Dairy Products

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**ABSTRACT:** The present study was undertaken to determine whether decreases in fat contents result in lower vitamin E contents. Milk samples of varying fat contents (half and half, whole milk, reduced-fat milk, low-fat milk, and nonfat milk) were obtained from a local dairy on six different occasions. α-Tocopherol was the major form of vitamin E (>85%); γ-tocopherol and α-tocotrienol were present to a lesser extent. As the fat contents of milk products decreased from 11 to 0.3%, the vitamin E contents decreased. For example, raw milk as compared to nonfat milk had both higher α-tocopherol contents (45.5 ± 4.6 vs. 4.5 ± 0.5 μg/100 g; $P \leq 0.0001$) and higher total lipids (3.46 ± 0.49 vs. 0.30 ± 0.07 g/100 g; $P \leq 0.0001$). Vitamin E, cholesterol, and total lipids increased as cream was added back to nonfat milk during production. For every 1 mg cholesterol increase, there was an increase of approximately 4 μg of α-tocopherol; for every 1 g total lipids increase, the α-tocopherol content increased by 17 μg. These data demonstrate that removal of milk fat markedly decreases the vitamin E content of various milk products.

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In recent years, the term "antioxidant vitamins" has caught the attention of consumers. This is in part due to an increase in the research and understanding of the significant roles of antioxidant vitamins in disease processes. Vitamin E is one such essential lipid-soluble, chain-breaking antioxidant. Several prospective studies have suggested inverse associations between dietary intakes or plasma concentrations of antioxidants and cardiovascular disease (1,2). In some studies, this association has been observed for dietary vitamin E, but in other instances the relationship was seen only in persons taking high doses of vitamin E as supplements (3,4).

There have been three major intervention studies testing the effects of vitamin E on coronary heart disease risk. The Cambridge Heart Antioxidant Study, a double-blind placebo controlled study, looked at the effect of vitamin E (α-tocopherol) in the secondary prevention of coronary heart disease. The study demonstrated a 76% reduction in second nonfatal heart attacks in patients, who previously had had one heart attack (5). The GISSI-Prevenzione trial was a study of more than 11,000 individuals with a recent myocardial infarction (MI) who were randomly assigned to consume fish oil, vitamin E, or both, in a 2 × 2 factorial design study. The results were favorable for the fish oil supplement and neutral for vitamin E, but a *post-hoc* statistical analysis suggested a positive outcome for vitamin E supplementation (7). However, the Heart Outcomes Prevention Evaluation Trial of more than 9,000 subjects with vascular disease and diabetes also did not show a benefit for vitamin E, although the angiotension-converting enzyme inhibitor ramipril was shown to be beneficial in reducing cardiovascular risk (8). In contrast, in a trial with 196 patients with endstage renal disease, vitamin E (800 IU) compared to placebo showed a 46% reduction in the primary end point (cardiovascular disease including sudden death), and a reduction in MI by 70% (9). Thus, the role of vitamin E in decreasing coronary heart disease risk remains controversial.

Both vitamin E and fat are important in human health, but may have opposite effects. The American public has focused on decreasing dietary saturated fat to benefit health. However, modifying dairy product fat contents by either reducing total fat or altering the kind of fat may alter the vitamin E contents. Since vitamin E is a fat-soluble vitamin, it is likely to be removed by fat-modification of dairy foods. Is this detrimental because it reduces vitamin E intake? The present study was undertaken to analyze vitamin E, total lipid, and cholesterol in regular and fat-modified dairy products to assess whether decreases in fat result in lower vitamin E contents.

## MATERIALS AND METHODS

*Selection of analytical samples.* Milk samples were obtained from Loch Mead Dairy (Junction City, OR) on six different occasions. Briefly, raw milk was processed by the dairy to separate cream and nonfat milk. Depending on the type of milk being produced, a varying amount of fat in the form of

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Abbreviations: FDA, Food and Drug Administration; HPLC, high-performance liquid chromatography; MI, myocardial infarction.

cream was added back to the nonfat milk; homogenization and pasteurization followed immediately. The U.S. Food and Drug Administration (FDA) regulations define the names for various milk products based on the fat contents (half and half, 11%; whole milk, 3%; reduced-fat milk, 2%; low-fat milk, 1%; and nonfat milk, 0.5% fat).

Six aliquots of each milk sample were obtained for analysis from a single batch of raw milk being processed on the same day. All the samples were transferred from the dairy to the laboratory on ice and analyzed within 24 h from the time of collection. Six different batches were analyzed.

*Laboratory analyses. (i) Vitamin E.* The distribution of α- and γ-tocopherols and α- and γ-tocotrienols in half and half, raw, whole, reduced-fat, low-fat, and nonfat milk was determined as previously described (10). Briefly, the milk vitamin E was extracted with hexane [*n*-hexane high-performance liquid chromatography (HPLC) grade; EM Science, Cherry Hill, NJ] following saponification with ethanolic potassium hydroxide. An aliquot of the hexane layer was evaporated under nitrogen and the residue was resuspended in 1:1 ethanol/methanol. An appropriate aliquot was then injected onto the HPLC. The HPLC was configured with an SIL-10AD$_{VP}$ autoinjector with a sample cooler (Shimadzu, Kyoto, Japan) consisting of a SCL-10A system controller, a LC-10AD$_{VP}$ HPLC series isocratic pump, a Beckman Ultrasphere (ODS C-18 column, 4.6 mm i.d., 25 cm, 5 μm particle size) with a Waters® Spherisorb ODS guard column and detected using LC-4C amperometric detector with a glassy carbon electrode (Bioanalytical Systems Inc., Lafayette, IN). The mobile phase was a mixture of methanol/water (99:1, % vol/vol) and 0.1% (wt/vol) lithium perchlorate. The total run time for the assay was approximately 12 min. Peaks were integrated using Shimadzu Class-VP automated software program (Columbia, MD). Authentic α- and γ-tocopherols and α- and γ-tocotrienols were used as external standards for quantification of vitamin E (Cognis, La Grange, IL). No γ-tocotrienol was detected in the samples.

*(ii) Cholesterol.* The cholesterol content of the milk sample was measured using a cholesterol kit from Sigma Diagnostics (Procedure No. 352, St. Louis, MO) in an aliquot of hexane saved from the extract described for vitamin E analysis.

*(iii) Total lipids.* Total lipids were extracted from milk samples using methanol and chloroform (11). The total lipids were calculated from the weight of the dried aliquot and expressed as weight in grams per 100 g milk.

*Statistical analysis.* Data were analyzed using a one-way analysis of variance model using StatView (SAS Institute Inc., Cary, NC). Differences between means were considered statistically significant if $P < 0.05$. If significant differences were found, then Fisher's *post-hoc* tests were used for making pairwise comparisons. Here differences between means were considered statistically significant if $P < 0.01$.

## RESULTS

*Milk vitamin E, cholesterol, and total lipids.* Routine processing of milk involves separation of the cream from raw milk and later adding the cream back to nonfat milk in appropriate amounts depending on the dairy product. The total lipid, cholesterol, and vitamin E contents (α- and γ-tocopherols and α-tocotrienol) of half and half, raw, whole milk, reduced-fat milk, low-fat milk, and nonfat milk were analyzed and results are shown in Table 1. α-Tocopherol was the most abundant form of vitamin E in all types of milk—it represented from 84 to 92% of the vitamin E, while γ-tocopherol and α-tocotrienol were each roughly 5%.

The total lipids content of milk increased from 0.30 ± 0.07 g/100 g in nonfat to 11.6 ± 0.53 g/100 g in half and half with the addition of increasing amounts of cream to nonfat milk. The cholesterol contents of milk also varied with the amount of cream added, with the highest concentrations in the products with the highest fat contents. Similarly, the α- and γ-tocopherols and α-tocotrienol contents varied in the different products apparently depending on the fat content. Half and half contained the most fat and had the highest amount of α-tocopherol (193 ± 1.66 μg/100 g) among all the products tested. The other products had lower fat and α-tocopherol contents. For example, the α-tocopherol concentration of whole milk (43.9 ± 2.2 μg/100 g) was

**TABLE 1**
**Total Lipid, Cholesterol, and Vitamin E Concentrations in Milk**

| | Total lipids (g/100 g)[a] | Cholesterol (mg/100 g)[b] | α-Tocopherol (μg/100 g)[a] | γ-Tocopherol (μg/100 g)[a] | α-Tocotrienol (μg/100 g)[d] |
|---|---|---|---|---|---|
| Raw | 3.46 ± 0.49 | 16.0 ± 1.5 | 45.5 ± 4.6 | 1.92 ± 0.44[c] | 1.96 ± 0.51[c] |
| Whole | 3.40 ± 0.11 | 14.3 ± 1.4 | 43.9 ± 2.2 | 2.06 ± 0.38[d] | 1.76 ± 1.76[d] |
| Reduced-fat | 2.12 ± 0.15 | 7.06 ± 0.5 | 26.4 ± 3.9 | 1.34 ± 0.58[e] | 1.09 ± 0.31[e] |
| Low-fat | 1.16 ± 0.15 | 3.56 ± 0.4 | 14.2 ± 1.7 | 0.96 ± 0.31[f] | 0.59 ± 0.09[f] |
| Nonfat | 0.30 ± 0.07 | 1.74 ± 0.3 | 4.5 ± 0.5 | 0.62 ± 0.11[g] | 0.14 ± 0.03[g] |
| Half and half | 11.6 ± 0.53 | 47.7 ± 1 | 193 ± 1.7 | 12.1 ± 2.17[c,d,e,f,g] | 7.00 ± 2.74[c,d,e,f,g] |

[a]For both total lipid and α-tocopherol concentrations, pairwise comparisons between means for each of the products shown were significantly different ($P < 0.0001$) (except between raw and whole milk which was not significantly different).
[b]For cholesterol concentrations, pairwise comparisons between means were significantly different for all milk products ($P < 0.0001$); except $P < 0.009$ between raw and whole milk and $P < 0.004$ between reduced-fat and nonfat milk.
[c]For γ-tocopherol concentrations, pairwise comparisons between means were significantly different ($P < 0.0001$) only between [c,d,e,f,g]half and half and [c]raw, [d]whole, [e]reduced-fat, [f]low-fat, and [g]nonfat milks.
[d]For α-tocotrienol concentrations, pairwise comparisons between means were significantly different ($P < 0.0001$) only between [c,d,e,f,g]half and half and [c]raw, [d]whole, [e]reduced-fat, [f]low-fat, and [g]nonfat milks.

**TABLE 2**
**Cholesterol and Vitamin E Contents of Milk Relative to Total Lipids**

| | Cholesterol per total lipids (mg/g)[a] | α-Tocopherol per total lipids (μg/g)[b] | γ-Tocopherol per total lipids (μg/g)[c] | α-Tocotrienol per total lipids (μg/g) |
|---|---|---|---|---|
| Raw | 4.64 ± 0.39[b,d] | 13.2 ± 1.1 | 0.57 ± 0.16[b] | 0.57 ± 0.11 |
| Whole | 4.22 ± 0.44[a] | 12.9 ± 0.8 | 0.60 ± 0.11[a] | 0.52 ± 0.06 |
| Reduced-fat | 3.34 ± 0.18[c,d] | 12.4 ± 1.2 | 0.63 ± 0.26 | 0.51 ± 0.14 |
| Low-fat | 3.08 ± 0.25 | 12.3 ± 1.2[a] | 0.83 ± 0.25 | 0.51 ± 0.09 |
| Nonfat | 6.13 ± 1.86[a,b,c] | 15.7 ± 3.9 | 2.22 ± 0.80[a,b] | 0.50 ± 0.17 |
| Half and half | 4.11 ± 0.12 | 16.7 ± 0.6[a] | 1.04 ± 0.19 | 0.60 ± 0.24 |

[a]For cholesterol per total lipids, pairwise comparisons between means were significantly different between [a,b]nonfat and [a]whole or [b]raw milks ($P < 0.0001$), or between [c,d]reduced-fat and [c]nonfat or [d]raw milks ($P < 0.01$).
[b]For α-tocopherol per total lipids, pairwise comparisons between means were significantly different between [a]low-fat milk and [a]half and half ($P < 0.001$).
[c]For γ-tocopherol per total lipids, pairwise comparisons between means were significantly different between [a,b]nonfat milk and [a]whole or [b]raw milks ($P < 0.0001$).

higher than reduced-fat milk (26.4 ± 3.6 μg/100 g), low-fat milk (14.2 ± 1.7 μg/100 g), or nonfat milk (4.5 ± 0.5 μg/100 g).

*Vitamin E and cholesterol concentrations relative to total lipids.* Table 2 provides the vitamin E and cholesterol contents of milk relative to the total lipids for all the milk samples. The α-tocopherol per total lipids in nonfat milk was higher than in raw milk. Similarly, cholesterol per total lipids was higher in nonfat milk (6.13 ± 1.86 μg/g) as compared with raw milk (4.64 ± 0.39 μg/g, $P < 0.0003$). The α-tocopherol and the total lipids in different dairy products were correlated (Fig. 1); for every 1 g increase in total lipids, the α-tocopherol content increased by 17 μg.

*Vitamin E content relative to cholesterol content.* Table 3 provides the vitamin E content of milk relative to the cholesterol content for the milk samples analyzed. The α-tocopherol per cholesterol content was greatest in half and half (4.0 ± 0.1 μg/mg) and lowest in nonfat milk (2.6 ± 0.3 μg/mg, $P < 0.0001$). Interestingly, the γ-tocopherol per cholesterol content in nonfat milk was higher than that in raw milk ($P < 0.0001$). The α-tocopherol and the cholesterol contents in the different dairy products were also correlated (Fig. 2); for every 1 mg of cholesterol there was an increase of approximately 4 μg of α-tocopherol.

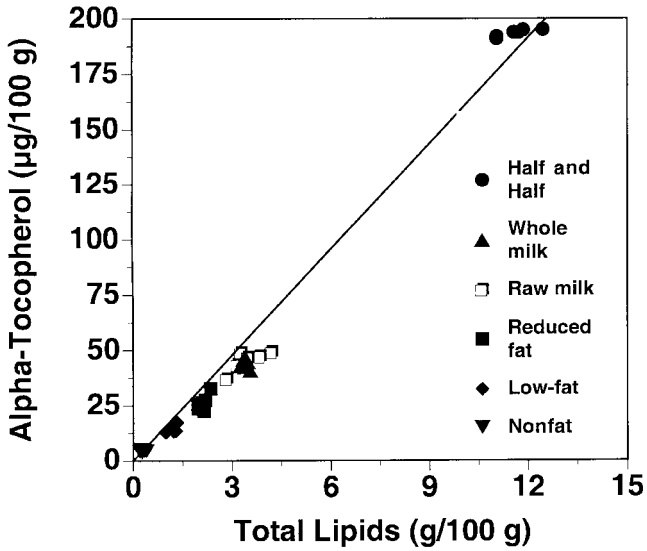


**FIG. 1.** Relationship between α-tocopherol and total lipids in milk samples of varying lipid contents. [$f(x) = 17.0x - 7.8$; $R^2 = 0.985$].

## DISCUSSION

α-Tocopherol was the major vitamin E form found in this study of cow's milk products of varying fat contents; γ-tocopherol and α-tocotrienol were found to lesser extent, while γ-tocotrienol was not detected. As the fat contents of the dairy products decreased from (11 to 0.3%), the vitamin E content also decreased. The α-tocopherol content of whole milk reported in this study was 44 μg/100 g. Previously, whole milk (3.3%) was reported to contain 30 μg α-tocopherol/100 g, while milk containing 2% fat (protein-fortified) contained 40 μg α-tocopherol/100 g (12). Most dietary vitamin E is found in fats and oils. α-Tocopherol is found predominantly in canola, olive, and sunflower oils (14). Indeed, the major food source of vitamin E in the diet of Americans, as a result of its high fat contents, is desserts (15). Importantly, decreasing fat intake also decreases vitamin E intake (16).

The milk products examined in this study contained typical lipid levels. The fat contents ranged from 11.6% total lipid for half and half to 0.3% for nonfat milk. These total lipid values meet the FDA food labeling requirements for the products described. The total lipids in the raw milk that we obtained were typical for raw milk (3 to 5% total lipids) as previously reported (17).

Cholesterol is the major form of sterol found in dairy products and it, like vitamin E, varied with the total lipid contents of the dairy products. In our study, the cholesterol contents of different dairy products were: half and half 47.7 mg/100 g, whole milk 14.3 mg/100 g, reduced-fat milk 7.06 mg/100 g, low-fat milk 3.56 mg/100 g, and nonfat milk 1.74 mg/100 g. These values are similar to the values reported in the nutrition analysis software "The Food Processor" by ESHA Research (Salem, OR) (18).

The unique finding in our study was that dairy products

**TABLE 3**
**Vitamin E Content of Milk Relative to Cholesterol Content**

| | α-Tocopherol per cholesterol (μg/mg)[a] | γ-Tocopherol per cholesterol (μg/mg)[b] | α-Tocotrienol per cholesterol (μg/mg)[c] |
|---|---|---|---|
| Raw | 2.8 ± 0.1[a] | 0.12 ± 0.03[a,e] | 0.12 ± 0.03 |
| Whole | 3.1 ± 0.2[b,d] | 0.15 ± 0.03[b] | 0.12 ± 0.02 |
| Reduced-fat | 3.7 ± 0.3[d] | 0.19 ± 0.09[c] | 0.15 ± 0.05[b] |
| Low-fat | 4.0 ± 0.2 | 0.27 ± 0.10[a,b] | 0.17 ± 0.03[a] |
| Nonfat | 2.6 ± 0.3[c,d] | 0.37 ± 0.10[c,d] | 0.08 ± 0.01[a,b,c] |
| Half and half | 4.0 ± 0.1[a,b,c,d,e] | 0.25 ± 0.05[d,e] | 0.15 ± 0.06[c] |

[a]Pairwise comparison for α-tocopherol per cholesterol were statistically significant ($P < 0.0001$) between [a,b,c]half and half and [a]raw or [b]whole and [c]nonfat milks; between [d]nonfat and [d]whole milk ($P < 0.0006$).
[b]Pairwise comparisons for γ-tocopherol per cholesterol were statistically significant between [a,b]low-fat and [a]raw ($P < 0.001$), and [b]whole milk ($P < 0.004$); between [c,d]nonfat and [c]reduced-fat ($P < 0.0015$) and [d]half and half (0.004); between [e]half and half and [e]raw milk ($P < 0.01$).
[c]For α-tocotrienol significant differences were observed between [a,b,c]nonfat and [a]low-fat ($P < 0.01$), [b]reduced-fat milk ($P < 0.01$); or [c]half and half ($P < 0.005$).

contain α-tocotrienol. This particularly piqued our interest because of the potent antioxidant properties of α-tocotrienol. Although α-tocotrienol has just one-third the biological activity of α-tocopherol in rats (19), it has equal (20) or higher antioxidant activity compared with α-tocopherol (21,22). The α-tocotrienol contents of dairy products ranged from 1.76 μg/100 g for whole milk to 0.14 μg/100 g for nonfat milk (Table 1). Other studies have not reported the presence of tocotrienols in dairy products. This may be because we used an extremely sensitive method for detection of tocopherols and tocotrienols (10). Nonetheless, γ-tocotrienol, a predominant vitamin E form in palm oil (14), was not found in milk. Alternatively, the presence of α-tocotrienol in milk may be attributed to the tocotrienol content of the feed, specifically grasses consumed by the cows. Grasses of green pastures in the early stage of growth have an increased α-tocotrienol content (23). Our milk samples were all obtained in late spring from the neighborhood dairy where cows were allowed to eat grass.

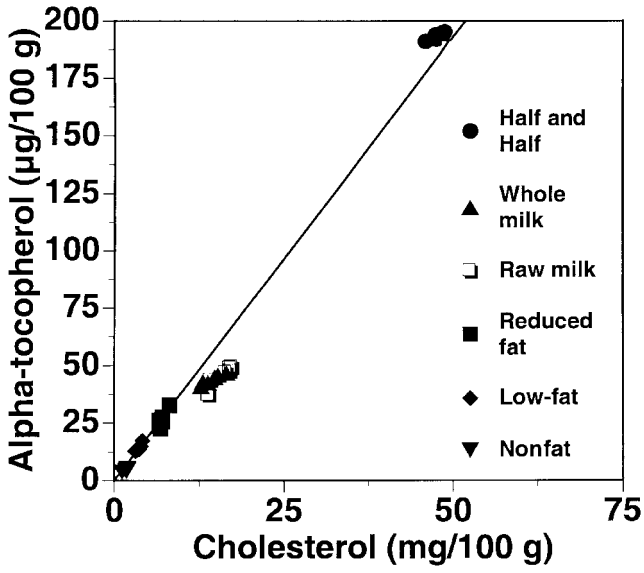


**FIG. 2.** Relationship between α-tocopherol and cholesterol in milk samples of varying lipid contents. [f($x$) = 4.1$x$ – 6.8; $R^2$ = 0.985].

It is clear that increased amounts of fat in the milk products result in increases in both cholesterol and vitamin E contents; therefore, the different forms of vitamin E were normalized to total lipid and cholesterol contents. The total lipids-adjusted cholesterol was highest in nonfat milk (Table 2). A possible explanation for this observation could be the disruption of milk fat globules during the centrifugation procedure, to isolate cream thereby contributing to residual fat globule membranes disproportionately rich in cholesterol, which accumulate in the nonfat milk (24). The cholesterol-adjusted α-tocopherol content was found to be lowest in nonfat milk (Table 3), suggesting association of α-tocopherol with the fat droplets rather than the membranes. Milk production typically involves separation of cream from raw milk and later adding the cream back to nonfat milk in varying amounts depending on the dairy product. Although statistically significant differences were found in the ratios between the vitamin E forms and lipids or cholesterol, in general, these differences were relatively minor and not likely to represent significant differences to the consumer.

Vitamin E was present in all the dairy products analyzed. Given in Table 4 is the vitamin E content of different milk products, when a quart is consumed, compared to rich sources of dietary vitamin E, almonds and frozen spinach (14). A single serving of almonds nearly provides the recommended daily allowance for vitamin E , while even a quart of whole milk does not provide an equivalent amount of α-tocopherol. For example, one serving of almonds provides 15 g fat and 12 mg α-tocopherol, while a cup of frozen spinach provides 1.5 g fat and 3.4 mg α-tocopherol. Even though spinach has one-tenth the fat content of almonds, it provides a significant amount of vitamin E as compared to quart of whole milk, which provides 33 g fat and 0.4 mg α-tocopherol. Although, milk cannot be considered a primary food source of vitamin E, it is the most commonly consumed dairy product and if large enough amounts of milk products are consumed they become a significant source of vi-

**TABLE 4**
**Comparison of Milk Products, Almonds and Frozen Spinachs as Vitamin E Sources**

| | Amount consumed | Calories (kcal) | Total fat (g) | α-Tocopherol (mg) | γ-Tocopherol (mg) | α-Tocotrienol (mg) |
|---|---|---|---|---|---|---|
| Whole milk | 1 quart | 599 | 33 | 0.42 | 0.019 | 0.017 |
| Reduced-fat milk | 1 quart | 485 | 19 | 0.25 | 0.013 | 0.010 |
| Low-fat milk | 1 quart | 409 | 10 | 0.13 | 0.009 | 0.006 |
| Nonfat milk | 1 quart | 342 | 2 | 0.04 | 0.006 | 0.001 |
| Spinach, frozen | 1 cup | 54 | 1.5 | 3.4 | — | — |
| Almonds | 2 Tbsp. | 166 | 15 | 12 | 0.51 | 0.56 |

tamin E. However, according to the 1994–1995 Continuing Survey of Food Intakes by Individuals data, nonfat milk consumption is increasing, while whole milk intake is decreasing (25). Because the vitamin E content of nonfat milk is one-tenth that of whole milk, we suggest that vitamin E fortification of milk might be a reasonable approach to restore α-tocopherol intakes to those seen with whole milk. This is especially important because children consuming relatively low-fat diets have decreased vitamin E intakes (26).

In conclusion, this study indicates that vitamin E, especially α-tocopherol, γ-tocopherol, and α-tocotrienol, is present in milk of various fat contents. Importantly, the α-tocopherol content of milk decreases along with cholesterol content as the fat content decreases.

## ACKNOWLEDGMENTS

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# Biosynthesis of Eicosapentaenoic Acid in the Sea Urchin *Psammechinus miliaris*

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**ABSTRACT:** The sea urchin *Psammechinus miliaris* (Gmelin) (Echinodermata: Echinoidea) was shown by using a deuterated tracer ($D_5$-18:3n-3) and quantitation by negative chemical ionization gas chromatography–mass spectrometry to convert 18:3n-3 to 20:5n-3. The rate of conversion was very slow, corresponding to 0.09 μg/g tissue/mg 18:3n-3 eaten over 14 d. Deuterated arachidonic acid ($D_8$-20:4n-6) was also included in the diet to give a measure of the relative amounts of diet eaten by the different animals. The recovery of this fatty acid in tissue lipids was 33.7% compared with only 0.95% recovery of $D_5$-18:3n-3 and its anabolites, indicating that the majority of the $D_5$-tracer was catabolized. Considerable elongation of $D_5$-18:3n-3 into 20:3n-3 and a trace of 22:3n-3 was found, and these were accompanied by minor amounts of the intermediates 18:4n-3 and 20:4n-3. No deuterated 22:6n-3 was found.

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The biosynthesis of polyunsaturated fatty acids (PUFA) in invertebrates is seldom studied, and no quantitative information is available on rates of formation. PUFA are abundant in the marine environment with 16-, 18-, 20-, and 22-carbon PUFA, all found in different classes of phytoplankton, while higher consumers, both invertebrate and vertebrate, retain and concentrate $C_{20}$ and $C_{22}$ PUFA (1). This abundance of dietary $C_{20}$ and $C_{22}$ PUFA may mask biosynthetic capability in consumers. Radiotracer studies have shown that some terrestrial invertebrates can synthesize PUFA *de novo* (2,3) and that *Artemia* can convert 18:2n-6 to 18:3n-3 and then to 20:5n-3 (4). These were formerly functions associated only with photosynthetic organisms.

Tracer experiments using [1-$^{14}$C]-18:2n-6 and [1-$^{14}$C]-18:3n-3 have been conducted in a number of invertebrates including the clam *Mesodesma mactroides* (5), the calanoid copepod *Paracalanus parvus* (6), and the prawn *Penaeus japonicus* (7) and have shown small percentage conversions to longer-chain more unsaturated products. However, a drawback of using [1-$^{14}$C]-labeled substrates is the possible loss of label by β-oxidation and reincorporation of labeled acetate. Thus results may be ambiguous beyond the first elongation step. None of the above studies using radiolabeled tracers quantitated the amounts of fatty acid moving through the pathway.

The sea urchin *Psammechinus miliaris* has recently been identified as a possible aquaculture species (8), as the roe (gonad of both males and females) is a valuable food product. As such there is a requirement for artificial diets which enhance gonad growth. In the wild, *P. miliaris* grazes on living and detached kelp (9), but it also feeds on a wide selection of other algae and encrusting invertebrates (10). Gonad growth in *P. miliaris* was enhanced by commercially prepared salmon feed (11), and gonad growth was also stimulated by diets containing lower levels of lipid (6–8%) of mainly vegetable origin (12). It was unclear whether *P. miliaris* is capable of producing $C_{20}$ and $C_{22}$ PUFA from shorter-chain precursor fatty acids, or indeed whether this species has a requirement for such fatty acids.

In this study we demonstrate, using a deuterated tracer and quantitation by gas chromatography–mass spectrometry (GC–MS), that *P. miliaris* can elongate and desaturate 18:3n-3 to produce 20:5n-3. This method is very sensitive and overcomes the problems of interpretation when using [1-$^{14}$C]-labeled fatty acids.

## MATERIALS AND METHODS

*Chemicals.* Chloroform, methanol, ethanol, isohexane, and diethyl ether were high-peformance liquid chromatrography-grade from Fisher (Loughborough, Leicestershire, United Kingdom). Butylated hydroxytoluene and standard fatty acids were obtained from Sigma (Poole, Dorset, United Kingdom) and diisopropylamine, anhydrous acetonitrile, and pentafluorobenzyl bromide from Aldrich (Gillingham, Dorset, United Kingdom). Deuterated tracer $D_5$ (17,17,18,18,18)-linolenic acid and $D_8$ (5,6,8,9,11,12,14,15)-arachidonic acid were purchased from Cambridge Isotope Laboratories (Andover, MA) as the fatty acid ethyl esters.

*Preparation of diet.* The experimental diet was based on a standard fish diet with vegetable meals and vegetable oils substituting for fish meal and fish oil to eliminate preformed $C_{20}$ and $C_{22}$ PUFA. The diet comprised (g/100 g): soy flour 39, wheat meal 31, corn gluten meal 11.4, lysine 0.3, methionine 0.3, mineral mix 4.0, vitamin mix 1.0, carboxymethylcellulose 2.0, and oil 11.0. The oil component consisted of, mg/g dry diet: $D_5$-18:3n-3 fatty acid ethyl ester 27, $D_8$-20:4n-6 fatty acid ethyl ester 5.9, and linseed oil 81. The diet was produced as a 1-mm diameter pellet.

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Abbreviations: $D_n$, deuterated; GC–MS, gas chromatography–mass spectrometry; PUFA, polyunsaturated fatty acid; tri23:0, tritricosanoyl.

*Animals. Psammachinus miliaris* were collected by SCUBA divers from a depth of 5 m in Loch Creran, Scotland (56°32′20″ N; 5°17′00″ W) in early September. Six urchins (horizontal test diameter 2–3 cm, wet weight 9–12 g) were selected at random and placed in aquaria that had an independent supply of 250 L filtered seawater at ambient temperature (11.8–15.3°C) and salinity. The photoperiod was maintained at a constant 16-h light/8-h dark cycle throughout the experiments. The urchins were fed on the macroalga *Laminaria saccharina ad libitum* for 2 wk to equilibrate their nutritional status. The seawater supply was then exchanged for a 5-µm filtered supply, and the urchins were starved for 48 h prior to feeding the labeled diet. The urchins were fed 0.54 g of labeled diet over the course of 3 h. Urchins were individually presented with pellets that they readily accepted, trapping them with their tube feet and rapidly manipulating them to their jaws. All the diet was ingested. The urchins were returned to a diet of *L. saccharina* and sacrificed 14 d later. They were weighed, the gonad and gut dissected, and the tissues, including the test, weighed. Samples were stored at –70°C prior to analysis.

*Lipid extraction.* The gonads and guts were thawed on ice then homogenized in 10 mL chloroform/methanol (2:1, vol/vol). Tritricosanoylglycerol (tri23:0 glycerol) standard (30 µg) was added to the gonad homogenates and 10 µg to the gut homogenates. The homogenates were shaken and then stood on ice for 1 h with periodic shaking. The eviscerated tests were crushed in a pestle and mortar, and the slurry was homogenized in 20 mL chloroform/methanol (2:1, vol/vol) with 100 µg tri23:0. The homogenate was left on ice for 2½ h with periodic shaking. The homogenates were then filtered and a Folch extract prepared (13). The final total lipid fraction was weighed and stored under argon at –20°C.

*Quantitation of deuterated fatty acids.* One milligram of total lipid was saponified with 2 mL of 0.1 M KOH in 95% (vol/vol) ethanol under nitrogen for 1 h at 78°C. Nonsaponifiable material was removed by extracting with isohexane/diethyl ether (2:1, vol/vol), the aqueous phase was acidified then free fatty acids were extracted with diethyl ether. Pentafluorobenzyl esters were then prepared from 100 µg free fatty acid using acetonitrile/diisopropylamine/pentafluorobenzyl bromide (1000:10:1, by vol) at 60°C for 30 min under nitrogen as described by Pawlosky *et al.* (14). Excess reagent and solvent were then removed under nitrogen, samples dissolved in isohexane, and stored at –20°C under argon until analysis. Calibration standards of individual fatty acids with 23:0 were prepared by varying the amount of unknown fatty acid while keeping the 23:0 constant and plotting the peak area ratio against the mass ratio of the fatty acids. Sample volumes for analysis were adjusted such that the amount of 23:0 injected onto the GC–MS was constant. Pentafluorobenzyl esters were chromatographed and quantitated on a Fisons MD 800 GC–MS fitted with an on-column injector and a Chrompack CP wax 52CB column (30 m × 0.32 mm i.d., 0.25 micron film thickness) (Burke Analytical, Alva, Clackmannanshire, United Kingdom) using helium as carrier gas (column head-pressure 7 psi) and running in negative chemical ionization mode with methane as reagent gas (pressure 7 psi). The temperature program was 80–190°C at 40°C/min, 190–240°C at 1.5°C/min, then 240°C for 10 min. Peaks were identified by selective ion scanning for the required masses using a dwell time of 80 ms and cycle time of 20 ms and quantitated by reference to the appropriate standard fatty acid.

## RESULTS

After 14 d, all three tissues contained $D_5$-18:3n-3 and its anabolites together with $D_8$-20:4n-6 (Table 1). The distribution of labeled fatty acids within the tissues varied between the animals with gonad, gut and test each having the majority of $D_5$-labeled fatty acids in two animals. However, $D_8$-20:4n-6 was predominantly found in the test (Table 1) (five out of six animals). When expressed as mg lipid or fatty acid per g tissue, gut was the most lipid-rich tissue for total lipid and labeled fatty acids (Table 1). The total recoveries of labeled fatty acids were 139 µg for $D_5$-18:3n-3 and metabolites and 1.076 mg of $D_8$-20:4n-6, corresponding to 0.95 and 33.7% of ingested material, respectively, suggesting that most of the tracer was catabolized.

There was a good correlation between the total $D_5$-fatty acids and $D_8$-20:4n-6 recovered in the six animals ($r$ = 0.9917). This indicated that the fatty acids were being processed similarly in the animals. The proportion of $D_8$-20:4n-6 recovered in individual animals was used to calculate the amount of $D_5$-18:3n-3 eaten by individuals. The data for fatty acid incorporation were then expressed as µg fatty acid/g tissue/mg $D_5$-18:3n-3 ingested.

**TABLE 1**
**The Distribution of Lipid and Deuterated Fatty Acids in the Different Tissues (mean ± 1 SD, *n* = 6)**

| | Tissue | | | |
|---|---|---|---|---|
| | Gonad | Gut | Test | Total |
| Lipid (mg) | 12.9 ± 5.7 | 5.3 ± 0.8 | 30.5 ± 10.4 | 48.7 ± 12.9 |
| mg lipid/g tissue | 24.8 ± 8.3 | 28.3 ± 4.5 | 5.6 ± 1.1 | 4.7 ± 0.9 |
| Total $D_5$-fatty acids (µg) | 6.4 ± 5.7 | 9.3 ± 8.8 | 7.5 ± 2.6 | 23.2 ± 14.2 |
| µg $D_5$-fatty acid/g tissue | 11.5 ± 8.9 | 49.2 ± 44.6 | 1.4 ± 0.3 | 2.2 ± 1.2 |
| $D_8$-20:4n-6 (µg) | 31.6 ± 26.2 | 70.6 ± 68.4 | 77.2 ± 30.4 | 179.4 ± 89.6 |
| µg $D_8$-20:4n-6/g tissue | 57.0 ± 40.5 | 373.6 ± 345.6 | 14.4 ± 4.8 | 17.1 ± 7.4 |

**TABLE 2**
**Fatty Acids Derived from $D_5$-18:3n-3 Present 14 d After a Pulse of 14.6 mg $D_5$-18:3n-3 (mean ± 1 SD, *n* = 6)**

| | μg $D_5$-Fatty acid/g tissue/mg $D_5$-18:3n-3 eaten | % Recovered |
|---|---|---|
| $D_5$-18:3n-3 | 0.33 ± 0.07 | 38.0 |
| $D_5$-20:3n-3 | 0.35 ± 0.05 | 39.9 |
| $D_5$-22:3n-3 | 0.021 ± 0.005 | 2.3 |
| $D_5$-18:4n-3 | 0.011 ± 0.004 | 1.1 |
| $D_5$-20:4n-3 | 0.076 ± 0.018 | 8.4 |
| $D_5$-22:4n-3 | Trace | <0.1 |
| $D_5$-20:5n-3 | 0.090 ± 0.015 | 10.2 |
| $D_5$-22:5n-3 | Trace | <0.1 |

[a]Trace = <0.001 μg $D_5$-fatty acid/g tissue/mg $D_5$-18:3n-3 eaten. Percentage recovered is percentage of total $D_5$-fatty acid recovered.

The initial substrate $D_5$-18:3n-3 and its immediate elongation product 20:3n-3 were the predominant labeled fatty acids recovered (Table 2), together accounting for 77.9% of the recovered label. Further elongation to 22:3n-3 accounted for 2.3% of deuterated fatty acids. Octadecatetraenoic acid (18:4n-3), the Δ6-desaturation product of 18:3n-3, was a minor component (Table 2), accounting for 1.1% of label. The subsequent elongation product 20:4n-3 was some seven times more abundant, and its Δ5-desaturation product 20:5n-3 accounted for 10.2% of recovered label. This represented 0.009% of the 18:3n-3 ingested (0.09 μg/g tissue/mg $D_5$-18:3n-3 eaten). Only trace amounts of 22:4n-3 and 22:5n-3 were found, and we could not detect any $D_5$-22:6n-3. The samples were analyzed also for the elongation and desaturation products of $D_8$-20:4n-6. There was some elongation to 22:4n-6 (5.7% of substrate) but no further elongation to 24:4n-6 or desaturation to 22:5n-6.

## DISCUSSION

Deuterated arachidonic acid ($D_8$-isomer) was added to the diets to give an indication of the amount of diet eaten. The reasoning behind this was that a $C_{20}$ PUFA, which is an important structural fatty acid of cell membranes (a major PUFA in this species, *ca.*10% of total fatty acids) and also a precursor of the eicosanoids (15), is less likely to be heavily catabolized than a $C_{18}$ PUFA, or indeed a saturated or monounsaturated fatty acid. The data supported this hypothesis as $D_8$-20:4n-6 was retained much better than $D_5$-18:3n-3 and its metabolites, with recoveries of 33.7 and 0.95%, respectively. The great majority of the $D_5$-18:3n-3 therefore either was not assimilated or was catabolized. It is likely that "nonessential" fatty acids were being oxidized to provide energy for either somatic growth or gonad recovery. Therefore, we used the relative amounts of $D_8$-20:4n-6 recovered in the different animals to deduce the amount of diet, and hence $D_5$-18:3n-3, ingested. Using this correction reduced the variability in the data.

*Psammechinus miliaris* has an annual reproductive cycle, peak gonad indices being obtained during June and July in the area where these urchins were collected (16). The urchins used in this study were in the postspawning or recovery phase of the reproductive cycle (17), and their gonads were therefore relatively depleted in lipid (2.5% wet weight) compared to 7.1% for urchins of the same species in the premature phase (18). In the recovery phase of the gametogenic cycle, urchins replenish the nutrient stores in the nutritive phagocytes of the gonad (17). Therefore, the urchins used in this study were likely to have been sequestering lipid.

The results clearly showed that the urchins were capable of synthesizing 20:5n-3 from 18:3n-3, even when reared on a natural diet (*L. saccharina*), which contains 20:4n-6 and 20:5n-3 as the major PUFA. The rate of formation of 20:5n-3 was slow, equivalent to only 0.009% of the $D_5$-18:3n-3 ingested over a 14-d period. In a preliminary experiment, we found that the amounts of $D_5$-18:4n-3, $D_5$-20:4n-3, and $D_5$-20:5n-3 in gonad approximately doubled between day 7 and day 14 post-dose, whereas the amount of substrate $D_5$-18:3n-3 decreased (data not shown). Of the label recovered from $D_5$-18:3n-3, 42.2% was in elongation products and 19.7% in Δ6-desaturation products. This indicates an active $C_{18}$–$C_{20}$ elongase that effectively reduces the substrate availability for the desaturase. There was only limited elongation of $C_{20}$ PUFA to $C_{22}$ PUFA; $D_5$-22:3n-3 (6.0% of substrate $D_5$-20:3n-3) and $D_8$-22:4n-6 (5.7% of substrate $D_8$-20:4n-6) were measurable while $D_5$-22:4n-3 and $D_5$-22:5n-3 arising from $D_5$-20:4n-3 and $D_5$-20:5n-3, respectively, were barely detectable. The much larger amounts of $D_5$-20:3n-3 and $D_8$-20:4n-6 allowed their elongation products to be measured. Urchins reared on a natural diet of *Laminaria* contained 11.3% 20:4n-6, 10.0% 20:5n-3, 0.1% 22:5n-6, and 1.5% 22:6n-3 (18), supporting the finding that conversion of $C_{20}$ PUFA to $C_{22}$ PUFA is low in this species. The urchins clearly contain an active Δ5-desaturase. This was suggested by earlier fatty acid compositional data (19) which showed a number of unusual Δ5-monounsaturated fatty acids and Δ5-nonmethylene-interrupted dienes. In *P. miliaris* feeding on salmon diet or the detritus from salmon cages that contains $C_{20}$ and $C_{22}$ monounsaturated fatty acids, the corresponding Δ5-20:2 and Δ5-22:2 nonmethylene-interrupted dienes were found (18).

The data show conclusively that *P. miliaris* can synthesize 20:5n-3 from 18:3n-3 and that this species therefore has the genetic apparatus necessary to bring about this conversion, i.e., a Δ6-desaturase, $C_{18}$ to $C_{20}$ elongase, and a Δ5-desaturase. However, further desaturation reactions do not occur while further elongation to $C_{22}$ PUFA is limited. The rate of formation of 20:5n-3 was very slow, about one-twentieth of the rate of 22:6n-3 formation measured in rainbow trout using the same method (Bell, M.V., unpublished data), and was well below that necessary to account for the amount of 20:5n-3 present in the animals which must have been accumulated from the diet. The ability to synthesize $C_{20}$ PUFA when the diet usually contains these fatty acids is perhaps surprising and suggests an essential role for $C_{20}$ PUFA in echinoderms, probably as prostanoid precursors. Several B series prostaglandins were reported in 10 species of Japanese echinoderms (20).

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# The First Naturally Occurring α-Methoxylated Branched-Chain Fatty Acids from the Phospholipids of *Amphimedon complanata*

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**ABSTRACT:** The phospholipid fatty acid composition of the sponge *Amphimedon complanata* was reinvestigated, and the 2-methoxy-13-methyltetradecanoic acid, 2-methoxy-14-methylpentadecanoic acid, and 2-methoxy-13-methylpentadecanoic acid were identified for the first time in nature. Structure characterization was accomplished by means of gas chromatographic retention times and gas chromatography–mass spectrometry. These acids could have originated from bacteria in symbiosis with the sponge.

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The only naturally occurring α-methoxy fatty acids known to date are those from the phospholipids of sponges (1–3). Earlier examples include normal-chain saturated 2-methoxy fatty acids between $C_{19}$–$C_{24}$ carbons and very long chain monounsaturated fatty acids such as (2*R*,21*Z*)-2-methoxy-21-octacosenoic acid, which was the first-reported α-methoxy fatty acid from a phospholipid (2). All of these acids were reported to have the *R* configuration at the chiral center. Recent efforts have concentrated on the $C_{16}$ family of α-methoxylated fatty acids, such as the 2-methoxy-5(*Z*)-hexadecenoic acid, 2-methoxy-6(*Z*)-hexadecenoic acid, and the 2-methoxyhexadecanoic acid, which were identified in the phospholipids of several Caribbean sponges such as *Amphimedon compressa* (4). Both 2-methoxy-5(*Z*)-hexadecenoic acid and 2-methoxyhexadecanoic acid occur more often in the phospholipids of sponges than their Δ6 analogs (5).

Despite these isolation efforts there are no reports in the literature as to any natural source for branched-chain α-methoxylated fatty acids although some mid-chain methoxylated fatty acids, such as (4*E*,8*E*)-7-methoxy-9-methylhexadeca-4,8-dienoic acid from *Lyngbya majuscula*, are known (6). We have now investigated the sponge *A. complanata* (7), collected at a different site, and found a novel series of iso and anteiso $C_{15}$–$C_{16}$ α-methoxylated phospholipid fatty acids that we describe herein. These novel compounds could have originated from the phospholipids of a novel bacterium in symbiosis with *A. complanata*.

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Abbreviations: DMDS, dimethyl disulfide; ECL, equivalent chain length; FCL, fractional chain length; GC–MS, gas chromatography–mass spectrometry; TLC, thin-layer chromatography.

## MATERIALS AND METHODS

*Instrumentation*. Gas chromatography–mass spectrometry (GC–MS) data were collected at 70 eV with a Hewlett-Packard 5972A MS ChemStation (Palo Alto, CA) equipped with a 30 m × 0.25 mm special performance capillary column (HP-5MS) cross-linked with 5% phenyl methylpolysiloxane. The temperature program for the analyses was as follows: 130°C for 2 min, then increased at 3°C/min to 270°C and maintained for 40 min.

*Sample collection*. *Amphimedon complanata* was collected on February 27, 2000, at El Natural, Aguadilla, Puerto Rico, at a depth of 25 ft (7.6 m). The sponge was washed in seawater, carefully cleaned of all nonsponge debris, and lyophilized. A voucher specimen is available at the Department of Chemistry of the University of Puerto Rico, Río Piedras campus.

*Extraction and isolation of phospholipids*. The freeze-dried sponge (48.2 g) was extracted with 2 × 250 mL of chloroform/methanol (1:1, vol/vol) yielding the total lipids (11.9 g). The neutral lipids, glycolipids, and phospholipids (1.2 g) were separated by column chromatography on silica gel (60–200 mesh) using the procedure of Privett *et al*. (8). The phospholipid classes were investigated by preparative thin-layer chromatography (TLC) using silica gel and $CHCl_3$/MeOH/$NH_4OH$ (65:35:5) as solvent and comparing with authentic samples. The principal phospholipids were phosphatidylethanolamine and phosphatidylserine, as previously described (7).

*Preparation and isolation of fatty acid derivatives*. The fatty acyl components of the phospholipids were obtained as either their methyl or ethyl esters by reaction of the phospholipids with methanolic or ethanolic HCl followed by column chromatography (9). The double-bond positions in the monounsaturated acids were determined by dimethyl disulfide (DMDS) derivatization following the standard procedure previously described (10). *N*-Acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine-HOAc (10:1) in a capped vial (24 h at 100°C). The methyl esters were hydrogenated in 10 mL absolute methanol in the presence of catalytic amounts of platinum oxide ($PtO_2$). Mass spectral data for the novel methoxylated acids and some of their derivatives follow.

*Methyl 2-methoxy-13-methyltetradecanoate*. Equivalent chain length (ECL) = 15.86; GC–MS *m/z* (relative intensity) $M^+$ 286 (1), 270 (1), 236 (2), 228 (17), 227 (100), 213 (1),

199 (1), 185 (1), 171 (1), 153 (1), 152 (2), 139 (3), 138 (3), 125 (11), 123 (6), 117 (1), 111 (27), 109 (10), 104 (6), 99 (4), 97 (58), 95 (21), 87 (17), 85 (16), 83 (68), 81 (31), 71 (61), 69 (68), 67 (37), 58 (15), 57 (50), 55 (70).

*Ethyl 2-methoxy-13-methyltetradecanoate*. ECL = 15.79 (calculated using ethyl esters); GC–MS *m/z* (relative intensity) $M^+$ 300 (1), 270 (1), 237 (3), 235 (1), 228 (1), 227 (100), 213 (1), 211 (1), 194 (4), 185 (1), 171 (1), 155 (3), 152 (4), 139 (5), 138 (3), 125 (13), 118 (6), 111 (33), 109 (13), 101 (13), 97 (69), 95 (25), 88 (14), 85 (23), 83 (83), 81 (35), 71 (69), 69 (82), 67 (40), 58 (17), 57 (68), 55 (84).

N-*2-Methoxy-13-methyltetradecanoylpyrrolidine*. GC–MS *m/z* (relative intensity) $M^+$ 325 (1), 310 (10), 295 (30), 266 (2), 252 (4), 228 (8), 227 (33), 196 (2), 168 (2), 156 (2), 143 (55), 128 (15), 126 (24), 113 (22), 111 (29), 97 (63), 95 (12), 85 (14), 83 (61), 82 (12), 72 (19), 69 (64), 67 (20), 57 (47), 55 (100).

*Methyl 2-methoxy-14-methylpentadecanoate*. ECL = 16.86; GC–MS *m/z* (relative intensity) $M^+$ 300 (1), 250 (2), 242 (9), 241 (55), 167 (1), 153 (2), 152 (2), 139 (2), 125 (8), 111 (24), 109 (10), 104 (4), 99 (5), 97 (59), 95 (21), 85 (24), 83 (55), 81 (27), 68 (22), 67 (30), 58 (15), 57 (61), 55 (73).

*Methyl 2-methoxy-13-methylpentadecanoate*. ECL = 16.94; GC–MS *m/z* (relative intensity) $M^+$ 300 (1), 250 (3), 242 (12), 241 (70), 239 (2), 180 (2), 167 (1), 153 (2), 152 (3), 139 (5), 138 (5), 125 (10), 123 (7), 111 (30), 104 (10), 99 (5), 97 (66), 95 (37), 87 (9), 85 (23), 83 (81), 81 (37), 70 (13), 69 (81), 67 (38), 58 (18), 57 (100), 55 (94).

## RESULTS

As previously reported, the main phospholipids from *A. complanata* were phosphatidylethanolamine and phosphatidylserine, with lesser amounts of phosphatidylcholine (7). Transesterification with either 1.0 N HCl/MeOH or HCl/EtOH permitted the characterization of the fatty acids as methyl or ethyl esters and the aldehydes as dimethyl or diethyl acetals using GC–MS. A total of 72 phospholipid fatty acids were identified. DMDS derivatives were used to locate the double bonds in the monounsaturated methyl esters, whereas pyrrolidides were mainly used to locate methyl branching. The total phospholipid fatty acid composition of *A. complanata* is presented in Table 1. Saturated normal-chain fatty acids between $C_{12}$ and $C_{29}$ predominated in these phospholipids (42%), in particular the acids 16:0 and 18:0, which were the most abundant in this family (12.3%). Monounsaturated fatty acids between $C_{16}$ and $C_{30}$ were the second-most abundant series of compounds (26.8%) and noteworthy among these was vaccenic acid. Branched-chain iso and anteiso fatty acids accounted for only 7% of the total composition, but most of these acids had chain lengths between $C_{15}$ and $C_{19}$, which are the typical chain-lengths of iso and anteiso bacterial fatty acids.

Attention was centered on six α-methoxylated fatty acids that constituted 1.7% of the total phospholipid fatty acid composition. Three of these acids, namely, 2-methoxy-5(*Z*)-hexadecenoic acid, 2-methoxy-6(*Z*)-hexadecenoic acid and 2-

**TABLE 1**
**Identified Phospholipid Fatty Acids from *Amphimedon complanata***

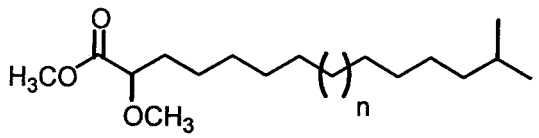
| Fatty acids | Relative abundance[a] (wt%) |
|---|---|
| Dodecanoic (12:0) | 0.4 |
| Tetradecanoic (14:0) | 4.0 |
| 12-Methyltetradecanoic (i-15:0) | 1.5 |
| 13-Methyltetradecanoic (ai-15:0) | 0.9 |
| Pentadecanoic (15:0) | 1.0 |
| 11-Hexadecenoic (16:1n-5) | 1.1 |
| 2-Methoxy-13-methyltetradecanoic (2-OMe-i-15:0)[b] | 0.9 |
| Hexadecanoic (16:0) | 6.2 |
| 2-Methoxy-5-hexadecenoic (2-OMe-5-16:1) | 0.01 |
| 2-Methoxy-6-hexadecenoic (2-OMe-6-16:1) | 0.01 |
| 2-Methoxy-14-methylpentadecanoic (2-OMe-i-16:0)[b] | 0.2 |
| 2-Methoxy-13-methylpentadecanoic (2-OMe-ai-16:0)[b] | 0.4 |
| Heptadecanoic (17:0) | 1.0 |
| 2-Methoxyhexadecanoic (2-OMe-16:0) | 0.2 |
| 7-Methyl-6-hexadecenoic (17:1) | 1.3 |
| 15-Methylhexadecanoic (i-17:0) | 1.9 |
| 14-Methylhexadecanoic (ai-17:0) | 0.8 |
| 9-Heptadecenoic (17:1n-8) | 0.4 |
| Heptadecanoic (17:0) | 2.4 |
| 9,12-Octadecadienoic (18:2n-6) | 0.5 |
| 9-Octadecenoic (18:1n-9) | 0.8 |
| 11-Octadecenoic (18:1n-7) | 4.1 |
| Octadecanoic (18:0) | 6.1 |
| Methyloctadecanoic (19:0) | 1.5 |
| 11-Methyloctadecanoic (19:0) | 1.1 |
| 17-Methyloctadecanoic (i-19:0) | 1.0 |
| 16-Methyloctadecanoic (ai-19:0) | 0.7 |
| Nonadecenoic (19:1) | 0.8 |
| Nonadecenoic (19:1) | 1.1 |
| Nonadecanoic (19:0) | 2.4 |
| 5,8,11,14-Eicosatetraenoic (20:4n-6) | 2.8 |
| Eicosadienoic (20:2) | 1.9 |
| 11,14-Icosadienoic (20:2n-6) | 2.7 |
| 11-Eicosenoic (20:1n-9) | 1.9 |
| 13-Eicosenoic (20:1n-7) | 1.4 |
| Eicosanoic (20:0) | 1.9 |
| Methyleicosanoic (21:0) | 0.5 |
| Heneicosanoic (21:0) | 1.9 |
| 4,7,10,13,16,19-Docosahexaenoic (22:6n-3) | 0.9 |
| 7,10,13,16-Docosatetraenoic (22:4n-6) | 2.4 |
| 4,7,10,13,16-Docosapentaenoic (22:5n-6) | 1.4 |
| 13-Docosenoic (22:1n-9) | 0.5 |
| 15-Docosenoic (22:1n-7) | 1.0 |
| 17-Docosenoic (22:1n-5) | 0.3 |
| Docosanoic (22:0) | 7.5 |
| 16-Tricosenoic (23:1n-7) | 1.4 |
| 17-Tricosenoic (23:1n-6) | 1.8 |
| 18-Tricosenoic (23:1n-5) | 0.7 |
| Tricosanoic (23:0) | 4.0 |
| 2-Hydroxydocosanoic (2-OH-22:0) | 1.1 |
| 17-Tetracosenoic (24:1n-7) | 1.3 |
| 18-Tetracosenoic (24:1n-6) | 1.4 |
| 19-Tetracosenoic (24:1n-5) | 1.8 |
| Tetracosanoic (24:0) | 3.0 |
| 18-Pentacosenoic (25:1n-7) | 0.8 |
| 19-Pentacosenoic (25:1n-6) | 0.8 |
| 5,9-Hexacosadienoic (26:2n-17) | 0.7 |
| 17-Hexacosenoic (26:1n-9) | 0.5 |
| 19-Hexacosenoic (26:1n-7) | 0.9 |
| Heptacosenoic (27:1) | 0.1 |
| 5,9,19-Octacosatrienoic (28:3n-9) | 0.2 |
| 5,9,21-Octacosatrienoic (28:3n-7) | 0.1 |
| 5,9-Octacosadienoic (28:2n-19) | 0.1 |
| 19-Octacosenoic (28:1n-9) | 0.1 |
| 21-Octacosenoic (28:1n-7) | 0.3 |
| Octacosanoic (28:0) | 0.01 |
| Nonacosanoic (29:0) | 0.01 |
| 5,9,21-Nonacosatrienoic (29:3n-8) | 0.02 |
| 5,9,23-Nonacosatrienoic (29:3n-6) | 0.1 |
| 5,9,23-Triacontatrienoic (30:3n-7) | 4.8 |
| 21-Triacontenoic (30:1n-9) | 0.1 |
| 23-Triacontenoic (30:1n-7) | 0.1 |

[a]The following aldehydes were also identified in the sponge: 16:0, 17:0, 18:0, 19:0, 20:0, and 25:0.
[b]These acids are unprecedented in nature.

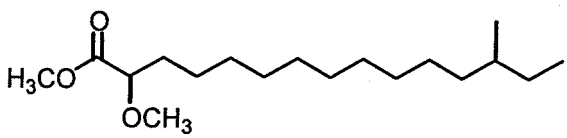
methoxyhexadecanoic acid, were previously reported by us from several Caribbean sponges (3,4). The finding in *A. complanata* that has never been observed before is that all three of these acids were present (at low levels) in the same sponge. Characterization of the remaining three saturated methoxylated acids (as either methyl or ethyl esters) was possible using GC–MS as well as gas chromatographic ECL values as compared to synthetic standards (4). For example, the mass spectrum of one of these methyl esters, **1a** (Scheme 1), displayed a molecular ion peak at *m/z* 286 and a strong $M^+ - 59$ peak at *m/z* 227 (100%), together with a small peak at *m/z* 104 (McLafferty rearrangement) typical of an α-methoxylated saturated methyl ester (4). On the other hand, both methyl esters **1b** and **2** yielded molecular ion peaks at *m/z* 300 and strong $M^+ - 59$ peaks at *m/z* 241, together with small peaks at *m/z* 104, confirming that both were α-methoxylated $C_{16}$ saturated methyl esters (4). The natural origin of the α-methoxy substitution was further confirmed when the corresponding ethyl esters were directly prepared from the phospholipids, since α-methoxylated ethyl esters were obtained. For example, in the mass spectrum of the ethyl ester of **1a**, the molecular ion peak shifted to *m/z* 300 and the McLafferty rearrangement peak to *m/z* 118, but the *m/z* 227 peak remained the same. In the mass spectrum of ethyl esters **1b** and **2**, the $M^+$ shifted to *m/z* 314 and the McLafferty rearrangement peak to *m/z* 118, but the *m/z* 241 peak remained the same. Therefore, this simple transesterification reaction (HCl/EtOH) proved that the methoxylated fatty acids are not artifacts arising from the HCl/MeOH reaction.

Although the α-methoxy substitution in these novel $C_{15}$–$C_{16}$ methyl esters was confirmed, the GC retention times suggested that all of these methoxylated methyl esters were branched. The position of the methyl branching was determined from ECL values and mass spectral data. For example, methyl esters **1a** and **1b** presented ECL values of 15.86 and 16.86, respectively, whereas **2** had an ECL value of 16.94 on the nonpolar capillary column. These values suggested methyl branching near the end of the chain, in particular when compared to a calculated ECL value of *ca.* 16.2 expected for a normal-chain methyl 2-methoxypentadecanoate and an ECL value of 17.2 reported for methyl 2-methoxyhexadecanoate (4). From the ECL values it was also clear that **1a** and **1b** had the same methyl substitution, while the methyl substitution in **2** was different. Additional data further favored an iso branching for **1a** and **1b**, and an anteiso branching for **2**. For example, the methyl branching in methyl 2-hydroxy-13-methyltetradecanoate was determined by actually synthesizing the methoxylated ester **1a** and further looking in its mass spectrum at the intensity of the *m/z* 43 fragment (11). Comparing the intensity of the *m/z* 43 fragment (loss of a propyl unit) with that of other fragments and further comparing their respective intensities to those observed in the mass spectrum of a normal-chain α-methoxy methyl ester are good ways to confirm the iso branching (11). In fact, the intensity of the *m/z* 43 fragments in **1a** and **1b** was increased compared to other mass peaks, such as the *m/z* 57 fragments. For this comparison the mass spectrum (measured under the same conditions as **1a** and **1b**) of methyl 2-methoxyhexadecanoate, previously synthesized and also present in this sponge, was used. In the case of **2,** a distinctive ion at $[M - 61]^+$ (*m/z* = 239) was observed, indicative of anteiso branching. Another piece of evidence favoring the iso branching for **1a** and **1b** and the anteiso branching for **2** was obtained from their GC retention times when gas chromatographic ECL values were calculated using only the α-methoxy methyl esters as standards. In such a calculation **1a** and **1b** present fractional chain-length (FCL) values of 0.67, while **2** has an FCL of 0.74, in reasonable agreement with those values reported for the traditional iso and anteiso fatty acid methyl esters (12). Therefore, it can be concluded that the α-methoxylated methyl esters in question are the iso methyl 2-methoxy-13-methyltetradecanoate (**1a**) and methyl 2-methoxy-14-methylpentadecanoate (**1b**), as well as the anteiso methyl 2-methoxy-13-methylpentadecanoate (**2**).

Pyrrolidide derivatives were also synthesized in an attempt to determine the methyl branching in the methoxylated acids by GC–MS, but fragmentations involving the polar methoxy group obscured the typical fragmentation pattern expected from a branched pyrrolidide (13). For example, in the mass spectrum of *N*-2-methoxy-13-methyltetradecanoylpyrrolidine a molecular ion peak ($M^+$) at *m/z* = 325 was observed together with strong fragmentations at *m/z* = 295 ($M^+ - 30$), *m/z* = 227 ($M^+ - 98$), and the typical McLafferty rearrangement at *m/z* = 143 confirming the α-methoxy functionality. However, a strong ($M^+ - 15$) peak at m/z = 310 was also identified, and the intensity of this ($M^+ - 15$) peak was higher than the corresponding peak in the mass spectrum of *N*-2-methoxyhexadecanoyl pyrrolidine, which we also synthesized. This observation also supported the iso-branching for **1a**. Therefore, pyrrolidide derivatization has limited usefulness in locating methyl branching in α-methoxylated fatty acids.




**1a, n=1**
**1b, n=2**




**2**

**SCHEME 1**

## DISCUSSION

This specimen of *A. complanata* contains a very complex phospholipid fatty acid profile typical of sponges belonging

to the genus *Amphimedon* (4,5,7). Noteworthy is its ability to extend the n-7 and n-9 families of even-numbered chain monounsaturated fatty acids from 9-18:1 and 11-18:1 to 21-30:1 and 23-30:1, respectively. In fact, this is the first report of both 21-triacontenoic acid and 23-triacontenoic acid from the phospholipids of an *Amphimedon* sponge. These $C_{30}$ fatty acids were reported before from sponges such as *Trikentrion loeve* (14). The n-6 and n-7 families predominated in the odd-numbered chain monounsaturated fatty acid series, but they mainly reached $C_{25}$–$C_{27}$ chain lengths.

The interesting feature of this *Amphimedon* is that it contains all of the $C_{16}$ short-chain α-methoxylated fatty acids reported to date plus a series of novel iso-anteiso α-methoxylated $C_{15}$–$C_{16}$ fatty acids. These are the first branched α-methoxylated phospholipid fatty acids from any natural source. An analogy to the corresponding iso-anteiso α-hydroxylated $C_{15}$–$C_{16}$ fatty acids is unavoidable, and in fact the methoxylated acids could have originated, although not necessarily, from the corresponding α-hydroxylated fatty acids. For example, the 2-hydroxy-13-methyltetradecanoic acid is known as a characteristic lipid constituent of gliding bacteria such as *Flexibacter elegans* (11); it has been identified in the phospholipids of Gram-positive Flavobacteria such as *Flavobacterium meningosepticum* and also, in addition to α-OH iso-$C_{16}$, in the Actinomycetales such as *Arthrobacter simplex* (15–17). The occurrence of α-OH anteiso-$C_{16}$ is rare, but it is known to be a constituent of the ceramide dihexosides from the spermatozoa of the starfish *Asterias amurensis* and a phospholipid constituent of *Arthrobacter simplex* (18,19). In addition, α-OH anteiso-$C_{16}$ has been identified in Antartic lake sediment (19).

The identification of small amounts of α-methoxylated iso-anteiso $C_{15}$–$C_{16}$ fatty acids in *A. complanata* suggests that these acids are probably constituents of a novel bacterium associated with the sponge. There are several pieces of information that point in this direction. One is their low abundance in the sponge, which indicates that they are not major sponge phospholipid constituents. The typical sponge fatty acids are the Δ5,9 acids, and several biosynthetic experiments tend to indicate that the shorter-chain $C_{15}$–$C_{19}$ fatty acids are actually of bacterial origin (21). A structural comparison of these α-methoxylated fatty acids with known α-hydroxylated fatty acids also suggests a bacterial origin, as most of the α-OH iso and anteiso $C_{15}$–$C_{17}$ acids are indeed bacterial in nature (22). In fact, some myxobacteria contain phosphatidylethanolamines as the major phospholipid with α-hydroxy iso-$C_{17:0}$ fatty acids in the 2-position and nonhydroxy fatty acids in the 1-position (22). Therefore, it is very likely that in *A. complanata* a novel symbiotic marine bacterium could actually contain novel phosphatidylethanolamines with iso and anteiso branched α-methoxylated fatty acids at the 2-position, similar to what is known for the α-hydroxy iso-$C_{17:0}$ fatty acids in myxobacteria (22).

## ACKNOWLEDGMENTS

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METHOD

# A Fast and Reliable Spectroscopic Method for the Determination of Membrane–Water Partition Coefficients of Organic Compounds

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**ABSTRACT:** Partition coefficients ($K_p$) between egg yolk phosphatidylcholine multilamellar vesicles and water were determined for two nonsteroidal anti-inflammatory drugs (indomethacin and acemetacin) using two independent methodologies: derivative spectrophotometry and variation of the experimental acidity constant in the presence of increasing vesicle concentration. Second-derivative spectrophotometry allowed for total elimination of background signal effects arising from lipid vesicles, without the need for separation techniques that may disturb equilibrium states. By using a model based on a simple partition, the values of $K_p^T$ can be obtained directly; furthermore, by performing determinations at two different pH values it is possible to calculate partition coefficients for the neutral and negatively charged forms of the drugs ($K_p^{AH}$ and $K_p^{A}$). In the other methodology, values of apparent acidity constants ($K_{app}$) were determined by spectrophotometry at different pH values and different lipid concentrations, and an increase in $K_{app}$ with decreasing lipid concentration was observed for both drugs, and from this dependence it was possible to calculate $K_p^{AH}$ and $K_p^{A^-}$ for each drug. These values were used as a check for those obtained by derivative spectroscopy, which has proven to be a reliable and more expeditious method to obtain $K_p^{AH}$ and $K_p^{A^-}$.

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The hydrophobicity of an organic compound is one of its physicochemical characteristics that extensively controls its absorption, biomembrane transport, bioaccumulation, and even environmental accumulation. Traditionally, the octanol–water partition coefficient has been used to measure compound hydrophobicity, which has then been correlated with drug activity, toxicity, and distribution processes (1). The octanol–water system is a good membrane model when polar group interactions between the solute and the phospholipid bilayer are minimal or absent, but better systems are needed for molecules that can establish this type of interactions (1–3), because octanol can only model nonpolar molecular interactions between solutes and membranes. Biomembranes and other lipid phases in organisms have a more complex structure than octanol; and hydrophobic, electronic, and steric forces contribute to the overall interaction with a molecule (1). Thus, the partitioning of solutes into bilayer membranes is argued to occur by a mechanism that is different from that into an oil phase (2,4). To overcome this problem, alternative systems have been developed to model the partitioning of a molecule into a lipid phase. Among these, polymer–water, micelle–water and cell–water, as well as systems using artificial membranes, such as immobilized artificial membranes or liposome suspensions, have been developed (2–9). Those with liposome suspensions not only present a good correlation with the behavior observed in membranes but also are easy to prepare and to handle.

Drug interactions with heterogeneous media typically induce changes in some physicochemical properties of the drug (spectroscopic properties, solubility, apparent acidity), and by monitoring these changes it is possible to quantify drug binding, which is normally expressed by two related quantities, drug/liposome association constants or partition coefficients between aqueous and lipid phases (5,7,10–13). Partition coefficients are important in defining the characteristics of absorption, distribution, metabolism, and excretion of a drug because they provide an indication of the extent of drug interaction with membranes and/or drug penetration in cells. If the drug's site of action is the membrane itself, then the determination of partition coefficient can help elucidate the drug's mechanism of action.

Partition coefficients are dependent on variables such as the nature of the membrane, temperature, and, when the drug and/or membrane carries ionizable groups, pH. Several methods have been devised to measure partition coefficients, namely, ultracentrifugation (4,9), hygroscopic desorption (14,15), microdialysis (6), chromatography (7,8), fluorimetry (10), and spectrophotometry (11–13,16). As most methods rely on phase separation, a process that can disturb the equilibrium state of the samples (12,13), it is of great interest to develop methods that may circumvent this time-consuming process. Direct application of spectrophotometric methods has been hampered by the intense background signals originating from light scattered by liposomes. Derivative spectrophotometry can eliminate background signals and improve the resolution of overlapping bands, and its application to liposome media, which avoids separation procedures, has been reported (11–13,16).

In this work we present two independent spectrophotometric methods to determine partition coefficients between aqueous and lipid pseudophases for two nonsteroidal anti-inflammatory drugs (indomethacin and acemetacin) in egg yolk phosphatidylcholine (EPC) multilamellar liposomes. The in-

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Abbreviation: EPC, egg yolk phosphatidylcholine.

teraction of indomethacin with EPC has previously been studied by differential scanning calorimetry (17), but the extension of the interaction was not quantified. Of these two spectrophotometric methods, one is based on the shift in drug acidity constant caused by an increase in phospholipid concentration, whereas the other relies on direct application of derivative spectrophotometry. The latter method not only provides a fast and reliable path to obtain partition coefficients for both forms (neutal and negatively charged) of the drugs but also requires only two determinations at different pH values. Its reliability was confirmed by the similarity of the values so obtained with those of the former method.

## EXPERIMENTAL PROCEDURES

*Reagents.* The anti-inflammatory drugs indomethacin and acemetacin and EPC were from Sigma, and the other chemicals from Merck (analytical grade; Darmstadt, Germany); all were used as received. Solutions were prepared with double-deionized water (conductivity less than 0.1 μS $cm^{-1}$).

*Vesicle preparation*. Multilamellar liposomes were prepared by hydration of a lipid film (18). In this method a known amount of EPC was taken in chloroform/methanol (9:1), and the suspension was evaporated to dryness under a stream of nitrogen. The lipid film was then hydrated, either with the appropriate buffer [Hepes (10 mM, $I$ = 0.1 M NaCl), acetate buffer (29.6 mM acetic acid, 70.4 mM sodium acetate; $I$ = 0.1 M NaCl)] or aqueous solution ($I$ = 0.1 M NaCl), and the mixture was stirred in a vortex. The EPC concentration in vesicle suspensions was determined by phosphate analysis using the phosphomolybdate method (20).

*Potentiometric measurements*. All potentiometric measurements were carried out with a Crison 2002 pH meter and 2031 piston buret controlled by a personal computer, which was also used for data manipulation. The electrode assembly was made up of an Orion 900029/4 Ag/AgCl reference electrode and a Russel SWL glass electrode, and the titrations were made in a double-wall cell. System calibration was performed by the Gran method (19) in terms of hydrogen ion concentration, using strong acid-strong base titration [HCl (0.001 M)/NaOH (≈0.02 M)] with solutions whose ionic strength was adjusted to 0.1 M with NaCl. Titrations were always carried out under a nitrogen atmosphere, and the temperature was kept constant at 25.0 ± 0.1ºC by circulating thermostated water in the cell.

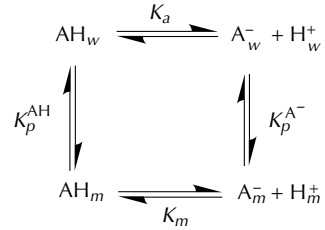
*Spectrophotometric determination of acidity constants*. All absorption spectra were recorded at 25.0 ± 0.1ºC with a Hitachi U-2000 dual-beam spectrophotometer, using quartz cells with a 1-cm path length. The temperature was kept constant by circulating thermostated water in the cell holder. Acidity constants of the anti-inflammatory drugs were obtained from ultraviolet/visible data in aqueous solution and in liposome media, and in all cases the ionic strength was adjusted to 0.1 M with NaCl. Drug/EPC suspensions were prepared by addition of drug to pre-formed EPC suspensions. Data were collected for at least six different drug concentrations in the range 20 to 80 μM, and for each drug concentration at least eight different suspensions of EPC were used (concentration between 0 and 360 μM). After an equilibration period of 2 h in the dark, aliquots of strong base or strong acid were added to these suspension to adjust –log [$H^+$] to the desired value (–log [$H^+$] range used: 3–9); –log [$H^+$] measurements and system calibration were performed by potentiometry as described above. Throughout this work the pH values referred are actually values of –log [$H^+$]. Acidity constants were calculated with the program SQUAD (21) by using data from at least two independent experiments, each with more than eight solutions, and in the wavelength range from 210 to 400 nm, at 2-nm intervals.

*Determination of partition coefficients by derivative spectrophotometry*. Partition coefficients were determined at pH 3.0, 5.0, and 7.4; at the extreme values the drugs are predominantly in the neutral and deprotonated form. At each pH, two sets of solutions containing different concentrations of EPC in the range 0–360 μM were prepared: solutions without drug, and solutions to which a fixed amount of drug was added (in each experimental run, as described above, drug concentration was kept constant and the different values used ranged from 20 to 80 μM). First-derivative spectra were calculated from the recorded spectra after blank subtraction, whereas second-derivative spectra were calculated directly from the recorded spectra. For each drug at least six independent experiments were performed at each pH.

In the experiments at pH 7.4, for which the pH was kept constant with Hepes buffer (10 mM, $I$ = 0.1 M NaCl), two methods were used to prepare drug/liposomes suspensions. In the incubation method, the lipid film was hydrated with Hepes and the drug dissolved in the same buffer was added after liposome formation; the suspensions were then incubated for 2 h. In the encapsulation method, the lipid film was hydrated with a buffer solution (Hepes) to which the drug had been previously added. At pH 3.0 and 5.0, only the incubation method was used: the drug was added to a suspension of liposome ($I$ = 0.1 M NaCl) in the appropriate buffer. The pH was adjusted to 3.0 by addition of a strong acid solution, and to 5.0 using an acetate buffer (29.6 mM acetic acid, 70.4 mM sodium acetate; $I$ = 0.1 M NaCl).

## RESULTS

In drug/EPC suspensions the acid-base equilibria in Scheme 1 must be considered (22) where $K_a$ and $K_m$ are drug acidity




**SCHEME 1**

constants in the aqueous and liposome pseudophases, $K_p^{AH}$ and $K_p^{A^-}$ are the partition coefficients of neutral (AH) and negatively charged ($A^-$) forms of the drug; the subscripts *w* and *m* stand for species in aqueous phase and in the liposome phase, respectively. The different equilibrium constants are defined next, where $V_L$ and $V_w$ stand for the lipid and aqueous solution volumes:

$$K_a = \left[H_w^+\right]\frac{\left[A_w^-\right]}{\left[AH_w\right]} \quad [1]$$

$$K_m = \left[H_w^+\right]\frac{\left[A_m^-\right]}{\left[AH_m\right]} \quad [2]$$

$$K_{app} = \left[H_w^+\right]\frac{\left[A_w^-\right]+\left[A_m^-\right]}{\left[AH_w\right]+\left[AH_m\right]} \quad [3]$$

$$K_p^{AH} = \frac{\left[AH_m\right]/V_L}{\left[AH_w\right]/V_w} \quad [4]$$

$$K_p^{A^-} = \frac{\left[A_m^-\right]/V_L}{\left[A_w^-\right]/V_L} \quad [5]$$

$$K_p^T = \frac{\left[AH_m\right]+\left[A_m^-\right]/V_L}{\left[AH_w\right]+\left[A_w^-\right]/V_w} \quad [6]$$

A few comments must be made regarding acidity constant definitions: $K_m$ corresponds to the value of $[H_w^+]$ of the bulk aqueous phase when $[A_m^-] = [AH_m]$, and the apparent acidity constant $K_{app}$ to the value of $[H_w^+]$ of the bulk aqueous phase when $[A_m^-] + [A_w^-] = [AH_m] + [AH_w]$. Finally, it must be stressed that $K_p^T$ is a global drug partition coefficient that accounts for the partition of both forms of the drug between the two pseudo-phases.

*Determination of partition coefficients using shifts in* $pK_{app}$. Any substance that ionizes in water exists normally in more than one form, and when in the presence of organized media (micelles or liposomes) these distinct forms have different binding constants (or partition coefficients; these two quantities are related). The concentrations of "free" (unbound) deprotonated and neutral forms are thus dependent on the extent of their interactions with the organized media, and consequently their apparent acidity constant, $K_{app}$, changes with lipid concentration (22). The values of $K_{app}$ are calculated directly from spectrophotometric data with the program SQUAD by assuming that all forms of the drug exist free in aqueous solution.

Shifts in $K_{app}$ arise from changes in the electrostatic potential and in the local dielectric constant of the liposome microenvironment, and can provide indirect information on the type of drug/liposome interactions (23). A number of different mathematical models have been developed to quantify micellar or membrane effects on equilibrium constants of different substances, and in these models the organized and aqueous media are assumed to form two separate pseudo-phases, with the drugs partitioned between them, eventually also exchanged with a counterion (23–27). In this work, the model proposed by Berezin (24) was used to provide a quantitative interpretation of changes in the apparent acidity constants of anti-inflammatory drugs in EPC liposomes (23–25). This model neglects ion exchange and it was chosen because it is the simplest model able to describe adequately the experimental results. Berezin and coworkers (24) proposed that the species formed upon dissociation of weak acids are partitioned differently between the liposomic and aqueous phases, and they expressed the apparent acidity constant, $K_{app}$, as

$$K_{app} = K_a \frac{1 + K_p^{A^-} C_L}{1 + K_p^{AH} C_L} \quad [7]$$

which, after linearization becomes:

$$\frac{\left(1 - \frac{K_{app}}{K_a}\right)}{C_L} = K_p^{AH}\frac{K_{app}}{K_a} - K_p^{A^-} \quad [8]$$

where $C_L$ represents EPC concentration. The values of $K_p$ obtained from these equations have dimensions of $M^{-1}$, and can be transformed to those in Equations 4–6 dividing by the lipid molar volume, $V_\Phi = 0.688\ M^{-1}$. The molar volume is obtained from the specific volume (0.983 mL/g) (28) and mean molecular weight (700) of EPC through equation $V_\Phi = V_L/(V_w \times C_L)$ (18).

The dependence with $C_L$ of the values of apparent acidity constants determined from spectrophotometric data, both in aqueous and in EPC media, calculated using the program SQUAD, are plotted in Figure 1. The values of $pK_{app}$ increase with increasing EPC concentration but level out to a constant value at high EPC concentrations. In aqueous solutions, the values of $pK_a$ determined for indomethacin and acemetacin were, respectively, 4.46 and 3.76. We note that the acidity constant obtained in aqueous solution for indomethacin is similar to that reported in the literature ($pK_a = 4.5$) (29,30).

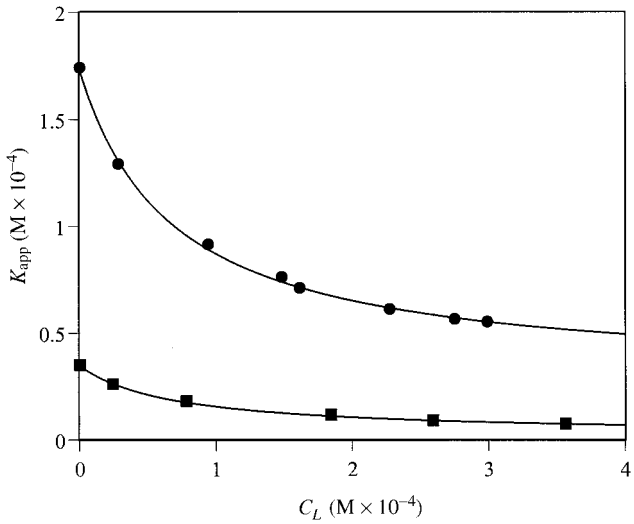



**FIG. 1.** The dependence of the apparent acidity constant, $K_{app}$, on egg yolk phosphatidylcholine (EPC) concentration for indomethacin (■) and acemetacin (●); the curve is the best fit by the Berezin model (Eq. 7).

Plots of $\left(1 - K_{app}/K_a\right)/C_L$ vs. $K_{app}/K_a$ for both drugs yielded straight lines and provided the corresponding partition coefficients. For indomethacin the slope is $1.40 \times 10^4$ and the intercept $7.28 \times 10^2$ ($R^2 = 0.98$), and for acemetacin the slope is $1.59 \times 10^4$ and the intercept $2.83 \times 10^3$ ($R^2 = 0.99$). The values of $K_p^{AH}$ and of $K_p^{A^-}$ are obtained by dividing the slope and the intercept, respectively, by $V_\Phi$. For indomethacin, $K_p^{AH} = 20{,}350$ and $K_p^{A^-} = 1{,}060$, and for acemetacin the values are 23,110 and 4,110, respectively. The data were then fitted by Equation 7 using nonlinear regression methods (DeltaGraph 4.0) ($R = 0.999$) and in Figure 1 the calculated curves are superimposed on the experimental points. The calculated values for the partition coefficients and the respective standard deviations are included in Table 1.

Alternatively, from the values of $K_p^{AH}$ and of $K_p^{A^-}$ determined by shifts in $K_{app}$ and from the $pK_a$ it is possible to calculate $K_p^T$ at any pH; the calculated values at pH 3.0, 5.0, and 7.4 are also included in Table 1.

*Determination of partition coefficients using derivative spectroscopy.* Normally, it is not possible to obtain partition coefficients using direct spectrophotometric methods, owing to the intense background signals that arise from light scattered by lipid vesicles. Thus, partition coefficients of lipid suspensions are typically determined in the aqueous phase after lipid separation. However, as the separation procedures are time-consuming and may disturb the equilibrium state of sample suspensions, an alternative procedure is to use derivative spectrophotometry, as it has been shown to eliminate the background signal and to improve the resolution of overlapping signals. This technique has been applied to the direct determination of drug partition coefficients in aqueous lipid suspensions without disturbing the equilibrium state (11–13).

Partition coefficients of indomethacin and acemetacin between lipid and aqueous phases were determined by derivative spectrophotometry in the presence of different concentrations of EPC (0–360 μM). Drug concentration was kept constant in each experimental run, and the partition coefficients obtained for experiments with different drug concentrations (between 20 and 80 μM) were found to be concentration-independent. Furthermore, as the drugs are carboxylic acids, experiments were performed at pH 3.0 and 7.4, values for which the drug exists predominantly in the neutral and deprotonated form, respectively, as well as at intermediate pH 5.0.

Light absorbance of both drugs decreases with an increase in EPC concentration. Figure 2 shows the second derivative spectra for acemetacin. The existence over a wide range of EPC concentration of three isosbestic points in second-derivative spectra provides an indication that (i) scattering due to the liposome is completely eliminated (12) and (ii) the system has only two states: drugs in polar bulk aqueous and in nonpolar EPC bilayer phases (11,12). Additional support for this latter assertion can be understood by noting the bathochromic (red) shift observed in $\lambda_{max}$ when the anti-inflammatory drugs are moved from aqueous solution to liposomic suspensions, as part of the drug molecules are transferred to nonpolar media (11,12).

The partition coefficient (Eqs. 4–6) can also be expressed in mole fractions:

$$K_p^T = \frac{\left(\left[F_m\right]/\left[F_t\right]\right)/C_L}{\left(\left[F_w\right]/F_t\right)/C_w} \quad [9]$$

where $[F_w]$ and $[F_m]$ stand for drug concentration in the aqueous solution and in the liposomes, respectively, $C_L$ is the concentration of EPC, and $C_w$ that of water. By representing total drug concentration by $[F_t]$, ($[F_t] = [F_w] + [F_m]$), and the mole fraction of drug bound to lipid by θ, it is possible to write Equation 9 as [the partition coefficient in this equation has dimension $M^{-1}$, see above]

$$K_p^T = \frac{\theta/C_L}{(1-\theta)/V_w} \quad [10]$$

Similarly with what is found for absorbance, the derivative intensity ($D_t$) at a specific wavelength is the sum of the derivative intensities in aqueous phase ($D_w$) and in the liposome phase ($D_m$), and is given by

$$D_t = E_w\left[F_w\right] + E_m\left[F_m\right] \quad [11]$$

in which $E_w$ and $E_m$ are the drug molar derivative intensities in water and in the lipid bilayer. The mole fraction of drug bound to the lipid, θ, is thus given by

$$\theta = \frac{\left[F_m\right]}{\left[F_t\right]} = \frac{D_w - D_t}{D_w - D_m} \quad [12]$$

From $[F_t] = [F_w] + [F_m]$, and by defining $E_t = E_m - E_w$ (12), Equation 11 becomes

$$D_t = E_w\left[F_t\right] + E_t\left[F_m\right] \quad [13]$$

From Equations 10, 12, and 13 it is possible to derive Equation 14, which allows the calculation of the partition coefficients of the protonated and neutral forms of indomethacin and acemetacin by nonlinear regression:

**TABLE 1**
**Partition Coefficients for Neutral and Negatively Charged Forms ($K_p^{AH}$ and $K_p^{A^-}$) of Indomethacin and Acemetacin in EPC Multilamellar Liposomes[a]**

| | Drug | |
|---|---|---|
| Partition coefficients | Indomethacin | Acemetacin |
| $K_p^{A^-}$ | 1,158 | 3,492 |
| $K_p^{AH}$ | 20,869 | 21,474 |
| $K_p^T$ (pH 3.0) | 20,208 | 18,811 |
| $K_p^T$ (pH 5.0) | 5,569 | 4,470 |
| $K_p^T$ (pH 7.4) | 1,181 | 3,496 |

[a]Obtained directly from apparent acidity constants experimental data. Global partition coefficients ($K_p^T$) calculated for different pH values assuming that $K_p^T$ is the weighted average of $K_p^{AH}$ and $K_p^{A^-}$. EPC, egg yolk phosphatidylcholine.

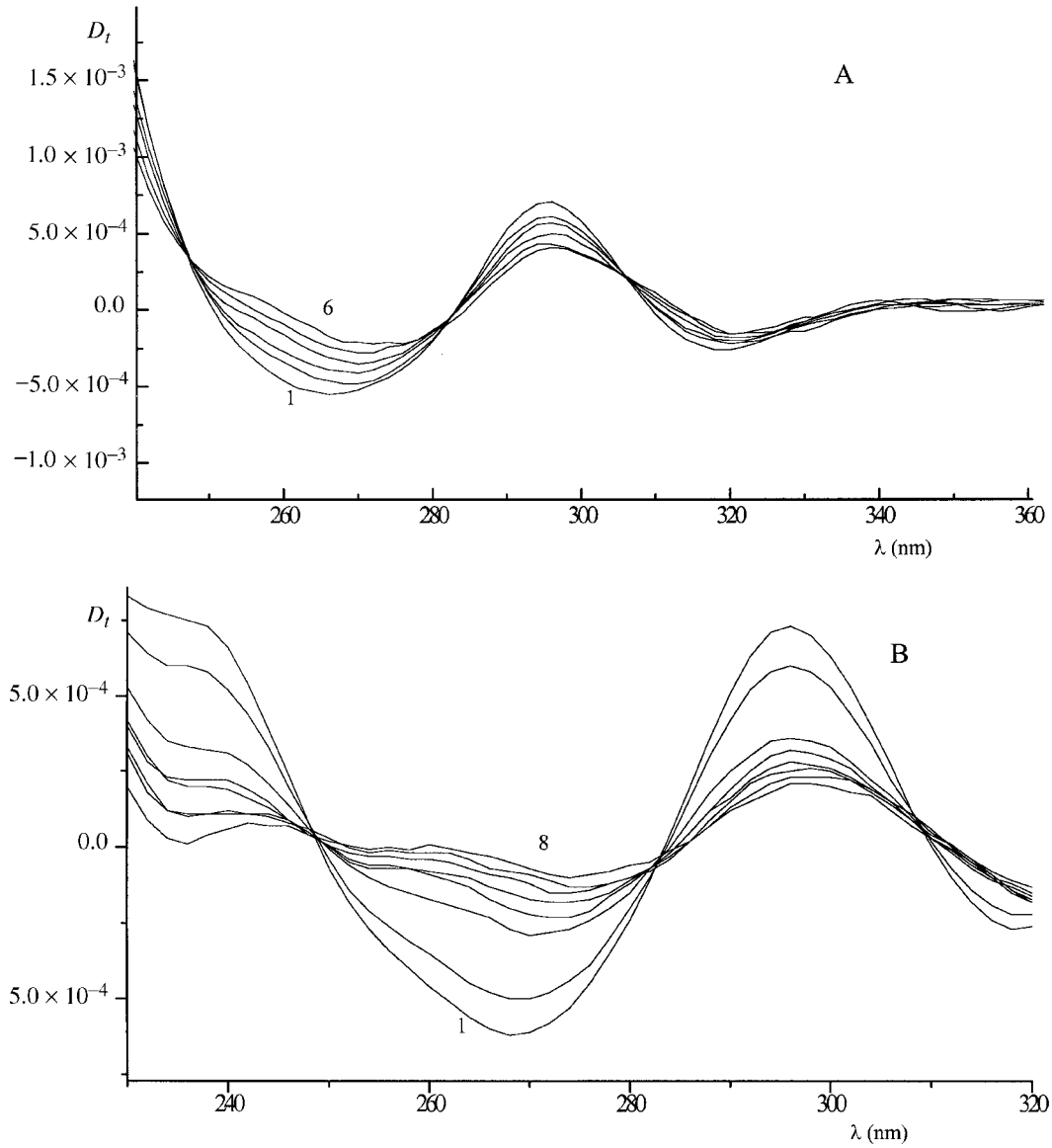



**FIG. 2.** Second-derivative spectra of acemetacin at different concentrations of EPC. At pH 3.0 (A); EPC concentration (μM): (1) 0; (2) 18; (3) 45; (4) 92; (5) 157; (6) 255. At pH 7.4 (B), EPC concentration (μM): (1) 0; (2) 19; (3) 77; (4) 121; (5) 147; (6) 171; (7) 229; (8) 276. For abbreviation see Figure 1.

$$D_t = D_w + \frac{E[F_t]K_p^T C_L}{1 + K_p^T C_L} \quad [14]$$

Equations 10 and 12 can be rearranged to yield a linear relation between $[1/(D_w - D_t)]$ and $(1/C_L)$:

$$\frac{1}{D_w - D_t} = \frac{1}{D_w - D_m} + \frac{1}{(D_w - D_m)K_p^T}\frac{1}{C_L} \quad [15]$$

and from a plot of $[1/(D_w - D_t)]$ vs. $1/C_L$, the intercept is $[1/(D_w - D_m)]$, and the slope allows for the determination of $K_p^T$.

Again, the linear form (Eq. 15) was used to test the applicability of the model and straight lines were obtained for both drugs. By using this approach, the following values were obtained for the total drug partition coefficients ($K_p^T$) (both forms): for indomethacin $1.84 \times 10^4$ at pH 3.0, $5.55 \times 10^3$ at pH 5.0, and $1.11 \times 10^3$ at pH 7.4; for acemetacin $1.95 \times 10^4$ at pH 3.0, $4.29 \times 10^3$ at pH 5.0, and $4.02 \times 10^3$ at pH 7.4. The experimental data were fitted to Equation 14 with correlation coefficients higher than 0.99, and the values for the partition coefficients, which are similar to those obtained from the double reciprocal plots, are included in Table 2. In Figure 3 the experimental values of $D_t$ of indomethacin and acemetacin at pH 3.0 are plotted as a function of $C_L$; superimposed is the curve that best describes the experimental data. The values of $K_p^T$ were found to be independent of drug concentration in the range studied (20 to 80 mM), and of the method used in liposome preparation.

It must be pointed out that by subtracting from the first-derivative spectra, those absorption spectra of the drug-free liposome solutions, it is possible to obtain spectra with much

**TABLE 2**
**Global Partition Coefficients ($K_p^T$) for Indomethacin and Acemetacin in EPC Multilamellar Liposomes[a]**

| Global partition coefficients | | Drug: Indomethacin | Drug: Acemetacin |
|---|---|---|---|
| $K_p^T$ (pH 3.0) | | 20,834 ± 1490 | 20,465 ± 1970 |
| $K_p^T$ (pH 5.0) | | 5,755 ± 390 | 4,500 ± 420 |
| $K_p^T$ (pH 7.4) | | 1,150 ± 80 | 3,583 ± 340 |
| Calculated partition coefficients | | | |
| pH 3.0 and 5.0 | $K_p^{A^-}$ | 1,211 | 3,411 |
| | $K_p^{AH}$ | 21,514 | 23,429 |
| pH 3.0 and 7.4 | $K_p^{A^-}$ | 1,128 | 3,578 |
| | $K_p^{AH}$ | 21,517 | 23,400 |
| pH 5.0 and 7.4 | $K_p^{A^-}$ | 1,127 | 3,580 |
| | $K_p^{AH}$ | 21,806 | 20,495 |

[a]Obtained directly from derivative spectrophotometry experimental data at different pH values. Partition coefficients for neutral and negatively charged forms ($K_p^{AH}$ and $K_p^{A^-}$) calculated assuming that $K_p^T$ is the weighted average of $K_p^{AH}$ and $K_p^{A^-}$.

reduced scattering. These can be used to determine partition coefficients by the process described above and yield similar results. However, second-derivative spectroscopy is the preferred method, not only because the results exhibit a smaller dispersion but also because it is less time consuming, as there is no need to obtain spectra of drug-free solutions.

The values of $K_p^T$ obtained from derivative spectroscopic data are related to total drug, and not to the individual partition coefficients of neutral and anionic forms. However, from the values of $K_p^T$ determined at two different pH values it is possible to obtain individual partition coefficients, because any of the anti-inflammatory drugs studied exist as a conjugate pair in solution. As their acidity constants ($K_a$) are known, by solving the two equations resulting from applying the Henderson-Hasselbalch at each of the two different pH for which the values of $K_p^T$ are known, it is possible to obtain the mole ratios [AH]/([AH] + [$A^-$]) and [$A^-$]/([AH] + [$A^-$]). Furthermore, as $K_p^T$ is the weighted average of $K_p^{AH}$ and $K_p^{A^-}$, from the values of $K_p^T$ and the calculated mole ratios at two pH values, it is straightforward to calculate $K_p^{AH}$ and $K_p^{A^-}$. The values calculated from data obtained at three pH values are included in Table 2.

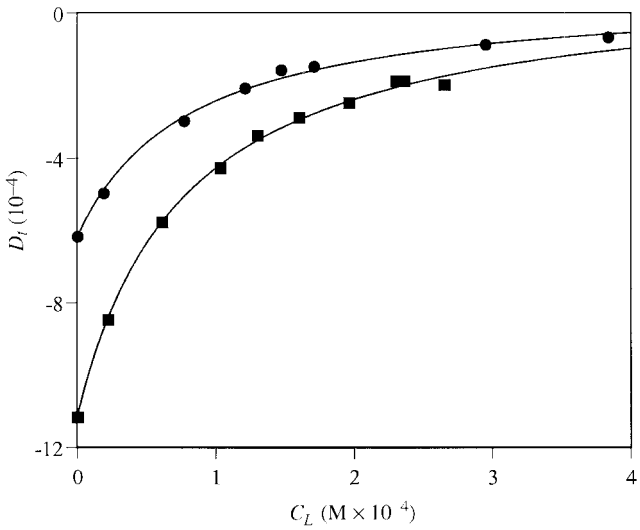



**FIG. 3.** Second-derivative spectrophotometric data at λ = 268 nm for indomethacin (■) and acemetacin (●) at pH 3.0 at different EPC concentrations; the curves represent the best fit by Equation 14. For abbreviation see Figure 1.

*Comparison of the results obtained by the two methodologies.* We start by pointing out that the values of $K_p^{AH}$ and $K_p^{A^-}$ calculated from derivative spectrophotometry using the Henderson-Hasselbalch equation are very similar to those obtained from shifts in $K_{app}$ (Tables 1 and 2).

By inserting the values of $K_a$ and of $K_p^{AH}$ and $K_p^{A^-}$ in the cycle of Scheme 1, it is possible to calculate the acidity constant of the drugs in the liposome pseudo-phase. Values of 5.72 for indomethacin and of 4.55 for acemetacin were obtained for p$K_m$ using values from shifts of p$K_{app}$; data from derivative spectrophotometry yielded values of 5.73 and 4.52. The fact that acidity constants are smaller in the liposome pseudo-phase is a consequence of preferentially binding by the neutral (protonated) form to the liposome. Furthermore, as the difference p$K_m$ – p$K_a$ is roughly 1.3 for indomethacin and only 0.8 for acemetacin the deprotonated form of the latter interacts more extensively with EPC than the deprotonated form of indomethacin.

To assess the quality of the data and the agreement of the results from the two methodologies, we note that by using the values of $K_p^T$ obtained from derivative spectrophotometry and the acidity constant it is possible to calculate values of $K_{app}$ for different concentrations of EPC by using Equation 3, which states that p$K_{app}$ = –log [$H^+$] when [$A_m^-$] + [$A_w^-$] = [$AH_m$] + [$AH_w$]. In Figures 4A and 4B such a plot is presented for acemetacin, and the values obtained are identical, within experimental error, to the measured values of p$K_{app}$.

Finally, to assess the invariability of $K_m$ with EPC content, the values of $K_p^{AH}$ and of $K_p^{A^-}$ can be used to calculate $K_m$ (Eq. 2) at several EPC concentrations, as $K_m$ = –log [$H^+$] when [$A]_m$ = [$AH_m$]. In Figure 4C the fraction of bound neutral and deprotonated forms of acemetacin are plotted vs. pH, and it is clear that the values of $K_m$ so calculated are independent of EPC concentration.

## DISCUSSION

First, it must be stressed that the values of $K_p^{AH}$ and $K_p^{A^-}$ obtained using the two methodologies are identical within experimental error (Tables 1 and 2), which supports the use of derivative spectroscopy in the determination of partition coefficients. With regard to drug interaction with EPC, our data show that the neutral form interacts more strongly with EPC than the charged form, an observation that supports the suggestion of Hwang and Shen (17) for indomethacin that the interaction of the drug takes place predominantly with the nonpolar matrix of the bilayer, and we propose that the anti-in-

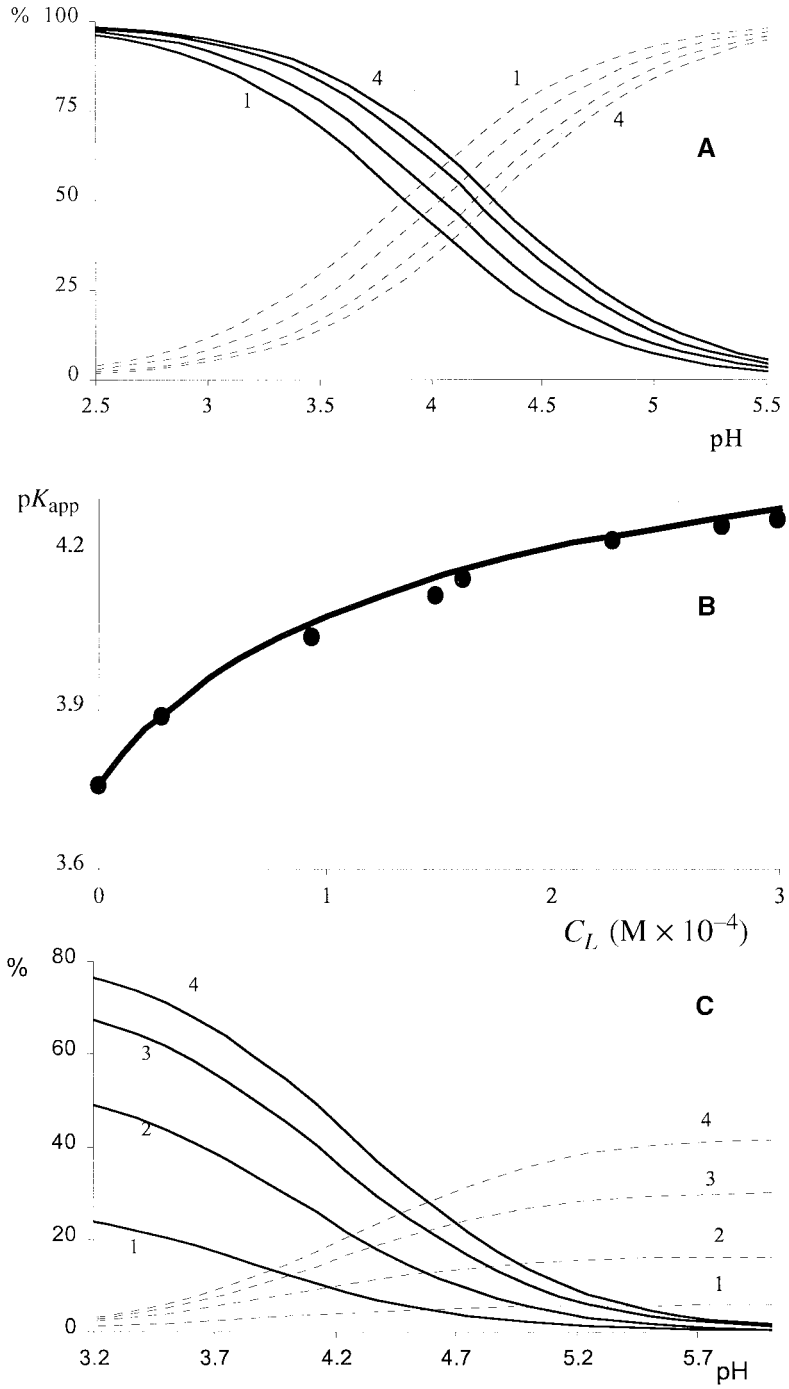



**FIG. 4.** (A) Distribution diagram for total (aqueous and lipid bound) neutral (solid) and deprotonated (dashed) forms of acemetacin at different EPC concentrations [(1) 25; (2) 80; (3) 180, and (4) 300 μM]. (B) Values of $pK_{app}$ calculated from the pH at which the curves in (A) intercept (solid curve) and experimental values obtained by direct spectrophotometry (●). (C) Distribution diagram for lipid bound forms of acemetacin (neutral: solid; deprotonated: dashed) calculated from derivative spectrophotometry values at several concentration of EPC [(1) 25; (2) 80; (3) 180, and (4) 300 μM]. The intercept of the curves yields $K_m$. For abbreviation see Figure 1.

flammatory molecules must be interdigitated between the hydrocarbon chains of the phospholipid. This model accounts for the smaller partition coefficients obtained for the charged drug forms, as the negatively charged carboxylic groups must be at the surface and facing the aqueous solution, which reduces the extent of interdigitation between the hydrophobic chains of the phospholipid and creates a repulsion between the charged groups of the drug and the negatively charged heads of the phospholipid, and thus renders drug incorporation less favorable.

The determination of partition coefficients from shifts in $pK_{app}$ is a well-established method, albeit somewhat tedious, as it requires extensive data sets and a very precise potentiometric system. It has been used to provide partition coefficients that have been used as a check for those obtained by derivative spectroscopy. This latter methodology, on the other hand, is a fast and easily implemented method to obtain values of $K_p^T$ for drugs in vesicles; and by using the approach outlined above, which assumes $K_p^T$ to be the weighted average of $K_p^{AH}$ and $K_p^A$, it is straightforward to calculate these latter partition coefficients from values of $K_p^T$ at two different pH values. As a final comment, we point out that the use of derivative spectroscopy has proved to be a fast and reliable method to obtain partition coefficients for drugs in vesicles, thus addressing the obvious limitations of the water–octanol method.

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# A Novel Assay Method for Glycosphingolipid Deacylase by Enzyme-Linked Immunochemical Detection of Lysoglycosphingolipid

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**ABSTRACT:** Lysoglycosphingolipids consist of a sphingoid long-chain base and monosaccharide or complex sugar, and they lack the fatty acyl group present in native glycosphingolipids. Less than 1 pmol of lyso-Forssman glycolipid and lysoganglioside GM1 were detected on a thin-layer chromatogram by an enzyme-linked immunochemical coloration method with anti-Forssman glycolipid antibody (FOM-1) and cholera toxin B subunit, respectively. Each spot between 1 and 100 pmol lyso-Forssman glycolipid was immunostained as densely as that of the same amount of native Forssman glycolipid. The density of the lyso-Forssman glycolipid spots increased proportionally with increment in the amount of lysoglycolipid. The density of spots of 0.2–100 pmol lysoganglioside GM1 was also proportional to the amount of each lyso-GM1 spot. These results indicated that less than 1 to 100 pmol of deacylated glycosphingolipid was quantifiable by the immunochemical coloration method with sugar chain-specific antibodies. Glycosphingolipid deacylase, which cleaved an amide bond between the sphingoid long-chain base and fatty acyl chain in ceramide of glycosphingolipid, was assayed by detecting the lyso-Forssman glycolipid produced. Lipophilic compounds, recovered from an aliquot of the reaction mixture of Forssman glycolipid and crude enzyme at appropriate times, were analyzed by thin-layer chromatography. It was found that lyso-Forssman glycolipid was produced in the first 1–2 h by the enzyme and production increased with incubation time. This coloration method is more sensitive and specific than the visualization method with a nonspecific reagent such as orcinol-sulfuric acid reagent.

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Glycosphingolipidoses are metabolic disorders that occur accompanying an abnormal storage of glycosphingolipids. Lysoglycosphingolipids, which consist of a sphingoid long-chain base and monosaccharide or complex sugar, have been reported to be present in the lipid storage. Svennerholm *et al.* (1) found galactosylsphingosine in brains taken from patients with Krabbe disease. They also found glucosylsphingosine in brains, spleens, and livers of patients with Gaucher disease (2,3). Accumulation of lysosulfatide has been found in the brain of a patient with metachromatic leukodystrophy (4,5) and in a normal brain (5). Lysoganglioside GM2 has been detected in the brain of a patient with Tay-Sachs disease (6), and lysogangliosides GM1 and GM2 have been detected in tissues of patients with Sandhoff disease, Tay-Sachs disease, and GM1 gangliosidoses (7).

Lysoglycosphingolipids lack the fatty acyl group present in native glycosphingolipids. Glycosphingolipid deacylase (8–10) and sphingosine ceramide deacylase (11) have been shown to hydrolyze the amide bond between the sphingosine base and fatty acid in the ceramide moiety of glycosphingolipid and to release lysoglycosphingolipid and fatty acid. The existence of these enzymes has been proven only in bacteria. Glycosphingolipid deacylase has been found on the bacterial membrane of *Rhodococcus* sp. (8), *Nocardia* sp. (9), and *Streptomyces* sp. (10), and sphingosine ceramide deacylase has been found in the culture broth of *Pseudomonas* sp. (11). Glycosphingolipid deacylase and sphingosine ceramide deacylase have different substrate specificities, although both enzymes have been shown to hydrolyze glycosphingolipid into lysoglycosphingolipid and fatty acid. Glycosphingolipid deacylase hydrolyzed only glycosphingolipid with a long sugar chain (more than three sugars), and sphingosine ceramide deacylase hydrolyzed glycosphingolipid with more than one sugar and sphingomyelin. The existence of the enzymes was demonstrated by proving the production of lysoglycosphingolipids on a thin-layer chromatogram (TLC) visualized with resorcinol-hydrochloric acid reagent or orcinol-sulfuric acid reagent (8–11). The release of fatty acid from radioisotope-labeled glycosphingolipid was also reported to assay the enzyme (8,9). The existence of deacylase in eucaryotes, such as mammals, however, has not yet been proven, probably because the products of the enzymes are minor among the abundant glycolipids of mammalian cells and are hydrolyzed by glycosidases.

Antiglycosphingolipid antibodies and lectins that recognize the sugar chains of glycosphingolipids have been developed. Higashi *et al.* (12) reported a highly sensitive method for detecting glycosphingolipids on TLC by an enzyme-immunochemical staining method. Rosengren *et al.* (5) quantified lysosul-

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Abbreviations: FOM-1, anti-Forssman glycolipid antibody, manufactured by Biomedicals AG (August, Switzerland); $Gb_4Cer$, globoside; HPTLC: high-performance thin-layer chromatography; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone; TLC, thin-layer chromatography.

fatide and *N*-acetyllysosulfatide by radioimmunoaffinity TLC. In the present study, we demonstrated that lysoglycosphingolipids with pentose as the hydrophilic moiety were detectable at the same detection limit as that of the native glycosphingolipid by an immunostaining method with glycosphingolipid-specific antibodies. In this paper, we describe a new method for detecting glycosphingolipid deacylase by visualization of lysoglycosphingolipid formed by the enzyme.

## EXPERIMENTAL PROCEDURES

*Materials and reagents.* Anti-Forssman glycolipid antibody (FOM-1) was a product of Biomedicals AG (August, Switzerland), and the biotin-labeled second antibody (biotin-rabbit anti-rat immunoglobulin M) and horseradish peroxidase (HRP)-labeled third antibody (HRP-mouse anti-biotin) were purchased from Zymed (San Francisco, CA). Cholera toxin B subunit-HRP conjugate (Research Biochemicals International, Natick, MA) was used to stain ganglioside GM1. A Polygram Sil G TLC plate (No. 805013; Macherey-Nagel, Düren, Germany) was used for coloration of glycosphingolipids with specific antibodies.

The bacterium, *Rhodococcus* sp. strain GL-26, isolated in our laboratory (8), was cultivated in a medium of 5 g/L of phytone peptone (BBL, Cockeysville, MO), 5 g/L of yeast extract (BBL), and 5 g/L of glucose (pH 7.0). The cells were harvested, washed twice with 50 mM tris-HCl buffer (pH 7.4), suspended in the same buffer, and incubated in the presence of globoside ($Gb_4Cer$) at 2 mg/mL to induce glycosphingolipid deacylase. Induced cells were harvested, washed, and frozen at −80°C until use. After the cells had been disrupted by sonication, the cell membranes were collected by ultracentrifugation at 100,000 × *g* for 1 h.

Ganglioside GM1 and lysoganglioside GM1 were purchased from Sigma (St. Louis, MO). $Gb_4Cer$, the inducer of glycosphingolipid deacylase, was extracted from porcine erythrocyte membrane and purified by Iatrobeads column chromatography (13). Forssman glycolipid ($IV^3GalNAc\alpha$-$Gb_4Cer$) was extracted from equine kidney, purified by Iatrobeads column chromatography, acetylated (14,15), purified by Iatrobeads column chromatography again, and deacetylated. The purity of Forssman glycolipid was checked with high-performance TLC (HPTLC; No. 5631, Merck, Darmstadt, Germany) by developing with the solvent system of chloroform/methanol/water/acetic acid (60:35:7:1, by vol) or chloroform/methanol/2.5 M ammonia (5:4:1, by vol) and visualizing with orcinol-sulfuric acid reagent. Forssman glycolipid gave a single band on the HPTLC with each solvent system.

Deacylation of Forssman glycolipid was performed by a procedure similar to that used for the induction of GL-26 cells using Forssman glycolipid as the inducer instead of $Gb_4Cer$. Whole lipophilic compounds, recovered from the deacylation mixture of Forssman glycolipid and the bacteria, were applied on a reversed-phase HPLC column (STR HPLC Column Pre-ODS, 250 × 20 mm, i.d.; Shimadzu, Kyoto, Japan). Lyso-Forssman glycolipid was eluted from the column with a linear gradient of methanol and chloroform/methanol/distilled water (60:30:4.5, by vol). The purity of lyso-Forssman glycolipid was checked by the same method as that used to check the purity of Forssman glycolipid. Lyso-Forssman glycolipid also gave a single band.

*Immunochemical coloration of glycosphingolipids on TLC.* Sensitive coloration of glycosphingolipids with a glycosphingolipid-specific antibody was performed according to the procedure reported by Higashi *et al.* (12) with a minor modification. Forssman glycolipid and lyso-Forssman glycolipid were separated on a Polygram Sil G TLC plate by developing with a solvent system of chloroform/methanol/distilled water/acetic acid (60:40:8:1, by vol). After complete removal of the solvent from the TLC by leaving the plate in air followed by blowing hot air, the plate was soaked in a solution of 1% ovalbumin (2× crystallization; Seikagaku Corp., Tokyo, Japan) and 1% polyvinylpyrrolidone (PVP) in phosphate-buffered saline (PBS) in an appropriate-sized plastic box. The plate was shaken gently (about 30 rpm) for 1 h at 37°C followed by washing five times with PBS. Forssman glycolipid and lyso-Forssman glycolipid on the plate were detected by subsequent immunochemical reactions with FOM-1, biotin-labeled second antibody, and HRP-labeled anti-biotin antibody. HRP-conjugates on the plate were stained blue by peroxidase-specific chromogenic reagents (HP-1000; Konica, Tokyo, Japan).

Ganglioside GM1 and lysoganglioside GM1, separated with the solvent system of chloroform/methanol/distilled water/acetic acid (50:40:9:1, by vol), were detected with cholera toxin B subunit-HRP conjugate and the chromogenic reagents.

The plate image was replicated with a scanner (Epson GT-9600), a computer (Macintosh G4), and the software program Adobe Photoshop 5.5 (Sanvosa, CA). The plate image data was saved in a PICT file, which was then opened using the software NIH Image 1.62. Each blue spot was enclosed with an ellipse, and the area and mean density of the ellipse were computed using the software. The total density of each spot was calculated by multiplying the area and mean density. The relative density was calculated from the total density by dividing by that of 10 pmol of native glycosphingolipid.

## RESULTS

*Immunochemical coloration of lyso-Forssman glycolipid.* Lyso-Forssman glycolipid ($R_f$, 0.18) was separated from Forssman glycolipid ($R_f$, 0.41) by developing on a Polygram Sil G TLC plate with a solvent system of chloroform/methanol/distilled water/acetic acid (60:40:8:1, by vol). Both the lyso-Forssman glycolipid and native Forssman glycolipid were visualized in blue spots by the immunochemical coloration method as described in the Experimental Procedures section. Although spots of 0.2 and 0.5 pmol lyso-Forssman glycolipid and a spot of 0.2 pmol Forssman glycolipid were not distinguishable from the background or control (0 pmol), more than 1 pmol of lyso-Forssman glycolipid and more than 0.5 pmol Forssman glycolipid were stained visibly by this method (Fig.

1A). Each spot of 1 to 100 pmol of lyso-Forssman glycolipid was shown to be as dense as that of the same amount of Forssman glycolipid. The density of each spot was computed, and the relative density was calculated with a spot of 10 pmol of Forssman glycolipid as the standard spot (Figs. 1B and 1C). The relative density of lyso-Forssman glycolipid was shown to increase proportionally with the increment in the amount of glycolipid applied to the TLC, although spots of 0.2 and 0.5 pmol lyso-Forssman glycolipid and 0.2 pmol Forssman glycolipid, which were not distinguishable from the background, did not appear on the calibration line. The amount of lyso-Forssman glycolipid between 1 and 100 pmol was demonstrated to be computable from the density.

*Immunochemical coloration of lysoganglioside GM1.* Lysoganglioside GM1 ($R_f$, 0.23) was clearly separated from ganglioside GM1 ($R_f$, 0.32) by Polygram Sil G TLC. Ganglioside GM1 and lysoganglioside GM1 were detected with cholera toxin B subunit-HRP conjugate and peroxidase-specific chromogenic reaction. Spots of 0.05 pmol of deacylated or native ganglioside GM1 were indistinguishable from the background, but faint spots of 0.1 pmol of both lysoganglioside GM1 and native ganglioside GM1 were observed (Fig. 2A).

From the calculation of relative density, each spot of between 0.2 and 100 pmol of lysoganglioside GM1 was immunostained as densely as that of the same amount of ganglioside GM1 (Figs. 2B and 2C). The relative density of lysoganglioside GM1 was shown to increase proportionally with increment in the amount of glycolipid.

*Quantitative analysis of glycosphingolipid deacylase by determining lyso-Forssman glycolipid production.* Glycosphingolipid deacylase hydrolyzes Forssman glycolipid, concluding production of lyso-Forssman glycolipid. The amount of lyso-Forssman glycolipid in the reaction mixture of the glycolipid and the enzyme was determined in high sensitivity by the enzyme-linked immunochemical detection method with the antibody FOM-1. As shown in Figure 3, it was observed that lyso-Forssman glycolipid was produced in the first 1–2 h of incubation with a GL-26 membrane. It was also demonstrated that the amount of lyso-Forssman glycolipid increased with incubation time and with the amount of enzyme source. Glycosphingolipid deacylase (120 μg membrane protein) was calculated to liberate 4.61 pmol of lyso-Forssman glycolipid per hour (38.5 pmol/h/mg membrane protein).

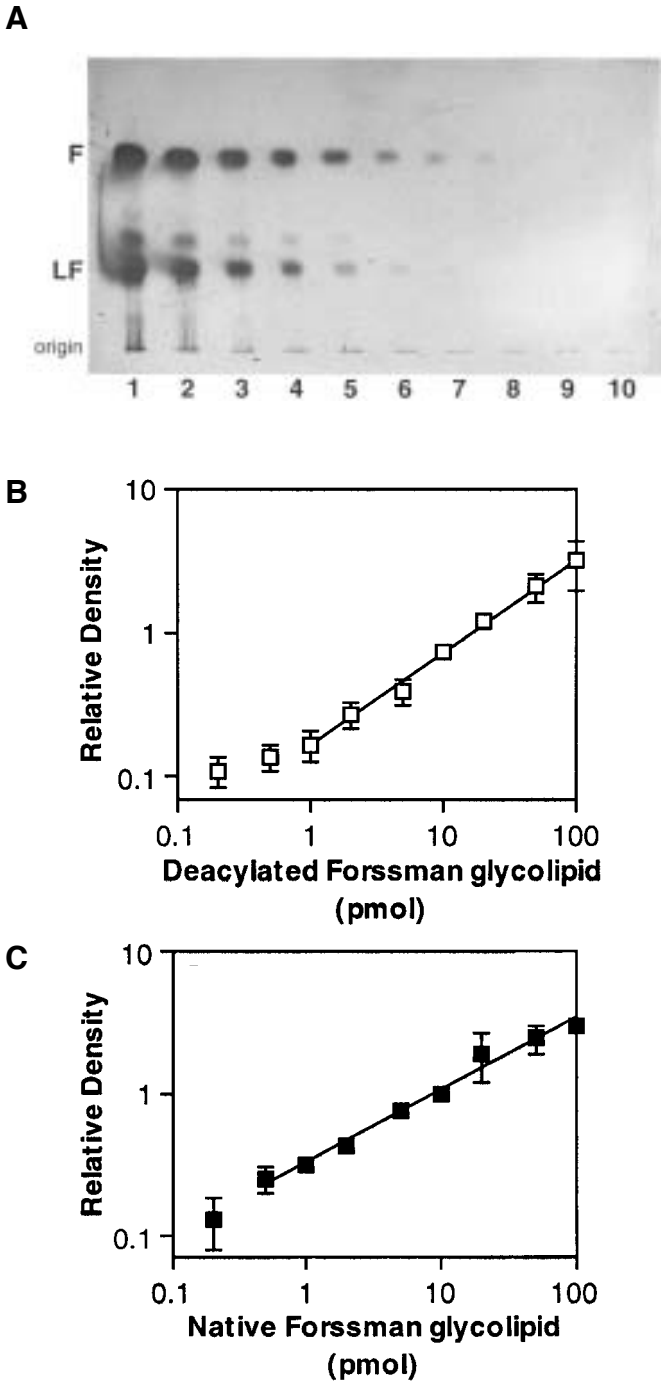


**FIG. 1.** Calibration curves of thin-layer chromatography (TLC)-immunostaining of lyso-Forssman glycolipid (LF). The amount of LF in the original mixture was determined by densitometric scanning of high-performance TLC visualized with orcinol-sulfuric acid reagent spray with Forssman glycolipid (F) as the standard. (A) One of four immunostained TLC plates of LF and F. Serially decreasing amounts of F and LF were applied on the same plate of Polygram Sil G. The plate was developed and stained by the enzyme-linked immunochemical method as described in the Experimental Procedures section. The amounts were as follows (pmol); lane 1, 100; lane 2, 50; lane 3, 20; lane 4, 10; lane 5, 5; lane 6, 2; lane 7, 1; lane 8, 0.5; lane 9, 0.2; lane 10, 0. (B) Standard curve for LF (□). (C) Standard curve for F (■). Symbols represent the average of the relative density (total density of each spot/total density of 10 pmol F) of four TLC plates; bars represent the standard deviation.

## DISCUSSION

It has been demonstrated that it is possible to determine the density of a spot using the combination of a computer, scanner, and software (PhotoShop and NIH Image), all commonly available these days.

It was also demonstrated that it is possible to calculate the amount of deacylated glycosphingolipid by computing the density of spots that had been stained by the enzyme-linked immunochemical coloration method. The detection limits of both native glycosphingolipid and its deacylated compound on immunostained plates were the same. These results con-

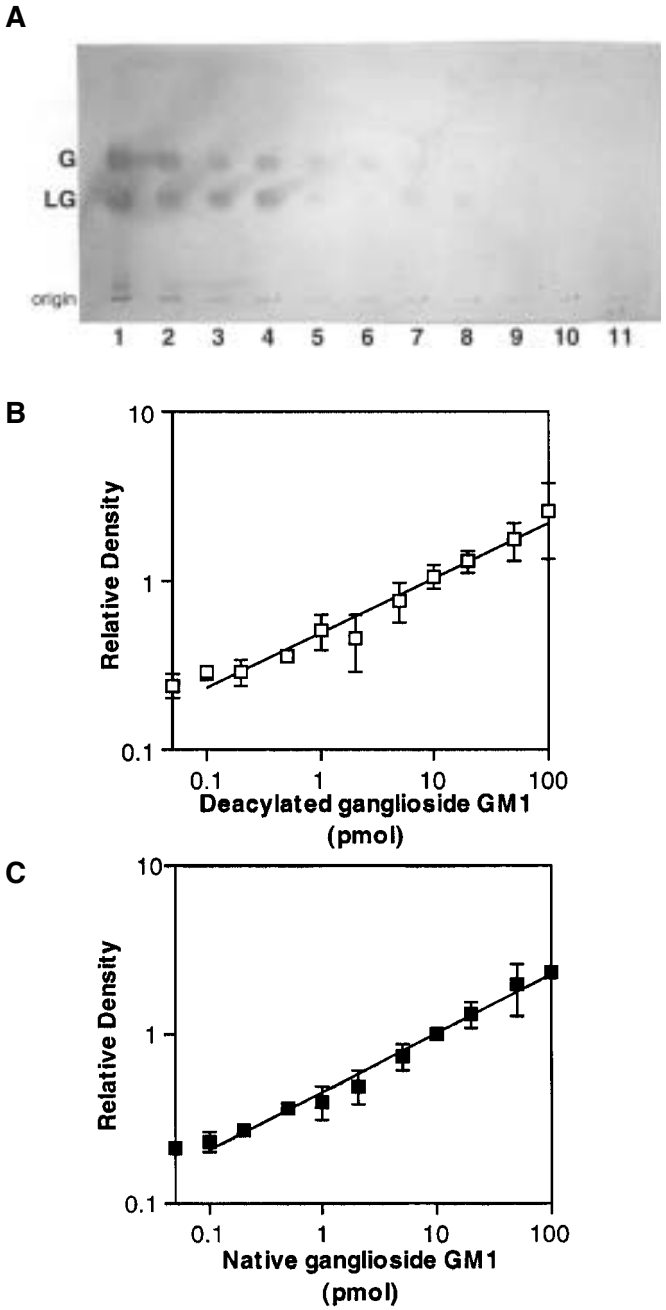


**FIG. 2.** Calibration curves of TLC-immunostaining of lysoganglioside GM1 with cholera toxin B. (A) One of three immunostained TLC plates of lysoganglioside GM1 (LG) and ganglioside GM1 (G). Serially decreasing amounts of G and LG were applied on the same TLC plate and immunostained as described in the Experimental Procedures section. The amounts were as follows (pmol); lane 1, 100; lane 2, 50; lane 3, 20; lane 4, 10; lane 5, 5; lane 6, 2; lane 7, 2; lane 8, 0.5; lane 9, 0.2; lane 10, 0.1; lane 11, 0.05. (B) Standard curve for LG (□). (C) Standard curve for G (■). Symbols represent the average of the relative density (total density of each spot/total density of 10 pmol G) of three TLC plates, and bars represent the standard deviation. For abbreviations see Figure 1.

firmed that sugar chains of Forssman glycolipid and ganglioside GM1 were recognized by FOM-1 and cholera toxin B subunit, respectively, and that modification of the ceramide part of the glycolipids had no effect on antibody recognition.

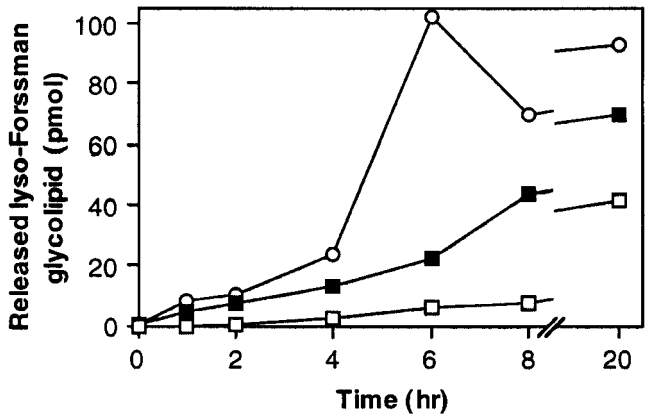


**FIG. 3.** Enzymatic production of LF by glycosphingolipid deacylase. F (1.6 μg, *ca.* 1.10 nmol) was incubated with the membrane of *Rhodococcus* sp. strain GL-26 in the presence of 3-(*N*-morpholino)propane sulfonic acid (40 μmol), cholate (0.4 μmol), and $CaCl_2$ (8 μmol) in 0.8 mL. An aliquot of 100 μL was withdrawn at each time and applied on a Sep Pak $C_{18}$ cartridge (1 μL; Waters, Milford, MA). After salts had been removed by washing the cartridge column with 1 mL of distilled water, lipophilic compounds were recovered by eluting 0.5 mL of methanol and 1 mL of chloroform/methanol (2:1, vol/vol). The combination of the eluted solution was dried *in vacuo* and applied on a Polygram Sil G plate. The plate was developed and immunostained as described in the Experimental Procedures section. F (10 pmol) was developed on the same plate as the standard material to calculate relative density. □, 60 μg of protein; ■, 120 μg of protein; ○, 180 μg of protein in the aliquot. See Figure 1 for abbreviation.

From less than 1 to 100 pmol of glycosphingolipid as well as the native glycosphingolipid were sustained on the Polygram Sil G plate during the procedure of blocking with ovalbumin; immunochemical reactions with the primary, second, and third antibodies, or lectin; and washing steps. Although deacylated glycosphingolipid, which possessed only a sphingoid long-chain base as the hydrophobic moiety and lacked a fatty acyl chain, was more hydrophilic than the native glycosphingolipid, no lysoglycosphingolipids seemed to be lost during the procedure.

Glycosphingolipid deacylase cleaved the amide bond between the sphingoid long-chain base and fatty acyl chain in ceramide of glycosphingolipid (8–10). These enzymes, which hydrolyzed only glycosphingolipids with more than three saccharides in the hydrophilic moiety, existed on the bacterial membrane and have never been solubilized with a variety of detergents. The membranes or bacteria themselves were the enzyme source for the assay. The enzymes were demonstrated to produce lysoglycosphingolipids, which were visualized on TLC with resorcinol-hydrochloric acid reagent or orcinol-sulfuric acid reagent. From the assay mixture of deacylase from strain GL-26, all lipophilic compounds, including Forssman glycolipid (substrate), lyso-Forssman glycolipid and fatty acid (products), and lipids of GL-26 cells were recovered by the SepPak $C_{18}$ (Waters, Milford, MA) cartridge technique. It is often difficult to analyze a small amount of released product by visualization on TLC with a nonspecific reagent of orcinol-sulfuric acid reagent or resorcinol-hydrochloric acid

reagent, because some bacterial lipids might be visualized by the reagent. Detection of the release of fatty acid from glycosphingolipid labeled with radioisotopic fatty acid is a more sensitive and specific assay method for deacylase. Labeled glycosphingolipid was obtained by the synthesis of lysoglycosphingolipid and radioisotopic fatty acid. The detection limit of this method might be improved by prolongation of exposure to radioisotopes. The immunochemical staining method described in this paper is also a highly sensitive and highly specific method. A few picomoles of lysoglycosphingolipid were detected, although most of the contaminating bacterial lipids were insensitive to the antibody and remained invisible. Therefore, a small amount of glycosphingolipid deacylase is detectable by this immunostaining method of lysoglycosphingolipid. It was difficult to clarify the substrate specificity in this immunostaining method as well as in the radioisotopic method. However, various antibodies against glycosphingolipids have recently been extensively investigated, and they should be applicable to the detection of lysoglycosphingolipids and the deacylase assay. The development and use of more sensitive primal antibodies, experimentation with various combinations of secondary (or third and more) antibodies, and the evolution of chromogenic reaction will enable detection of a smaller amount of glycosphingolipid on TLC.

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COMMUNICATION

# Variation in Molecular Species of Polar Lipids from *Thermoplasma acidophilum* Depends on Growth Temperature

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**ABSTRACT:** Five types of molecular species of $C_{40}$ isoprenoid chains, having different numbers of cyclopentane rings, were detected in the ether core lipid of *Thermoplasma acidophilum*. The average cyclization number of the hydrocarbon chains in the lipids increased with increasing growth temperatures.

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The membrane lipid structures of Archaea are composed of different lengths of isoprenoid chains with an ether linkage to glycerol in the *sn*-2,3 configuration (1,2). The hydrocarbon chain constituents of ether lipids are commonly the $C_{20}$, $C_{25}$, and $C_{40}$ isoprenoid chains. In the thermoacidophilic Archaea, caldarchaeol (2,2′,3,3′-tetra-*O*-dibiphytanyl-*sn*-diglycerol), which contains 2 mol of $C_{40}$ isoprenoid, is the major core lipid (3,4). Five types of molecular species, classified by the number of cyclopentane rings, have been detected in the core lipid of *Thermoplasma acidophilum* and other extreme thermophiles including *Sulfolobus solfataricus* (5–7); these are the acyclic-, monocyclic-, bicyclic-, tricyclic-, and tetracyclic-$C_{40}$ hydrocarbons. De Rosa *et al.* (8) reported that cyclization of the chain increased systematically with the growth temperature from 75 to 89°C in *S. solfataricus,* which is an extreme thermoacidophilic Crenarchaeon belonging to the order Sulfolobales. *Sulfolobus solfataricus* can grow from 50 to 87°C and optimally at 87°C. *Thermoplasma acidophilum* belongs to the Euryarchaeota, a subdomain of Archaea, and grows from 45 to 62°C (optimally at 59°C) (9). The cell is wall-less, and the major core lipid of the cell membrane is caldarchaeol (10).

In contrast to the report on *S. solfataricus* (8), Yang and Haug *et al.* (11) reported that the average cyclization number of the total lipids of *T. acidophilum* grown at 37°C was greater than that grown at 56°C. Recently, at least five types of neutral lipids and eight types of acidic lipids have been identified in *T. acidophilum,* and most of their structures have been characterized (12–14).

This report describes the effect of growth temperature on the degree of cyclization of the major lipids, five neutral lipids, and four acidic lipids in *T. acidophilum*, in all of which the core lipid is caldarchaeol.

## EXPERIMENTAL PROCEDURES

*Thermoplasma acidophilum* (ATCC 27658) was grown aerobically with low-speed stirring at 40, 50, and 60°C (±1°C) at pH 2.0. The medium (1.5 L, wt/vol) consisted of inorganic salts, 0.1% yeast extract, and 1% glucose in 2-L flasks, as previously described (13). Cells grown at 60°C were used as inoculum. Lipid extraction from the cells and the preparation of the neutral lipids and acidic lipids from the total lipids were carried out as previously described (13). As complete lipid extraction is necessary for reproducible results, the whole cells were treated with acidic methanolysis during the first step. Five neutral glycolipids (GL-1a, GL-1b, GL-2a, GL-2b, and GL-2c), and four acidic phosphoglycolipids (GPL-A, GPL-B, GPL-C, and GPL-D) were fractionated by the combination of column chromatography and preparative thin-layer chromatography (TLC). All of the core lipids from these intact lipids were co-chromatographed with caldarchaeol ($R_f$ = 0.55) by TLC analysis. Each lipid was purified by preparative TLC using boronic acid-impregnated high-performance TLC (HPTLC) plates of silica gel (Merck, Darmstdt, Germany). The HPTLC plates were developed with either chloroform/methanol/water (75:25:2, by vol) for purification of each neutral lipid, chloroform/methanol/0.2% $CaCl_2$ (55:45:7, by vol) or chloroform/methanol/1 M aqueous ammonia (65:35:5, by vol) for each acidic lipid.

The core lipids were obtained from whole cells or intact lipids by acid methanolysis. The core lipids bound to the intact polar lipids were completely cleaved and extracted by a chloroform/methanol mixture from the methanolyzate. TLC analysis of the core lipids was performed using an HPTLC plate of silica gel and developed with hexane/diethyl ether/acetic acid (60:40:2, by vol). Core lipids were detected by spraying with 18 M $H_2SO_4$ followed by heating at 150°C. Preparation of the hydrocarbon chains from the core lipids were obtained by HI degradation as described previously (13). The hydrocarbon chains were analyzed by gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC–MS). GLC was performed on a Hitachi 163 gas chromatograph (Hitachi, Tokyo, Japan) equipped with a flame-ionization detection system. Hydrocarbon chains were analyzed on 3% Dexsil 300 in a glass column (3 mm × 1 m)

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Abbreviations: EI, electron ionization; GC–MS, gas chromatography–mass spectrometry; GLC, gas–liquid chromatography; HPTLC, high-performance thin-layer chromatography; TLC, thin-layer chromatography.

**TABLE 1**
**Distribution of Cyclopentane Rings in Core Lipids of Whole Cell, Neutral Lipids, and Acidic Lipids from *Thermoplasma acidophilum* Grown at 40–60°C**

| Temperature of growth | % of $C_{40}$ hydrocarbon | | | | | Average degree |
|---|---|---|---|---|---|---|
| (°C) | Acyclic | Monocyclic | Bicyclic | Tricyclic | Tetracyclic | of cyclization[a] |
| Whole cell | | | | | | |
| 40 | 4.9 | 32.7 | 62.4 | ND[b] | ND | 1.6 ± 0.06 |
| 50 | 2.3 | 20.7 | 71.9 | 5.2 | ND | 1.8 ± 0.09 |
| 60 | 0.7 | 7.0 | 77.6 | 14.6 | <0.1 | 2.1 ± 0.04 |
| Neutral lipids | | | | | | |
| 40 | 16.9 | 36.1 | 46.6 | 0.4 | ND | 1.3 ± 0.12 |
| 50 | 7.8 | 23.0 | 66.3 | 2.9 | ND | 1.6 ± 0.12 |
| 60 | 2.9 | 5.1 | 81.8 | 10.2 | ND | 2.0 ± 0.08 |
| Acidic lipids | | | | | | |
| 40 | 6.0 | 31.2 | 55.3 | 7.6 | ND | 1.6 ± 0.02 |
| 50 | 1.7 | 14.8 | 76.1 | 7.4 | ND | 1.9 ± 0.07 |
| 60 | 0.3 | 4.4 | 76.9 | 17.8 | 0.6 | 2.1 ± 0.02 |

[a]Average degree of cyclization: (%monocyclic + 2 × %bicyclic + 3 × %tricyclic + 4 × %tetracyclic) × $10^{-2}$.
[b]ND, not detected (<0.01).

from 100 to 300°C at the rate of 15°C/min. GC–MS electron ionization (EI) was carried out using a gas chromatograph–mass spectrometer (JMS-AX505H; JEOL, Tokyo, Japan) with a capillary column (DB-1, 0.53 mm × 15 m). The accelerating voltage was 3.0 kV, and the primary beam for the bombardment was 6.0 keV of xenon.

**TABLE 2**
**Distribution of Cyclopentane Rings in Glycolipids (GL) and Glycophospholipids (GPL) from *T. acidophilum***

| Temperature of growth | % of $C_{40}$ hydrocarbon | | | | | Average degree |
|---|---|---|---|---|---|---|
| (°C) | Acyclic | Monocyclic | Bicyclic | Tricyclic | Tetracyclic | of cyclization[a] |
| GL-1a | | | | | | |
| 40 | 7.6 | 47.2 | 44.1 | 1.1 | ND | 1.4 |
| 50 | 3.5 | 29.7 | 64.8 | 2.0 | ND | 1.7 |
| 60 | 0.4 | 5.2 | 80.8 | 13.4 | 0.2 | 2.1 |
| GL-1b | | | | | | |
| 40 | 14.8 | 53.0 | 32.2 | ND | ND | 1.2 |
| 50 | 7.8 | 23.0 | 66.3 | 2.9 | ND | 1.6 |
| 60 | 9.3 | 21.3 | 69.4 | ND | ND | 1.6 |
| GL-2a | | | | | | |
| 40 | 1.3 | 29.7 | 67.3 | 1.7 | ND | 1.7 |
| 50 | 0.6 | 15.8 | 82.9 | 0.7 | ND | 1.8 |
| 60 | 0.4 | 2.7 | 91.4 | 5.5 | ND | 2.0 |
| GL-2b | | | | | | |
| 40 | 1.8 | 20.4 | 77.5 | 0.3 | ND | 1.8 |
| 50 | 1.8 | 8.5 | 85.1 | 4.6 | ND | 1.9 |
| 60 | 0.8 | 1.7 | 87.5 | 10.1 | ND | 2.1 |
| GL-2c | | | | | | |
| 40 | 44.2 | 19.6 | 36.3 | ND | ND | 0.9 |
| 50 | 36.8 | 13.0 | 47.1 | 3.2 | ND | 1.2 |
| 60 | 21.7 | 7.2 | 56.3 | 14.8 | ND | 1.6 |
| GPL-A | | | | | | |
| 40 | 3.4 | 44.6 | 52.1 | ND | ND | 1.5 |
| 50 | 1.5 | 8.8 | 85.0 | 4.7 | ND | 1.9 |
| 60 | Trace | 2.6 | 83.5 | 13.3 | 0.7 | 2.1 |
| GPL-B | | | | | | |
| 40 | 4.6 | 22.6 | 72.6 | 0.3 | ND | 1.7 |
| 50 | 2.0 | 8.1 | 86.6 | 3.3 | ND | 1.9 |
| 60 | Trace | 2.6 | 88.6 | 8.2 | 0.6 | 2.1 |
| GPL-C | | | | | | |
| 40 | 5.6 | 35.8 | 58.7 | ND | ND | 1.5 |
| 50 | Trace | 14.0 | 82.1 | 3.9 | ND | 1.9 |
| 60 | 0.8 | 5.1 | 78.7 | 14.7 | 0.7 | 2.1 |
| GPL-D | | | | | | |
| 40 | Trace | 15.6 | 80.8 | 3.6 | ND | 1.9 |
| 50 | Trace | 11.4 | 81.9 | 6.8 | ND | 2.0 |
| 60 | Trace | 3.4 | 83.0 | 13.6 | ND | 2.1 |

[a]Average degree of cyclization: (%monocyclic + 2 × %bicyclic + 3 × %tricyclic + 4 × %tetracyclic) × $10^{-2}$. For abbreviations see Table 1.

## RESULTS AND DISCUSSION

Freeze-dried whole cells were directly methanolyzed, then prepared as hydrocarbon chains. The gas chromatogram of the hydrocarbon chain from the core lipid (caldarchaeol) showed five peaks corresponding to the $C_{40}$ hydrocarbon chains detected in *S. solfataricus* (8). The peaks for $[M]^+$ obtained from the GC–MS EI spectra at *m/z* 562, 560, 558, 556, and 554 were identified as $C_{40}H_{82}$ (acyclic), $C_{40}H_{80}$ (monocyclic), $C_{40}H_{78}$ (bicyclic), $C_{40}H_{76}$ (tricyclic), and $C_{40}H_{74}$ (tetracyclic), respectively. The distribution of molecular species of the $C_{40}$ isoprenoid and the average cyclization in the whole cells and fractionated lipids of *T. acidophilum* grown at 40, 50, and 60°C, are shown in Table 1. From these results, the average cyclization of the $C_{40}$ hydrocarbon chains increased with the increasing growth temperature. These results are similar to those from *S. solfataricus*, but opposite to that of a previous report on *T. acidophilum* (11).

Distribution of the molecular species of the $C_{40}$ isoprenoid and average cyclization from the neutral glycolipids and acidic phosphoglycolipids are shown in Table 2. An increasing degree of cyclization was seen in all the lipids examined. The existence of a lipopolysaccharide with 24 mannose residues in its polar head portion has been reported in *T. acidophilum* (15,16). In our experiments, the lipopolysaccharide was not detected on the TLC plate, probably because of its behavior based on high molecular weight and high polarity. The efficiency of extraction for the high molecular weight lipids depends on the extraction solvents, which should be affected by the cyclization number in the total lipids. The previous contradictory result (11) from *T. acidophilum* might have been related to the extraction conditions. The phosphoglycolipids are more cyclized than the glycolipids. The reason for this might be that the physical volume of the polar head groups of the phosphoglycolipids are larger than that of the glycolipids; the volume of the core lipid as a membrane anchor part needs to become larger for controlling the distance between the polar heads.

Changing the number of cyclopentane rings in the core lipids of thermoacidophilic Archaea could maintain stable fluidity of the membrane against environmental temperature changes, similar to the change in the unsaturation of the fatty acylester lipids of Eukarya and Bacteria (17,18).

The main molecular species of the $C_{40}$ hydrocarbon chain of the major cellular lipid GPL-A and total lipids from *T. acidophilum* grown at its optimum of 60°C in 5 L of medium without stirring contains one cyclopentane ring ($C_{40}$ monocyclic). In the case of the cultivation carried out in a 1.5-L medium with stirring at 60°C, the main molecular species of the hydrocarbon chain was the bicyclic $C_{40}$ hydrocarbon. Thus cyclization might be a response not only to temperature but also to physical stimulation. *Thermoplasma acidophilum* is wall-less and its cell membrane directly faces into the environment. The stability of the membrane structure also might be controlled by cyclization of hydrocarbon chains against the physical stress, like stirring cultivation.

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ARTICLE

# Measurement of Human Chylomicron Triglyceride Clearance with a Labeled Commercial Lipid Emulsion

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**ABSTRACT:** Human chylomicron triglyceride (TG) kinetics has been difficult to determine directly owing to technical limitations. This report describes a new method for studying chylomicron metabolism. Healthy volunteers ($n$ = 10) sipped a drink providing 175 mg fat·kg$^{-1}$·h$^{-1}$ for 7.5 h to produce a steady-state chylomicronemia. A commercial 10% intravenous lipid emulsion was labeled with [$^3$H]triolein, purified by high-performance liquid chromatography, and sterilized. A trace amount of labeled emulsion was injected intravenously 30 min before (i.e., in the fasting state) and 5, 6, and 7 h after sipping began (i.e., triplicate determinations in the fed state). Chylomicron half-lives were calculated from the monoexponential decay curves, and apparent distribution volumes were estimated by back-extrapolation to time zero. Plasma and estimated chylomicron TG concentrations increased from 89 ± 13 and 0.8 ± 0.3 to 263 ± 43 and 91 ± 39 mg/dL (mean ± SEM), respectively, with feeding. Tracer-determined chylomicron TG half-lives were 5.34 ± 0.58 and 6.51 ± 0.58 min during the fasting and fed states, respectively ($P < 0.01$). The apparent distribution volume during the fasting state was 24% greater than plasma volume (4515 ± 308 vs. 3630 ± 78 mL, $P < 0.02$), consistent with significant margination of lipid emulsion particles to endothelial binding sites. Margination was reduced during the fed state, suggesting that native chylomicrons competed with lipid emulsion particles for endothelial lipoprotein lipase. The results indicate that a radiolabeled commercial lipid emulsion is metabolized in a fashion similar to native chylomicron TG, and thus can be used to study chylomicron TG kinetics.

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Chylomicrons are lipoproteins secreted by the intestine after fat ingestion and are thus the primary vehicle for lipid absorption. These particles, and especially their remnants, are thought to be major contributors to atherosclerosis since excessive postprandial lipemia is characteristic of patients at increased risk for myocardial infarction and stroke (1–5). Thus there is a growing need to understand the details of chylomicron metabolism.

Previous methods for determining chylomicron kinetics *in vivo* have been fraught with technical difficulties or used approaches of dubious physiological relevance. The ideal technique would be to inject endogenously labeled chylomicrons harvested from thoracic duct lymph (6), but this method has obvious limitations related to its invasiveness. Although an improvement, a duodenal fat perfusion test causes discomfort and requires fluoroscopic tube replacement (7,8). Others have used the intravenous fat tolerance test (8,9). This method alters triglyceride (TG) pool size, influences kinetic parameters (10), and has the potential to produce transient reticuloendothelial uptake of lipid (11). A radiolabeled lipid emulsion allows for an essentially massless injection with negligible pool perturbation. The noncommercial emulsion has been used with success (12–14) and is a reasonable approach. However, if a commercially available lipid emulsion could be easily labeled with a radioactive TG and injected, it could theoretically simplify the study of chylomicron TG metabolism in humans. The purpose of this study was to evaluate such a method.

## EXPERIMENTAL PROCEDURES

*Subjects.* Volunteers (ages 21–70 yr) were recruited who had body mass index of 22–30 kg/m$^2$, fasting serum low density lipoprotein (LDL) cholesterol concentrations less than 160 mg/dL, high density lipoprotein (HDL) cholesterol concentrations greater than 35 mg/dL, and TG concentrations less than 200 mg/dL. Individuals with known hepatic, renal, or gastrointestinal diseases, lactose intolerance, or those taking medications known to affect lipid metabolism or fat absorption were excluded. The study was approved by Saint Luke's Hospital Institutional Review Board, and informed, written consent was obtained from all participants.

*Protocol*. Subjects were provided with a low-fat meal (<30% energy from fat) the night before and asked to refrain from alcohol and strenuous exercise for 48 h before the test. They reported to the Metabolic Research Unit on the morning after an overnight (12 h) fast. Intravenous sampling cannula was place in a forearm vein and an injection cannulae in a contralateral vein; both were kept patent with infusions of 0.9% NaCl. Subjects consumed a priming dose (350 mg fat/kg) of a test drink made up of 87 parts of a 50:50 blend of cream and milk (Half and Half®) and 13 parts of chocolate syrup. Two hours later, they began to take a small portion of the drink every 15 min for the next 5.5 h in order to produce an overall fat ingestion rate of 175 mg·kg$^{-1}$·h$^{-1}$. The drink provided 25 kcal/kg (~68% of average daily allowance): 47%

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Abbreviations: HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; LpL, lipoprotein lipase; PL, phospholipid; TG, triglyceride; VLDL, very low density lipoprotein.

as fat, 16% as protein, and 37% as carbohydrate. A commercial lipid emulsion (see below) containing 4 μCi [$^3$H]triolein (140 mg TG in 2 mL) was injected 30 min before, and 5, 6, and 7 h after ingestion of the priming dose. Thus, the emulsion was injected four times: once in the fasting state and three times in the fed state. Blood samples (6 mL each) were drawn at 0, 1, 3, 5, 7, 9, 13, 17, 20, and 30 min after each bolus injection.

*Laboratory methods. (i) Purification of lipid emulsion.* The 9,10-$^3$H(N)-triolein (30 μCi/200 μL in ethanol; American Radiolabeled Chemicals, St. Louis, MO) was added to 1 mL of 10% Liposyn® (Abbott Laboratory, Chicago, IL) in a sterile vial, and the solution was incubated for 5 min in a 60°C water bath. Preliminary experiments indicated that maximal incorporation occurred within this time frame. We found that a substantial fraction (~40%) of the radioactivity was incorporated into TG-poor, phospholipid (PL)-rich vesicles present in the emulsion to enhance stability (15). In order to isolate only the large, chylomicron-sized particles for injection, the labeled emulsion was subjected to high-performance liquid chromatography (HPLC; Millipore Waters, Milford, MA) with a TSK Guard PW1 and three size-exclusion TSK G5000 PWx2 columns (TOSO Haas, Montgomeryville, PA) in series (16). Preliminary experiments showed that this procedure was able to isolate a fraction containing particles *ca.* 0.34 μm in diameter (the size of chylomicrons) as measured by both dynamic light-scattering (16) and ratio of TG/PL (17). A 4-mL fraction was collected into 4 mL of unlabeled 10% Liposyn® to stabilize the purified emulsion. The mixture was then autoclaved for 15 min prior to injection (a procedure routinely used to sterilize commercial lipid emulsions). In the preliminary *in vivo* experiments, $C^{14}$-labeled and autoclaved lipid emulsion was injected simultaneously with unautoclaved $H^3$-labeled emulsion in a subject, and their clearance rates were compared. The half-lives of $C^{14}$- and $H^3$-labeled emulsion were 5.1 and 5.5 min, respectively. We concluded that autoclaving did not materially alter the kinetic behavior of TG particles. We also verified that there was no exchange of radioactivity from the purified, labeled TG-rich particles to the smaller TG-poor vesicles of the unlabeled emulsion (16).

*(ii) Lipid profile*. Plasma was analyzed for total cholesterol, TG and HDL cholesterol concentrations using a Cobas Fara II with enzymatic reagents from Boehringer Mannheim (Indianapolis, IN) (18); very low density lipoprotein (VLDL) and LDL cholesterol concentrations were estimated by the Friedewald equation (19). Whole plasma, adjusted chylomicron (see below), HDL, and LDL TG concentrations were used to estimate VLDL TG concentrations. We assumed that HDL and LDL TG concentrations were the same during the fasting and fed states. Chylomicron TG concentration was determined by gas chromatography as described previously (16) in order to increase our ability to detect the very low chylomicron TG concentrations in the fasting samples. Our laboratory participates in the CDC NIH lipid standardization program and the Excel program from Pacific Biomed (Seattle, WA).

*(iii) Chylomicron isolation.* To isolate chylomicrons, 2 mL of plasma was underlayered below 8 mL of distilled water in an ultracentrifuge tube and spun in an SW41 rotor for 30 min at 25,000 rpm in a Beckman L7-65 ultracentrifuge (16). This procedure was repeated two more times to remove as much VLDL as possible from the chylomicron fraction. Preliminary experiments in which the labeled purified lipid emulsion was mixed with plasma and then subjected to this triple-spin technique indicated that the recovery of chylomicrons averaged 30% with a coefficient of variation of <0.5%. This factor was used to calculate adjusted chylomicron TG concentrations.

*(iv) Radioactivity of chylomicrons.* Chylomicron fractions were transferred to vials containing 10 mL Opti-fluor (Packard, Meriden, CT) and counted in a Wallac 1410 liquid scintillation counter (Pharmacia, Gaithersburg, MD) using appropriate quench curves established for dual label counting. Background counts at zero minutes were subtracted from each data point for each of the four injections.

*(v) Margination volume.* Margination volume is defined as the difference between the apparent distribution volume and the actual plasma volume (20). A comparison of these two volumes is a reflection of the extent of margination, or endothelial binding of the lipid emulsion particles. To measure distribution volume using the labeled emulsion as the tracer, lipids were extracted from postinjection plasma (500 μL) after adding [$^{14}$C]triolein in 5 μL of ethanol as an internal standard (21). The lipid extracts were dried under nitrogen, and the $^3$H and $^{14}$C radioactivity was determined as described above.

*Calculations.* Chylomicron TG half-lives ($t^{1/2} = 0.693/k$) were estimated from the monoexponential radiolabeled lipid disappearance curves ($y = \alpha e^{-kx}$; Microsoft Excel, version 4.0). Plasma $^3$H concentration at the moment of injection (from which apparent distribution volume was calculated) was estimated by back extrapolation of the monoexponential curve to time zero. The following formulae were used to calculate:

$$\text{apparent distribution volume (mL)} = \frac{\text{total dpm of lipid emulsion injected}}{\text{plasma radioactivity extrapolated to the moment of injection (dpm/mL)}} \quad [1]$$

where margination volume (mL) = apparent distribution volume – plasma volume, and plasma volume (mL) = 31.47 × body weight (kg) + 627 (7).

*Statistical analysis*. We used Repeated Measures analysis of variance with Student Newman Keuls test to compare tracer-determined half-lives, TG concentrations, and distribution volumes during the fasting and the fed states. The relationship between half-lives and plasma TG concentration was analyzed using linear regression. A two-tailed $P$ value of <0.05 was required for statistical significance.

## RESULTS

There were two female and eight male subjects in the study. Age and body mass index (kg/cm$^2$) of two women were 32 and 43, and 27 and 30, respectively. Average age and body mass index of men were 39 ± 5 and 27 ± 1, respectively. Con-

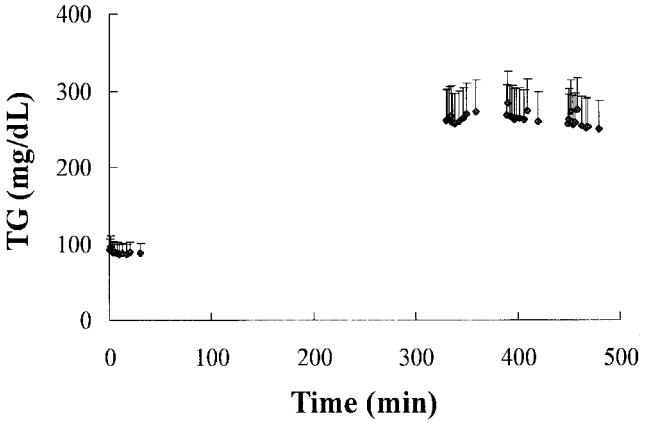


**FIG. 1.** Whole plasma triglyceride (TG) concentrations. Values are means ± SEM; $n = 10$.

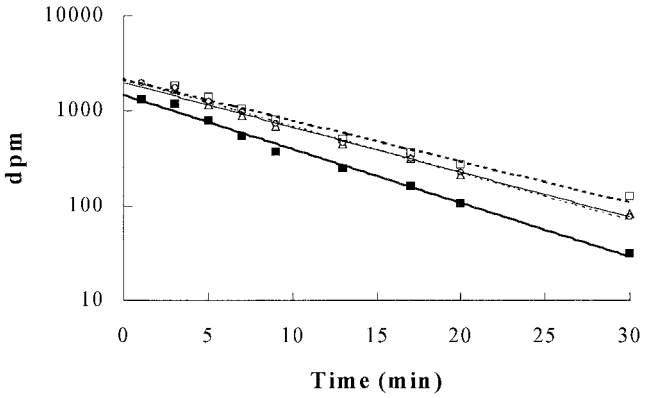


**FIG. 2.** Disappearance of lipid emulsion from chylomicron fraction during the fasting (—■—), and the fed states after injections one (---□---), two (—△—), and three (---○---). Monoexponential curves in logarithmic scale were $y = 1921.7e^{-0.1451x}$ ($R^2 = 0.9629$) during the fasting state, and $y = 2302.5e^{-0.1057x}$ ($R^2 = 0.9582$), $y = 2267.3e^{-0.1198x}$ ($R^2 = 0.9691$), and $y = 2345.1e^{-0.1235x}$ ($R^2 = 0.9781$) for the three injections during the fed state, respectively. $n = 10$.

centrations of fasting plasma LDL, HDL, and VLDL cholesterol were 103 ± 5, 39 ± 3, and 19 ± 3 mg/dL, respectively ($n = 10$). Fasting plasma TG concentrations at baseline were 89 ± 13 mg/dL and increased to 263 ± 43 mg/dL by the end of the study (Fig. 1). The unadjusted and adjusted chylomicron TG concentrations were 27.3 ± 11.7 and 91 ± 39 mg/dL during the fed state (Table 1). There were no significant differences on whole plasma, unadjusted and adjusted chylomicron, and VLDL TG concentrations between injections during the fed state, although there was a tendency for the 5-h chylomicron TG values to be higher than those at 6 and 7 h (Table 1).

Background counts before each injection were negligible (28 ± 8 dpm), consistent with the virtually complete removal of labeled TG after 30 min. Tracer-determined chylomicron TG half-lives were 22% longer when measured in the fed than in the fasting state ($P < 0.05$, Fig. 2 and Table 1). In addition, half-lives at the sixth and seventh hours were 13 and 19%, shorter, respectively, than those at the fifth hour ($P < 0.05$, Table 1). There was a positive correlation between estimated chylomicron TG half-lives and plasma TG concentrations during the fasting and the fed states (Fig. 3). Additionally, chylomicron TG half-lives were significantly correlated with VLDL TG concentration during the fasting state ($R^2 = 0.5452$, $P < 0.01$) and nearly during the fed states ($R^2 = 0.3482$, $P = 0.07$).

Apparent distribution volumes were calculated from the whole plasma (not chylomicron) clearance curves. Radiolabeled lipid emulsion disappeared more rapidly from whole plasma during the fasting than the fed states (Fig. 4). Apparent distribution volume was greater (24%, $P < 0.02$) than plasma volume in the fasting state, but was not different from plasma volume in the fed state (Fig. 5). There was also a significant (15%; $P < 0.02$) difference in apparent distribution volume between the fasting and the fed states. Margination volume was 2.4 times greater during the fasting than the fed states (885 ± 296 vs. 289 ± 217 mL; $P < 0.02$).

## DISCUSSION

We found that a labeled, commercial lipid emulsion produced estimates of chylomicron TG half-lives similar to those ob-

**TABLE 1**
**Chylomicron TG Half-lives and Whole Plasma, Chylomicron and VLDL TG Concentrations During the Fasting and Fed States[a]**

| Injection time (number) | Feeding state | Half-lives (min) | TG (mg/dL) Unadjusted chylomicron | Adjusted[b] chylomicron | Whole plasma | Estimated VLDL[c] |
|---|---|---|---|---|---|---|
| –30 min (1) | Fasting | 5.34 ± 0.58 | 0.2 ± 0.1 | 0.8 ± 0.3 | 89 ± 13 | 47 ± 11 |
| 5 h (2) | Fed | 7.16 ± 0.64[d] | 30.2 ± 13.2[d] | 100.6 ± 44.0[d] | 264 ± 43[d] | 120 ± 28[d] |
| 6 h (3) | Fed | 6.32 ± 0.56[d,e] | 26.5 ± 10.2[d] | 88.2 ± 33.9[d] | 267 ± 42[d] | 134 ± 24[d] |
| 7 h (4) | Fed | 6.04 ± 0.59[d,e] | 25.4 ± 11.6[d] | 84.6 ± 38.6[d] | 259 ± 43[d] | 130 ± 27[d] |
| | Average fed | 6.51 ± 0.58[d,e] | 27.3 ± 11.7[d] | 91.1 ± 38.8[d] | 263 ± 43[d] | 128 ± 26[d] |

[a]Mean ± SEM; $n = 10$. TG, triglyceride; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.
[b]Assumes a 70% loss during isolation (see chylomicron isolation section).
[c]Whole plasma TG – (adjusted chylomicron + HDL TG + LDL TG).
[d]Values differ ($P < 0.05$) from injection #1.
[e]Values differ ($P < 0.05$) from injection #2.

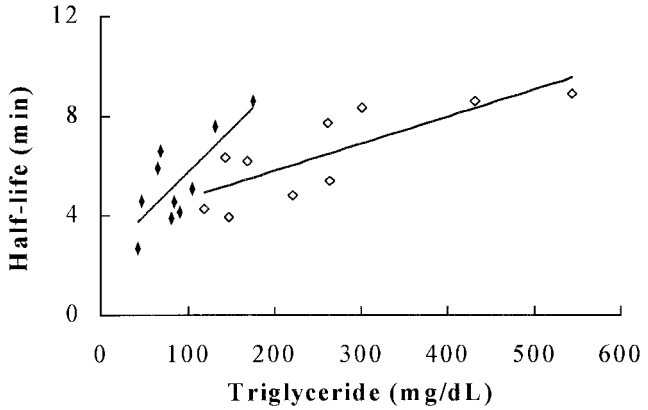


**FIG. 3.** Relationship between estimated chylomicron TG half-life and plasma TG concentration during the fasting (◆, $R^2 = 0.515$, $P < 0.05$) and the fed states (◇, $R^2 = 0.649$, $P < 0.01$. $n = 10$). See Figure 1 for abbreviation.

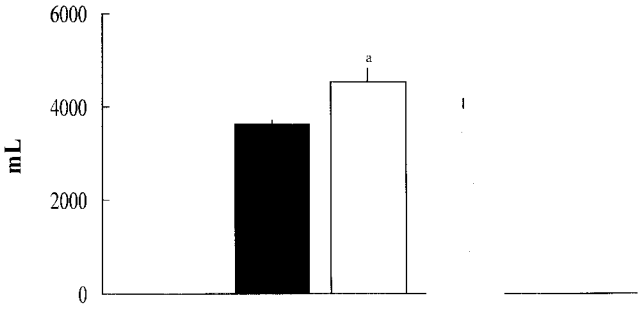


**FIG. 5.** Plasma volume (solid bar) and apparent distribution volumes during the fasting (open bar) and the fed (shaded bar) states. [a]Values differ ($P < 0.02$) from the plasma volume and the distribution volume during the fasting state. Mean ± SEM; $n = 10$.

served using more complex methods (6–8). Consistent with this are the margination data, which suggest that lipid emulsion particles compete with endogenous chylomicrons for binding to endothelial lipoprotein lipase (LpL). These two findings support the hypothesis that this preparation can serve as a tracer for chylomicron TG catabolism.

Over 35 years ago, Nestel *et al.* (6) infused harvested human chylomicrons and estimated chylomicron half-lives to be 5 to 8 min. Grundy and Mok (7) and Cohen (8) confirmed this estimate using a duodenal fat perfusion test. Since duodenal intubation may alter normal gastrointestinal physiology and can be unpleasant for the subject, we chose to utilize a chronic "sipping" procedure. We found that this more physiologic approach did achieve its goal of producing a relative steady-state hypertriglyceridemia (16) and was much less stressful for the subjects.

Since our radioactively labeled, commercial intravenous lipid emulsion had earlier been shown to accurately estimate chylomicron TG rate of appearance (16), it followed that it would also trace chylomicron TG catabolism. Previous attempts to use a bolus of unlabeled lipid emulsion produced estimates of chylomicron TG half-life 100% (9) to 200% (8) longer than that of native chylomicron TG. Compared to an unlabeled emulsion, a bolus injection of a trace amount of radiolabeled emulsion allows the administration of much less mass with less perturbation of the system. Other studies using labeled noncommercial emulsions have been useful in tracing not only chylomicron TG but also chylomicron particle clearance rates by labeling with both TG and cholesteryl esters. Using such emulsions, Nakandakare *et al.* (12) estimated the chylomicron TG residence time to be 5.9 min and Maranhão *et al.* (22) reported a chylomicron TG half-life of 6.6 min in rats. These results are similar to data from harvested chylomicrons (6,23) and duodenal fat perfusion studies (7,8). A similar observation was made in the present study using a labeled commercial emulsion. Plasma TG concentrations were unaltered after each bolus injection (since only 140 mg of TG was injected each time), and thus multiple injections of the labeled lipid emulsion are possible. In this setting, half-lives during the fed and the fasting states were 6.5 and 5.3 min, respectively.

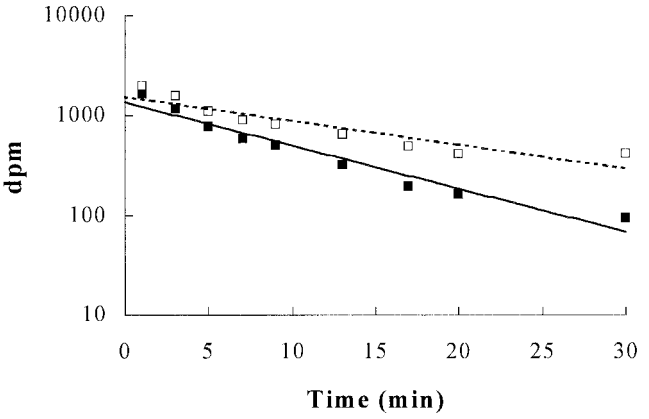


**FIG. 4.** Disappearance of lipid emulsion from whole plasma during the fasting (—■—) and the fed states (---□---); average of three tests. Monoexponential curves in logarithmic scale were $y = 1463.6e^{-0.1368x}$ ($R^2 = 0.9447$) during the fasting state and $y = 1574.6e^{-0.0766x}$ ($R^2 = 0.9189$) during the fed state. $n = 10$.

Hultin *et al.* (24) found that a labeled lipid emulsion was cleared 35–40% more slowly than native chylomicrons in rats. Slow clearance of emulsion particles in that study could also be explained by the presence of PL-rich vesicles which have been shown to inhibit lipolysis of TG-rich particles *in vivo* (25) and *vitro* (26). During emulsion production, PL is added in excess as a stabilizer. Preliminary studies with our emulsion confirmed that a substantial fraction of radiolabeled triolein (40%) added to the emulsion was associated with these vesicles (16). Since these particles can accumulate in the plasma after infusion (15), their kinetic behavior may differ from that of the large, TG-rich particles; thus they should be removed prior to injection if the kinetics of chylomicron TG is to be traced. It is also important that the particles in the infused emulsion be of similar size to chylomicrons because the affinity of LpL for TG-rich lipoproteins is influenced by particle size (27). Both of these goals were achieved using HPLC as described.

Preparation of a noncommercial emulsion requires special homogenization and multiple centrifugations to isolate the

proper-sized particles. Although the present method does not require these, it does require an HPLC system to obtain the proper particles for injection. Thus, both approaches have their own technical challenges.

Chylomicron TG half-lives are clearly influenced by background TG concentrations. Since LpL is saturable, one would expect a prolongation of half-life at higher TG concentration (28,29). Accordingly, we found that chylomicron TG half-lives were 22% longer during the fed than during the fasting state. In addition, there was a positive relationship between chylomicron TG half-lives and plasma TG concentrations (Fig. 3), and half-lives tended to plateau after TG levels of 300 mg/dL were reached. These observations suggest that the labeled particles in the commercial lipid emulsion used in our studies behave like nascent chylomicron particles.

We observed that chylomicron TG half-lives appeared to decrease during the latter portion of feeding protocol, between hours 5 and hours 6 and 7. Since we have no reason to believe that fat absorption was slower at 5 h than at 7 h, the decreasing chylomicron TG levels at the latter suggest that LpL activity may have been slowly increasing, possibly because more enzymes had been exposed on the endothelium, the affinity of existing LpL for its substrate had increased, or blood flow in LpL-rich tissues had increased. Karpe *et al*. (30) reported that preheparin (circulating) LpL activity was doubled 6 h after consuming an oral fat load and correlated as expected with an increase of serum free fatty acids (31). An alternative mechanism may involve the lipolysis-stimulated receptor which binds TG-rich lipoproteins when activated by free fatty acids generated locally by chylomicron TG hydrolysis (32). Further studies will be needed to explore this possible increase in lipolytic potential with prolonged feeding and the mechanisms behind it.

There is controversy as to whether the rise in plasma TG concentration following the ingestion of fat is due to chylomicrons or/and VLDL (33,34). Schneeman *et al*. (35) reported that 80% of the postprandial increase of TG-rich lipoprotein particle number is accounted for by VLDL, whereas 80% of the postprandial TG is carried by chylomicrons (36). In the present study, the postprandial TG increase was due to both chylomicrons and VLDL (90 and 81 mg/dL, respectively).

In an effort to explore other possible similarities between native chylomicrons and our emulsion, we determined margination volumes in the fasting and the fed states. We found that the apparent distribution volume in the fasting state was 24% greater than the plasma volume. Karpe *et al*. (20) reported that apparent distribution volume was 67% greater than plasma volume using plasmapheresis-derived, TG-rich lipoproteins ($S_f > 400$). They proposed that this was due to margination of TG-rich particles to endothelial LpL. The larger the difference between the apparent volume and the true plasma volume, the greater the vascular binding capacity (what we have called "margination volume"). The observation that there was no significant difference between the apparent distribution volume and plasma volume during the fed state is consistent with the presence of saturable endothelial binding sites. In other words, margination volume was small (i.e., LpL binding sites were few) when nascent chylomicrons were present. This suggests that when LpL binding sites are occupied by chylomicrons during steady-state absorption of a fat meal, intravenous lipid emulsion particles are unable to bind. This is consistent with the observation that an acute infusion of lipid emulsion after injection of retinyl palmitate-labeled chylomicrons induces a rapid release of chylomicrons from endothelial binding sites (20).

In summary, we found that a radiolabeled, commercial intravenous lipid emulsion was metabolized in a fashion similar to native chylomicrons. This conclusion is supported by three observations: (i) we found similar TG half-lives as have others using native chylomicrons; (ii) lipid emulsion TG half-lives were slower when chylomicrons were present; and (iii) margination volumes were reduced in the presence of chylomicrons. This technique could be useful in future studies investigating the effects of diets (high vs. low fat, different fatty acid compositions, fiber, etc.), drugs (pancreatic lipase inhibitors, bile acid reuptake receptor blockers, etc.), disease (obesity, diabetes, coronary heart disease, gastrointestinal disease, etc.), and physiological perturbations (exercise, pregnancy, lactation, aging, etc.) on chylomicron TG clearance.

## ACKNOWLEDGMENTS

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# Major Clofibrate Effects on Liver and Plasma Lipids Are Independent of Changes in Polyunsaturated Fatty Acid Composition Induced by Dietary Fat

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**ABSTRACT:** The effects of clofibrate on the content and composition of liver and plasma lipids were studied in mice fed for 4 wk on diets enriched in n-6 or n-3 polyunsaturated fatty acids (PUFA) from sunflower oil (SO) or fish oil (FO), respectively; both oils were fed at 9% of the diet (dry weight basis). Only FO was hypolipidemic. Both oil regimes led to slightly increased concentrations of phospholipids (PL) and triacylglycerols (TG) in liver as compared with a standard chow diet containing 2% fat. Clofibrate promoted hypolipidemia only in animals fed SO. Its main effect was to enlarge the liver, such growth increasing the amounts of major glycerophospholipids while depleting the TG. SO and FO consumption changed the proportion of n-6 or n-3 PUFA in liver and plasma lipids in opposite ways. After clofibrate action, the PUFA of liver PL were preserved better than in the absence of oil supplementation. However, most of the drug-induced changes (e.g., increased 18:1n-9 and 20:3n-6, decreased 22:6/20:5 ratios) occurred irrespective of lipids being rich in n-6 or n-3 PUFA. The concentration of sphingomyelin (SM), a minor liver lipid that virtually lacks PUFA, increased with the dietary oils, decreased with clofibrate, and changed its fatty acid composition in both situations. Thus, oil-increased SM had more 22:0 and 24:0 than clofibrate-decreased SM, which was significantly richer in 22:1 and 24:1.

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In rodents, the administration of clofibrate and of a wide range of structurally diverse compounds causes liver peroxisomes, mitochondria, and endoplasmic reticulum to proliferate in hepatic parenchymal cells (1–3). Treatment with these xenobiotics also leads to alteration in the level of expression of many enzymes, including peroxisomal and mitochondrial β-oxidation proteins (4), some enzymes involved in the microsomal synthesis of lipids (5) and fatty acids (6), and hydrolases, such as the carboxylesterases, capable of hydrolyzing lipophilic substances with ester, thioester, and amide groups (7). Most of these changes may in fact play a role in successful detoxification. Peroxisomal proliferators are known to act by binding to a receptor protein, a ligand-activated transcription factor being responsible for intensifying the transcription of specific nuclear genes (8). Sustained treatment with these drugs in high doses promotes liver hypertrophy and hyperplasia and may eventually lead to the development of tumors (9). Our previous work, focusing specifically on the lipids (10), showed that the enlargement induced in mouse liver by clofibrate was accompanied by an increase in the amount, though not in the concentration, of liver glycerophospholipids and a decrease in both the amount and the concentration of triacylglycerols (TG). Although the fatty acid composition of the newly formed phospholipids (PL) was less affected than that of TG, both had reduced percentages of polyunsaturated fatty acids (PUFA), especially those of the n-3 series, with respect to controls. The PUFA of liver TG were apparently consumed in the process of supporting the stimulated PL synthesis, but still were insufficient to provide for the increased energetic and biosynthetic demands imposed by the drug-induced cell growth and proliferation. It was suggested that the chow diet (CD), although adequate for most purposes, in this case may have been short of n-3 PUFA. In the present work we investigate the effects of clofibrate in mice whose liver and plasma lipids were considerably enriched in n-3 or n-6 PUFA.

## EXPERIMENTAL PROCEDURES

Male albino mice of the Balb-C strain, weighing 32 ± 2 g, were kept under standard laboratory conditions for 4 wk, and divided into SO (sunflower oil) and FO (fish oil) groups. An additional group of animals receiving a standard rodent chow diet (CD), containing 2% fat, was used for comparison. Each group consisted of five animals, of which four were analyzed. The experimental diets consisted of 23.2% protein, 62.8% carbohydrates, 4.9% vitamin and mineral supplements, and 9.1% of the corresponding oil, on a dry weight basis. From day 15 onward, half the animals from the SO and FO groups continued with their regimes, and the other half were fed the same diet but with the addition of clofibric acid (Sigma Chemical Co., St. Louis, MO), 0.5 g per 100 g of food (10). The protocol for the study conformed to accepted standards of animal care and experimental procedures. The fatty acid

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Abbreviations: CD, chow diet; CE, cholesterol esters; DPG, diphosphatidylglycerol; FO, fish oil; GC, gas chromatography; MFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SM, sphingomyelin; SO, sunflower oil; TG, triacylglycerols; TLC, thin-layer chromatography.

composition of CD, SO, and FO, in that order, was as follows: saturated fatty acids (SFA): 19.3, 14.1, and 25.8%; monounsaturated fatty acids (MFA): 22.4, 22.0, and 29.6%; n-6 PUFA: 57.9, 63.8, and 13.7%; and n-3 PUFA: 0.4, 0.1, and 30.9%, respectively. Taking into account the amount of fat in each diet, and the above fatty acid percentages, the content (w/w) of n-3 PUFA was similar in CD and SO (~0.8%), whereas the content of n-6 PUFA was similar in CD and FO (~1.2%). At day 30 the animals were killed after having been anesthetized with acepromazine and ketamine. Blood samples were obtained by cardiac puncture, collected using heparinized material, and centrifuged to separate plasma from blood cells. The livers were removed, rinsed in saline, weighed, cut into pieces, and homogenized in the solvents used for lipid extraction.

Plasma lipids were determined with commercial kits and the standard techniques used in clinical settings for human plasma. Lipid extracts were prepared from liver and plasma (11), and total lipid phosphorus was determined. Neutral lipids were separated by thin-layer chromatography (TLC) on silica using hexane/ether/acetic acid (80:20:2, by vol). The PL, TG, and cholesterol ester (CE) bands were scraped off after being located with dichlorofluorescein, and eluted (12). Liver PL were resolved into classes by TLC (13) and quantified by phosphorus measurement (14). For sphingomyelin (SM) fatty acid analysis, this lipid was isolated by TLC (15), eluted (12), dried under $N_2$, treated with 0.5 N NaOH in methanol at 50°C for 10 min, and subjected again to TLC. This ensured that SM was free of any possible contamination with traces of glycerophospholipids.

Fatty acids of all lipids were analyzed by standard gas chromatography (GC) of their fatty acid methyl esters on packed columns (10). Before GC, the methyl esters prepared from lipids were purified by TLC using hexane/ether (95:5, vol/vol) on silica plates that had been pre-washed with methanol/ether (75:25, vol/vol), and were recovered from the support by partition between water/methanol/hexane (1:1:2, by vol). Statistical analysis of the results was performed using the two-tailed Student's *t* test.

## RESULTS AND DISCUSSION

*Liver and plasma lipid concentrations.* Against our expectations, and despite the substantial amount of fat ingested on a long-term basis by our animals fed on both PUFA-rich diets, SO and FO, there was no proportional change in liver lipid content (Table 1). Both dietary oils resulted in unchanged body weight, and the small increase in liver weight was significant only when expressed in terms of the liver/body weight ratio. By contrast, the administration of clofibrate to animals receiving either SO or FO promoted a significant liver enlargement, similar in percentage to that previously reported in animals receiving CD, without affecting body weight (10).

**TABLE 1**
**Liver and Plasma Lipids of Mice Fed Diets with Sunflower Oil (SO) and Fish Oil (FO) and Changes Induced by the Simultaneous Administration of Clofibrate[a]**

| | Diet/clofibrate (+ or –) | | | | |
|---|---|---|---|---|---|
| | CD | SO | | FO | |
| | – | – | + | – | + |
| Weights (g) | | | | | |
| Body | 32.6 ± 1.3 | 29.9 ± 0.1 | 29.0 ± 0.9 | 30.0 ± 1.0 | 29.0 ± 1.0 |
| Liver | 1.3 ± 0.1 | 1.7 ± 0.1[b] | 2.4 ± 0.3[d] | 1.6 ± 0.1 | 2.5 ± 0.2[d] |
| Liver/body × 100 | 3.9 ± 0.5 | 5.7 ± 0.3[b] | 8.4 ± 1.1[d] | 5.2 ± 0.5 | 8.3 ± 0.7[d] |
| Liver lipids | | | | | |
| Phospholipids (mg/liver) | 21.8 ± 0.3 | 28.5 ± 0.6[b] | 48.6 ± 2.9[d] | 29.8 ± 1.2 | 48.0 ± 2.1[d] |
| (mg/g) | 16.1 ± 0.7 | 17.4 ± 0.3[b] | 20.1 ± 1.2 | 19.5 ± 0.7 | 19.4 ± 0.8 |
| Triacylglycerols (mg/liver) | 6.6 ± 1.7 | 14.8 ± 1.3[b] | 4.6 ± 1.1[d] | 22.9 ± 2.4[c] | 3.2 ± 1.5[d] |
| (mg/g) | 5.1 ± 2.2 | 8.7 ± 0.9 | 1.9 ± 0.7[d] | 14.3 ± 2.1[c] | 1.3 ± 0.4[d] |
| TG/PL × 100 | 30.3 ± 4.5 | 51.9 ± 3.4[b] | 9.5 ± 4.5[d] | 76.8 ± 5.3[c] | 6.7 ± 1.8[d] |
| Diacylglycerols (mg/g) | 0.13 ± 0.02 | 0.16 ± 0.01 | 0.14 ± 0.05 | 0.11 ± 0.01 | 0.13 ± 0.06 |
| Total cholesterol (mg/g) | 1.20 ± 0.2 | 1.10 ± 0.4 | 1.00 ± 0.2 | 1.30 ± 0.1 | 1.00 ± 0.2 |
| Cholesterol esters (mg/g) | 0.10 ± 0.02 | 0.09 ± 0.04 | 0.05 ± 0.02 | 0.09 ± 0.01 | 0.03 ± 0.01[d] |
| Esterified/total cholesterol × 100 | 8.3 ± 2.0 | 7.8 ± 3.0 | 5.3 ± 1.5[d] | 7.3 ± 0.7 | 2.7 ± 1.0[d] |
| Plasma lipids (mg/dL) | | | | | |
| Phospholipids | 158 ± 13 | 154 ± 10 | 160 ± 10 | 123 ± 17[c] | 117 ± 17 |
| Triacylglycerols | 135 ± 18 | 142 ± 12 | 101 ± 9[d] | 71 ± 12[c] | 79 ± 17 |
| Total cholesterol | 127 ± 4 | 107 ± 1[b] | 93 ± 5[d] | 48 ± 3[c] | 41 ± 10 |
| HDL-cholesterol | 85 ± 1 | 77 ± 7 | 51 ± 3[d] | 40 ± 3[c] | 25 ± 3[d] |
| LDL-cholesterol | 21 ± 4 | 15 ± 7 | 20 ± 5 | 13 ± 2[c] | 16 ± 2 |
| HDL/LDL cholesterol | 4.0 ± 1.0 | 3.3 ± 1.4 | 2.7 ± 0.5 | 2.2 ± 0.1[c] | 1.6 ± 0.4[d] |

[a]Plus and minus signs indicate the presence or absence of clofibrate. Results are presented as mean values ± SD ($n$ = 4 animals per group). CD, chow diet; SO, sunflower oil; FO, fish oil.
[b]Significant differences due to change in diet (SO or FO vs. CD; $P < 0.05$).
[c]Significant differences due to dietary oil type (SO vs FO; $P < 0.05$).
[d]Significant differences due to clofibrate (SO or FO without, vs. SO or FO with, clofibrate; $P < 0.05$).

The total amount of PL per liver was augmented slightly with the SO and FO diets, but increased considerably upon addition of clofibrate to the diets irrespective of oil type (Table 1). Since liver PL and liver weight increased simultaneously, the resultant PL concentration per gram of tissue was unchanged by the drug. In contrast, the amount and the concentration of TG increased after 1 mon on both PUFA-rich diets. The TG increase was modest, especially considering that irrespective of this enrichment, the TG were dramatically depleted by clofibrate treatment (Table 1). The diacylglycerols did not change in any of the experimental conditions. The CE, unaffected by dietary oils, were also reduced by clofibrate, whereas free cholesterol did not change.

In plasma, the concentrations of PL, TG, and total cholesterol, which were not affected by dietary SO, were decreased by FO supplementation (Table 1). Conversely, clofibrate did not much affect the lipids already reduced by FO, but it decreased plasma TG and cholesterol in animals receiving SO. In both diets clofibrate decreased high density lipoprotein-cholesterol.

The TG depletion induced by clofibrate may be ascribed to drug-induced changes that demand and consume fatty acids, such as peroxisomal and mitochondrial biogenesis to provide for drug substrate oxidation, or microsomal membrane formation to provide for conjugation; in turn, these processes cause hypertrophy. The latter, and the hyperplasia that ensues upon prolonged drug treatment, are processes that require PL and fatty acids. By having their fatty acids assist as substrates for oxidative and synthetic processes, liver TG may support PL formation and liver enlargement. A possible explanation for the failure of our high-fat diets to increase liver TG more than a few mg/g in the presence of unchanged body weight and unchanged or even decreased lipid plasma levels may be that the excess dietary fat may have modified the way the body metabolized fat. Even when tissue PL admit a given amount of n-6 or n-3 exogenous PUFA, the prolonged excess of these fatty acids may have resulted in the stimulated oxidation of the own fat provided. This possibility is supported by the fact that, in rats, the number of peroxisomes and mitochondria and the rates of β-oxidation increase under high-fat diets especially those containing a high n-3/n-6 PUFA ratio (16).

*Fatty acids of liver and plasma lipids.* The fatty acid profiles of principal lipids from liver and plasma (Table 2) were notably influenced by the type of oil given in the diet and by clofibrate. The effects of the two dietary oils tended to affect the n-6/n-3 PUFA ratios in opposite ways. The most marked qualitative changes were produced in the fatty acids of TG. In liver, SO or FO resulted in increased percentages of n-6 or n-3 PUFA, which partly replaced the major TG component, 18:1n-9. In turn, the most conspicuous outcome of clofibrate administration was that it decreased TG PUFA. Since TG levels were substantially reduced by the drug treatment (Table 1), it caused a greater decrease in the polyunsaturated than in the other acyl groups of TG. This was accompanied by a significant clofibrate-related increase in the percentage of 18:1n-9, to the point where this fatty acid again became the major TG acyl group.

The fatty acid composition of mouse liver and plasma CE (Table 2) and the changes induced by diet and clofibrate on such fatty acids are not extensively known. The content of liver CE was small, and low in PUFA; compared with the other lipids it was modified little in amount or fatty acid composition by the dietary manipulations. On the contrary, the abundant plasma CE changed from an n-6 to an n-3 fatty acid-rich lipid class as a consequence of dietary FO, the major circulating CE fatty acid becoming 20:5n-3 instead of 20:4n-6. Clofibrate tended to affect EC and its fatty acids in the same direction as those of TG.

In FO-consuming mice, while liver clofibrate-wasted TG were selectively depleted of PUFA, the major PUFA profiles of the enlarged PL fraction remained quite similar to those of their controls. This observation contrasts with previous results in which the mass of PL formed after clofibrate action was relatively impoverished in n-3 PUFA (10) and agrees with the interpretation that CD, even when not n-3 PUFA-deficient, was insufficient to provide for the stimulated PL biosynthesis imposed by the proliferator.

The increase in the percentage of 18:1n-9 in TG and in PL may be related to the fact that, like other peroxisomal proliferators, clofibrate induces hepatic stearoyl CoA desaturase (2, 16) among many other enzymes. Recent studies have shown that clofibrate increases, whereas PUFA decrease, the level of activity and the mRNA content of 9-desaturase (17). Another general change induced by clofibrate is a significant increase in the percentage of 20:3n-6 (Table 2), leading to a markedly increased 20:3n-6/20:4n-6 ratio in all lipids analyzed but especially in liver PL. With the present diets, the 22:4n-6/22:5n-6 and the 22:5n-3/22:6n-3 ratios in liver PL are increased by clofibrate just as in previous work (10), suggesting an action of the drug on PUFA desaturases. The administration of clofibrate thus resulted in qualitative changes in specific fatty acids that were independent of the type of PUFA predominating in the diet.

*Liver phospholipid classes and their fatty acids.* In plasma, dietary FO decreased total PL. Since this reduction mostly affected PC, the proportion of other circulating PL, including SM, increased with no apparent changes in concentration (data not shown). Clofibrate did not affect this situation, the plasma PL composition remaining similar to that in the corresponding SO or FO controls. In liver (Table 3), some small but significant differences in PL proportions were observed that indicated effects of dietary oils on specific lipid classes. Thus, SO and FO ingestion resulted in a different percentage of PC (48.0 ± 1.1 to 43.0 ± 1.7%, respectively) with an unchanged percentage of PE, and hence in slightly different phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratios (1.8 ± 0.1 to 1.5 ± 0.2, respectively). An interesting effect of SO and FO was that both tended to increase liver diphosphatidylglycerol (DPG) and SM relatively more than other PL, the increase in DPG being similar with both oils and that of SM being larger for FO. In the presence of clofibrate

**TABLE 2**
**Major Fatty Acids of Phospholipids, Cholesterol Esters, and Triacylglycerols from Liver and Plasma of Mice Fed Polyunsaturated Oils and Clofibrate[a]**

| | Diet/clofibrate (+ or –) | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Phospholipids | | | | | | | | | | Cholesterol esters | | | | | | | | | |
| | Liver | | | | | Plasma | | | | | Liver | | | | | Plasma | | | | |
| | CD | SO | | FO | | CD | SO | | FO | | CD | SO | | FO | | CD | SO | | FO | |
| | – | – | + | – | + | – | – | + | – | + | – | – | + | – | + | – | – | + | – | + |
| 16:0 | 23.2 | 20.3 | 23.3 | 28.2$^c$ | 26.6 | 23.7 | 25.2 | 24.7 | 25.1 | 26.7 | 51.2 | 61.8$^b$ | 55.1$^d$ | 60.5 | 66.9$^d$ | 3.6 | 5.2$^b$ | 4.3$^d$ | 6.3$^c$ | 6.4 |
| 17:0 | 0.3 | 0.4 | 0.1 | 0.6 | 0.2 | 0.7 | 0.6 | 0.7 | 1.0 | 0.3 | 0.8 | 1.0 | 0.8 | 1.1 | 1.0 | 0.3 | 0.3 | 0.2 | 0.2 | 0.3 |
| 18:0 | 15.8 | 21.9$^b$ | 11.1$^d$ | 18.9 | 11.4$^d$ | 15.1 | 16.7 | 19.3 | 16.3 | 15.6 | 6.1 | 7.6 | 5.9$^d$ | 8.2 | 6.8$^d$ | 1.3 | 2.7 | 1.0$^d$ | 1.7 | 2.2 |
| 16:1 | 1.0 | 0.7 | 1.6$^d$ | 1.9$^c$ | 1.8 | 3.7 | 3.2 | 2.8 | 4.2 | 5.5 | 6.4 | 3.1$^b$ | 5.6$^d$ | 4.8 | 5.3 | 1.7 | 2.0 | 2.2 | 4.8$^c$ | 5.0 |
| 17:1 | 0.2 | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.4 | 0.2 | 0.5 | 0.4 | 0.6 | 0.6 | 0.6 | 0.2 | 0.2 | 0.2 | 0.3 | 0.5 |
| 18:1 | 9.4 | 6.9$^b$ | 15.8$^d$ | 8.8 | 16.0$^d$ | 13.7 | 12.3 | 15.1 | 17.6 | 24.4 | 18.4 | 10.0$^b$ | 15.5$^d$ | 14.2$^c$ | 12.6 | 5.2 | 7.9$^b$ | 5.9$^d$ | 8.6$^c$ | 12.7$^d$ |
| 18:2n 6 | 14.2 | 18.4$^b$ | 13.8$^d$ | 7.8$^c$ | 6.8 | 17.6 | 21.1$^b$ | 19.0 | 8.2$^c$ | 6.5$^d$ | 8.7 | 10.6$^b$ | 9.7 | 3.6$^c$ | 3.0 | 30.1 | 31.6 | 36.1$^d$ | 17.5$^c$ | 18.7 |
| 18:3n-6 | 0.2 | 0.7$^b$ | 0.2$^d$ | 0.2$^c$ | 0.2 | 0.4 | 0.3 | 0.3 | 0.3 | 0.1 | 0.1 | 0.4 | 0.6 | — | — | 0.5 | 1.5$^b$ | 1.1 | 0.4 | 0.2$^d$ |
| 20:3n 6 | 0.5 | 0.7$^b$ | 5.3$^d$ | 0.4$^c$ | 2.1$^d$ | 0.5 | 0.6 | 3.7$^d$ | 0.4 | 1.9$^d$ | 0.1 | 0.3 | 0.6$^d$ | 0.2 | 0.2 | 0.3 | 0.3 | 2.3$^d$ | 0.2 | 0.7$^d$ |
| 20:4n 6 | 20.1 | 21.8$^b$ | 24.4$^d$ | 6.7$^c$ | 5.8 | 12.4 | 12.3 | 9.5$^d$ | 4.9$^c$ | 2.3$^d$ | 4.0 | 3.1 | 4.2 | 0.9$^c$ | 0.7 | 51.7 | 44.7$^b$ | 43.7 | 18.6$^c$ | 12.1$^d$ |
| 22:4n-6 | 0.2 | 0.3$^b$ | 0.1$^d$ | 0.3 | 0.1$^d$ | 0.4 | 0.4 | 0.3$^d$ | 0.6 | 1.1$^d$ | 0.1 | 0.1 | 0.2 | — | — | — | — | — | — | — |
| 22:5n-6 | 1.3 | 3.8$^b$ | 1.1$^d$ | 0.4$^c$ | 0.5 | 2.5 | 2.2 | 1.6$^d$ | 2.0 | 0.3$^d$ | 0.3 | 0.4 | 0.2$^d$ | 0.2$^c$ | 0.2 | 0.3 | 0.1 | 0.1 | — | — |
| 18:3n-3 | 0.2 | 0.1 | 0.1 | 0.3$^c$ | 0.1$^d$ | 0.5 | 0.2$^b$ | 0.2 | 0.5$^c$ | 0.4 | 0.4 | — | — | 0.3 | 0.3 | — | — | — | 0.1 | 0.2 |
| 20:5n 3 | 0.2 | 0.2 | 0.2 | 3.3$^c$ | 5.1$^d$ | 3.7 | 2.2$^b$ | 1.6 | 6.0c | 6.8 | 0.7 | 0.5 | 0.6 | 1.7$^c$ | 0.9$^d$ | 1.2 | 1.4 | 1.8 | 24.9$^c$ | 29.9$^d$ |
| 22:5n-3 | 0.3 | 0.1$^b$ | 0.1 | 1.0$^c$ | 1.5$^d$ | 0.2 | 0.1$^b$ | 0.1 | 1.0$^c$ | 1.0$^d$ | — | 0.1 | — | 0.1 | 0.3 | — | — | — | 0.5 | 0.3 |
| 22:6n 3 | 12.7 | 3.6$^b$ | 2.5$^d$ | 21.0$^c$ | 21.8 | 4.5 | 2.4$^b$ | 1.2 | 11.4$^c$ | 6.8$^d$ | 2.5 | 0.6$^b$ | 0.5 | 3.5$^c$ | 1.3$^d$ | 3.7 | 2.4$^b$ | 1.2$^d$ | 16.0$^c$ | 10.9$^d$ |
| SFA / MFA | 3.7 | 5.5$^b$ | 2.0$^d$ | 4.3 | 2.1$^d$ | 2.2 | 2.7$^b$ | 2.5 | 1.9$^c$ | 1.4 | 2.3 | 5.2$^b$ | 2.9$^d$ | 3.6$^c$ | 4.0 | 0.7 | 0.8 | 0.7 | 0.6 | 0.5 |
| n-6/n-3 PUFA | 2.7 | 11.6$^b$ | 16.0$^d$ | 0.6$^c$ | 0.5 | 3.8 | 7.5$^b$ | 11.4$^d$ | 0.9$^c$ | 0.8 | 3.8 | 12.8$^b$ | 13.7 | 0.9$^c$ | 1.5$^d$ | 17.1 | 20.9$^b$ | 28.2$^d$ | 0.9 | 0.8 |

| | Triacylglycerols | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Liver | | | | | Plasma | | | | |
| | CD | SO | | FO | | CD | SO | | FO | |
| | – | – | + | – | + | – | – | + | – | + |
| 16:0 | 22.3 | 21.4 | 20.3 | 26.8$^c$ | 26.4 | 15.6 | 14.6 | 15.6 | 15.5 | 17.2 |
| 17:0 | 0.3 | 0.2 | 0.1 | 0.1 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.5 |
| 18:0 | 2.3 | 1.8 | 1.2 | 2.8$^c$ | 2.5 | 4.1 | 4.3 | 3.5 | 4.7 | 4.5 |
| 16:1 | 3.6 | 2.8 | 6.6 | 6.7$^c$ | 6.5 | 1.6 | 2.4 | 3.4 | 2.7 | 3.1 |
| 17:1 | 0.3 | 0.1 | 0.2 | 0.9$^c$ | 0.5 | 0.3 | 0.3 | 0.3 | 0.4 | 0.5 |
| 18:1 | 31.5 | 22.0$^b$ | 35.5$^d$ | 20.3$^c$ | 35.9$^d$ | 26.5 | 26.2 | 36.2$^d$ | 20.9 | 44.0$^d$ |
| 18:2n-6 | 29.7 | 41.0$^b$ | 27.6$^d$ | 7.4$^c$ | 7.0 | 37.9 | 37.8 | 31.0 | 9.1$^c$ | 7.0 |
| 18:3n-6 | 1.1 | 1.8$^b$ | 1.0$^d$ | 0.5$^c$ | 0.7 | 0.9 | 1.5 | 0.9 | 0.8 | 0.2 |
| 20:3n-6 | 0.3 | 1.0$^b$ | 1.5$^d$ | 0.1$^c$ | 0.3 | 1.0 | 0.6 | 1.4$^d$ | 0.2$^c$ | 0.2 |
| 20:4n-6 | 3.7 | 4.2$^b$ | 3.6$^d$ | 1.0$^c$ | 0.8 | 6.6 | 6.8 | 4.4$^d$ | 1.3$^c$ | 0.7$^d$ |
| 22:4n-6 | 0.5 | 0.9$^b$ | 0.8 | 1.1$^c$ | 0.6 | 0.9 | 0.9 | 0.9 | 0.5 | 1.0 |
| 22:5n-6 | 0.7 | 1.5$^b$ | 0.3$^d$ | 0.2$^c$ | 0.1 | 2.3 | 1.8 | 0.6$^d$ | 0.5$^c$ | 1.0 |
| 18:3n-3 | 1.0 | 0.8 | 0.7 | 1.5 | 1.1 | 0.8 | 0.4$^b$ | 0.3 | 1.4$^c$ | 1.9$^d$ |
| 20:5n-3 | 0.4 | 0.2$^b$ | 0.1 | 7.6$^c$ | 3.1$^d$ | 0.1 | 0.2 | 0.2 | 16.0$^c$ | 4.7$^d$ |
| 22:5n-3 | 0.4 | 0.1$^b$ | 0.1 | 3.8$^c$ | 2.3$^d$ | 0.1 | 0.2 | 0.3 | 3.1$^c$ | 2.0$^d$ |
| 22:6n-3 | 2.2 | 0.4$^b$ | 0.4 | 19.3$^c$ | 12.1$^d$ | 0.9 | 1.5 | 0.8$^d$ | 22.5$^c$ | 11.6$^d$ |
| SFA/MFA | 0.7 | 0.9 | 0.5 | 1.1 | 0.7 | 0.7 | 0.7 | 0.5 | 0.9 | 0.5 |
| n-6/n-3 PUFA | 9.1 | 34.0$^b$ | 26.7$^d$ | 0.3$^c$ | 0.5 | 25.3 | 21.3 | 23.3 | 0.3$^c$ | 0.5 |

[a]Values are weight percent, as mean values ± SD ($n$ = 4 animals per group). SFA, saturated fatty acids; MFA, monoenoic fatty acids; PUFA, polyunsaturated fatty acids; for other abbreviations and for explanation of +/– signs see Table 1.
[b]Significant differences due to change in diet (SO or FO vs. CD; $P < 0.05$).
[c]Significant differences due to dietary oil type (SO vs. FO; $P < 0.05$).
[d]Significant differences due to clofibrate (SO or FO without, vs. SO or FO with, clofibrate; $P < 0.05$).

in both diets, the major hepatic glycerophospholipid classes (PC, PE, phosphatidylserine and phosphatidylinositol) increased to a similar extent in response to the growth imposed by the proliferator (Table 3), with respect to their corresponding controls. In contrast, the proportion of liver DPG, and even more markedly that of SM, tended to decrease with clofibrate, showing that the amount of these components increased less than that of other PL, or even decreased. SM was the only liver lipid whose concentration (Table 4) was significantly reduced in liver by clofibrate under both diets.

The increase in liver DPG may be correlated to the proliferation of mitochondria (16) that is induced by excessive

**TABLE 3**
**Liver Phospholipids from Mice Fed Polyunsaturated Oils and Clofibrate[a]**

| | Diet/clofibrate (+ or –) | | | | |
|---|---|---|---|---|---|
| | CD | SO | | FO | |
| Phospholipid | – | – | + | – | + |
| | mg PL/liver (%) | | | | |
| CGP | 10.1 ± 0.3 (46.5) | 13.7 ± 0.3 (48.0) | 23.2 ± 0.8[d] (47.7) | 12.9 ± 0.5 (43.4)[c] | 22.5 ± 1.1[d] (46.8) |
| EGP | 6.2 ± 0.2 (28.4) | 7.8 ± 0.4 (27.2) | 13.3 ± 0.6[d] (27.4) | 8.5 ± 0.4 (28.4) | 13.7 ± 0.3[d] (28.6) |
| LysoPC | 0.7 ± 0.2 (3.0) | 0.5 ± 0.1 (1.8) | 1.1 ± 0.1[d] (2.3) | 0.9 ± 0.2 (3.0) | 1.7 ± 0.5[d] (3.5) |
| PS | 0.9 ± 0.1 (3.9) | 1.1 ± 0.2 (3.8) | 2.0 ± 0.2[d] (4.2) | 1.2 ± 0.1 (3.9) | 1.8 ± 0.2[d] (3.7) |
| PI | 2.2 ± 0.2 (10.1) | 2.4 ± 0.2 (8.3) | 5.0 ± 0.1[d] (10.2) | 2.7 ± 0.2 (9.1) | 4.4 ± 0.2[d] (9.1) |
| DPG | 1.1 ± 0.02 (4.9) | 2.0 ± 0.2[b] (7.1) | 2.9 ± 0.2[d] (5.9) | 2.1 ± 0.2[b] (7.2)[b] | 2.7 ± 0.5[d] (5.6) |
| SM | 0.7 ± 0.1 (3.2) | 1.1 ± 0.1[b] (3.8) | 1.1 ± 0.2 (2.3)[d] | 1.5 ± 0.1[b,c] (5.0)[c] | 1.2 ± 0.2 (2.6)[d] |

[a]Phospholipid classes were determined by phosphorus analysis after thin-layer chromatography. Values are means ± SD ($n$ = 4 animals per group). CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; PS, phosphatidylserine; PI, phosphatidylinositol; DPG, diphosphatidylglycerol; SM, sphingomyelin; for other abbreviations and for explanation of ± see Table 1.
[b]Significant differences due to change in diet (SO or FO vs. CD; $P < 0.05$).
[c]Significant differences due to dietary oil type (SO vs. FO; $P < 0.05$).
[d]Significant differences due to clofibrate ($P < 0.05$).

**TABLE 4**
**Fatty Acids of Sphingomyelin from Livers of Mice Fed Polyunsaturated Oils and Clofibrate[a]**

| | Diet/clofibrate (+ or –) | | | | |
|---|---|---|---|---|---|
| | CD | SO | | FO | |
| Fatty acid | – | – | + | – | + |
| 16:0 | 6.3 ± 0.52 | 6.7 ± 0.86 | 7.1 ± 1.51 | 5.7 ± 1.13 | 7.5 ± 1.44 |
| 18:0 | 4.6 ± 0.52 | 3.7 ± 0.41 | 2.9 ± 0.48 | 2.4 ± 0.16[b] | 3.1 ± 0.15[d] |
| 20:0 | 3.5 ± 0.76 | 3.3 ± 0.42 | 2.8 ± 0.24 | 3.0 ± 0.28 | 3.4 ± 0.49 |
| 22:0 | 28.4 ± 0.95 | 38.9 ± 0.44[b] | 19.1 ± 1.61[d] | 38.0 ± 2.24[b] | 24.5 ± 1.61[d] |
| 24:0 | 16.5 ± 0.57 | 17.4 ± 2.58 | 13.9 ± 2.31 | 17.6 ± 1.85 | 11.2 ± 2.35[d] |
| SFA, even chain | 59.3 ± 2.47 | 70.7 ± 1.42[b] | 45.6 ± 2.36[d] | 66.4 ± 2.89[b] | 49.1 ± 3.42[d] |
| 17:0 | 0.2 ± 0.03 | 0.2 ± 0.13 | 0.2 ± 0.05 | 0.1 ± 0.04[b] | 0.1 ± 0.04 |
| 19:0 | 0.03 ± 0.01 | 0.1 ± 0.05 | 0.04 ± 0.04 | 0.07 ± 0.06 | 0.08 ± 0.01 |
| 21:0 | 0.1 ± 0.04 | 0.2 ± 0.10 | 0.1 ± 0.02 | 0.1 ± 0.01 | 0.1 ± 0.03 |
| 23:0 | 6.3 ± 0.72 | 5.2 ± 0.18 | 5.0 ± 0.37 | 5.9 ± 0.23 | 3.7 ± 0.45[d] |
| SFA, odd chain | 6.7 ± 0.74 | 5.6 ± 0.09 | 5.3 ± 0.37 | 6.2 ± 0.27 | 4.0 ± 0.35[d] |
| 16:1 | 0.6 ± 0.20 | 0.3 ± 0.06[b] | 0.3 ± 0.13 | 0.1 ± 0.11[b] | 0.2 ± 0.13 |
| 18:1 | 3.0 ± 0.90 | 1.0 ± 0.27[b] | 1.0 ± 0.48 | 0.7 ± 0.11[b] | 1.0 ± 0.34 |
| 20:1 | 0.4 ± 0.21 | 0.4 ± 0.02 | 0.2 ± 0.05[d] | 0.4 ± 0.03 | 0.2 ± 0.03[d] |
| 22:1 | 2.4 ± 0.27 | 2.0 ± 0.33 | 4.8 ± 0.76 | 2.2 ± 0.16 | 5.0 ± 0.89[d] |
| 24:1 | 22.2 ± 0.47 | 14.5 ± 1.10[b] | 37.4 ± 0.94[d] | 21.5 ± 2.90[c] | 37.9 ± 1.86[d] |
| MFA, even chain | 28.5 ± 0.63 | 18.3 ± 1.72[b] | 43.7 ± 0.94[d] | 24.9 ± 3.09[c] | 44.1 ± 2.23[d] |
| 17:1 | 0.03 ± 0.02 | 0.04 ± 0.02 | 0.03 ± 0.01 | 0.02 ± 0.01 | 0.08 ± 0.07 |
| 19:1 | 1.0 ± 0.44 | 0.4 ± 0.21 | 0.4 ± 0.39 | 0.2 ± 0.09 | 0.2 ± 0.13 |
| 23:1 | 1.9 ± 0.19 | 1.7 ± 0.31 | 1.1 ± 0.13[d] | 1.4 ± 0.06 | 1.3 ± 0.09 |
| MFA, odd chain | 2.9 ± 0.56 | 2.2 ± 0.47 | 1.5 ± 0.39[d] | 1.6 ± 0.07 | 1.6 ± 0.22 |
| 18:2 | 1.0 ± 0.21 | 1.4 ± 0.17 | 1.0 ± 0.01[d] | 0.3 ± 0.01[b,c] | 0.4 ± 0.02[d] |
| 24:2 | 1.6 ± 0.16 | 1.8 ± 0.15 | 2.9 ± 0.27[d] | 0.6 ± 0.16[b,c] | 0.8 ± 0.22 |
| Dienes | 2.6 ± 0.20 | 3.2 ± 0.30[b] | 3.9 ± 0.30 | 0.9 ± 0.20[b,c] | 1.2 ± 0.20 |
| Total SFA | 67.7 ± 1.6 | 75.3 ± 1.4[b] | 51.0 ± 2.4[d] | 72.8 ± 3.1 | 53.4 ± 2.7[d] |
| Total MFA | 33.3 ± 0.8 | 20.5 ± 2.2[b] | 44.9 ± 1.0[d] | 26.5 ± 3.1[b] | 45.2 ± 2.2[d] |
| SFA/MFA | 2.0 ± 0.1 | 3.7 ± 0.4[b] | 1.1 ± 0.1[d] | 2.8 ± 0.5[b] | 1.2 ± 0.1[d] |
| μmol SM/g | 0.64 ± 0.02 | 0.82 ± 0.01[b] | 0.57 ± 0.04[d] | 1.22 ± 0.05[b,c] | 0.62 ± 0.02[d] |

[a]Fatty acid composition is given as weight percent fatty acid, as mean values ± SD ($n$ = 4 animals per group). For abbreviations and explanation of ± see Tables 1 and 2.
[b]Significant differences due to change in diet (SO or FO vs. CD; $P < 0.05$).
[c]Significant differences due to dietary oil type (SO vs. FO; $P < 0.05$).
[d]Significant effects of clofibrate ($P < 0.05$).

PUFA consumption, since this glycerophospholipid is a well-known component of the inner mitochondrial membrane. Our DPG, which was exceedingly rich in 18:2n-6, admitted even more n-6 and a considerable amount of n-3 PUFA with the intake of SO or FO diets, respectively, while reducing the percentage of its monounsaturated fatty acids, mainly 18:1 and 16:1 (not shown). These diet-induced compositional changes could influence mitochondrial enzyme activity. Clofibrate affected the fatty acid composition of DPG as in other lipids, decreasing the percentage of PUFA and increasing that of 18:1n-9.

The fatty acid profile of liver SM (Table 4) was significantly affected by the consumption of both PUFA-rich diets, which resulted in an increase in SM very long chain SFA such as 22:0 and 24:0. By contrast, in clofibrate-treated mice there was a marked decrease in the percentage (and hence in the concentration) of the major SFA of SM; instead, very long chain MFA such as 22:1 and 24:1 became principal acyl chains.

The selective reduction of SM with clofibrate had also been observed in the absence of oil supplementation (10). More recently, several peroxisomal proliferators including clofibrate have been shown to increase liver PC and PE, while decreasing SM in rat liver (18) in agreement with our results. To our knowledge, the only previous report showing that the intake of PUFA can affect the fatty acids of liver SM is that by Bettger *et al.* (19). The mechanism(s) involved and the possible physiological effects of such diet-induced SM modification are still unknown. The fact that clofibrate affects the ratios between very long chain saturated and monoenoic very long chain fatty acids of SM had also been observed in animals fed the CD (10). Thus, this effect of clofibrate does not depend on the amount or type of fatty acids provided in the diet.

The diet-induced SM increase may arise from stimulated synthesis or inhibited degradation of this phospholipid, and conversely, the clofibrate-induced SM decrease may be the result of inhibited synthesis or activated degradation. The possibility that clofibrate affects enzymes involved in SM degradation is exciting, since ceramide and sphingosine are well-known regulators of protein kinase C and therefore mediators of important cell functions including proliferation, differentiation, and death (20). Clofibrate has been shown to inhibit apoptosis in liver (21). A potential consequence of the clofibrate-induced decrease in SM could be the generation of SM metabolites that could be responsible for the inordinate cell proliferation induced by the drug.

Since SM is a ubiquitous lipid, the cellular and subcellular locations of the contrasting effects of dietary oils and clofibrate need to be established. A possible relationship between SM changes and nuclear PL is worth investigating, since SM is an important lipid component of liver nuclear chromatin (22) and nuclear membrane (23). Neutral sphingomyelinase has also been characterized in liver hepatic chromatin fractions and nuclear membranes, and shown to be stimulated after the typical hepatocyte regeneration/proliferation induced by hepatectomy (24,25), a process that is comparable to the clofibrate-induced liver mass expansion seen in the present work.

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# Low Density Lipoprotein Receptor mRNA in Rat Liver Is Affected by Resistant Starch of Beans

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**ABSTRACT:** The effects of resistant starches of beans on serum cholesterol and hepatic low density lipoprotein (LDL) receptor mRNA in rats were investigated. Rats were fed a cholesterol-free diet with 150 g/kg corn starch (CS), 150 g/kg adzuki (*Vigna angularis*) starch (AS), 150 g/kg kintoki (*Phaseolus vulgaris*, variety) starch (KS), or 150 g/kg tebou (*P. vulgaris*, variety) starch (TS) for 4 wk. There were no significant differences in body weight among groups through the experimental period. The liver weight in the CS group was 1.1–1.2 times higher than that in the AS, KS, and TS groups. The cecum weight in the TS was 1.4 times higher than that in the CS group, and the cecal pH in the CS group was significantly higher than in the other groups. The serum total cholesterol, very low density lipoprotein + intermediate density lipoprotein + LDL-cholesterol and high density lipoprotein (HDL)-cholesterol concentrations in the bean starch groups were significantly lower than those in the CS group through the feeding period. The total cholesterol/HDL-cholesterol ratio in the bean starch groups was also significantly lower than that in the CS group at the end of the 4-wk feeding period. The hepatic cholesterol concentration in the TS group was significantly higher than in the CS group at the end of the 4-wk feeding period. The relative quantity of hepatic apo B mRNA in the AS group was 1.2 times higher than that in the CS group, and the hepatic LDL receptor mRNA levels in the AS and TS groups were 1.8–2.0 times higher than that in the CS group. The results of this study demonstrate that AS, KS, and TS lowered the serum total cholesterol level by enhancing the hepatic LDL receptor mRNA level.

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The possibility of lowering plasma cholesterol concentration by interfering with the absorption of cholesterol or bile acids has been extensively investigated using either compounds of natural origin (e.g., gel-forming fibers, resistant starch, phytosterols, and saponins) or synthetic sequestrants such as cholestyramine (1). Fiber is known to be a nonnutritional substrate. On the other hand, starch is generally the major constituent of the human diet and has long been regarded to be almost entirely digested in the upper part of the digestive tract. Soluble fibers are generally broken down by large intestine microflora, and the resulting production of short-chain fatty acids (SCFA) may be involved in the metabolic effects of fibers (2). Resistant starch is also broken down by the colonic microflora, essentially to SCFA, which allows the recovery of a part of the chemical energy by the host. Rats fed resistant starch had significantly lower plasma cholesterol (–32%) and triglyceride (–29%) concentrations than control rats fed a wheat starch diet or rats fed a cholestyramine diet (3). SCFA may be involved in lowering serum cholesterol concentration (4).

Sonoyama *et al.* (5) reported that the plasma cholesterol concentration was significantly lower in rats fed sugar beet fiber than in those fed fiber-free or cholestyramine diets, and this difference was due mainly to a lower high density lipoprotein (HDL)-cholesterol concentration. Fukushima *et al.* (6) also reported that serum very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) + low density lipoprotein (LDL)-cholesterol concentration was significantly lower in rats fed mushroom fiber and sugar beet fiber than in rats fed a cellulose fiber diet, and the hepatic LDL receptor mRNA level in the mushroom fiber and sugar beet fiber groups was significantly higher than that in the cellulose group. It was reported that resistant starch was more effective in lowering plasma HDL-cholesterol and LDL-cholesterol concentrations (3). However, the mechanism is not fully understood. In this study, we examined the effects of diets containing adzuki (*Vigna angularis*) starch, kintoki (*Phaseolus vulgaris*, variety) starch, and tebou (*P. vulgaris*, variety) starch on serum lipids, liver lipids, and hepatic mRNA.

## MATERIALS AND METHODS

*Animal and diets.* Male F344/DuCrj rats (8-wk old) were purchased from Charles River Japan Inc. (Yokohama, Japan). All animals were housed individually in cages on a 12-h light/dark cycle. Temperature and humidity were controlled at 23 ± 1°C and 60 ± 5%, respectively. The rats were divided into four groups of six animals each by randomization. There were no significant differences in body weights and serum total cholesterol concentrations between groups at the start of

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Abbreviations: Apo, apolipoprotein; AS, adzuki starch; CS, corn starch; DIG, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLC, gas–liquid chromatography; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; KS, kintoki starch; LDL, low density lipoprotein; PCR, polymerase chain reaction; RT, reverse transcription; SCFA, short-chain fatty acid; TG, triglyceride; TS, tebou starch; VLDL, very low density lipoprotein.

**TABLE 1**
**Composition of Experimental Diet**

| Components | Diet group | | | |
|---|---|---|---|---|
| | CS | AS | KS | TS |
| | (g/kg diet) | | | |
| Casein | 250 | 250 | 250 | 250 |
| Corn oil | 50 | 50 | 50 | 50 |
| Mineral mixture[a] | 35 | 35 | 35 | 35 |
| Vitamin mixture[b] | 10 | 10 | 10 | 10 |
| Choline chloride | 2 | 2 | 2 | 2 |
| Cellulose powder | 100 | 100 | 100 | 100 |
| Corn starch (CS) | 150 | — | — | — |
| Adzuki starch (AS) | — | 150 | — | — |
| Kintoki starch (KS) | — | — | 150 | — |
| Tebou starch (TS) | — | — | — | 150 |
| Sucrose to | 1000 | 1000 | 1000 | 1000 |

[a]AIN-76 mineral mixture (22).
[b]AIN-76 vitamin mixture (22).

the experimental period. The composition of each diet is shown in Table 1. Each starch was retrograded after each type of bean was boiled. The experimental groups were fed for 4 wk one of the following diets that contained 150 g/kg of adzuki starch (AS), kintoki starch (KS), and tebou starch (TS). The compositions of AS, KS, and TS (g/100 g) were as follows: moisture, 2.7, 1.4, and 2.7; total dietary fiber, 7.7 (insoluble fiber, 7.6; water-soluble fiber, 0.1), 16.6 (insoluble fiber, 15.9; water-soluble fiber, 0.7) and 23.5 (insoluble fiber, 23.1; water-soluble fiber, 0.4); protein (N × 6.25), 4.9, 4.3, and 4.2; lipid, 0.1, 0.2 and 0.2; carbohydrate, 81.5, 74.4, and 65.6; ash, 3.1, 3.1, and 3.8, respectively. Total dietary fiber, insoluble fiber, water-soluble fiber, protein, lipid, carbohydrate, moisture, and ash were determined by AOAC procedures (7). The control group consisted of rats fed 150 g/kg of corn starch (CS). The adzuki, kintoki, and tebou beans were kindly provided by the Hokkaido Tokachi Area Regional Food Processing Technology Center, Obihiro, Hokkaido, Japan. The rats were allowed free access to experimental diets and water for 4 wk. Body weight and feed consumption were recorded weekly and every day, respectively. All animal procedures described conformed to standard principles in *Guide for the Care and Use of Laboratory Animals* (8).

*Analytical procedures.* Blood samples (1 mL) were collected between 0800 and 1000 h from the jugular veins of fasting rats. The samples were taken into tubes without an anticoagulant. After the samples stood at room temperature for 2 h, serum was prepared by centrifugation at 1500 × *g* for 20 min. At the end of the experimental period of 4 wk, all fecal excretion during 2 d was collected. Fecal dry weights did not significantly differ among groups. The rats were killed by ether inhalation, and the livers and cecum quickly removed, washed with cold saline (9 g NaCl/L), blotted dry on filter paper, and weighed before freezing for storage.

*Chemical analysis.* Total cholesterol, HDL-cholesterol, and triglyceride (TG) concentrations in the serum were determined enzymatically using commercially available reagent kits (assay kits for the TDX system; Abbott Laboratory Co., Irving, TX). The VLDL + IDL + LDL-cholesterol concentration was calculated as follows: VLDL + IDL + LDL-cholesterol = total cholesterol – HDL-cholesterol.

Total lipids were extracted from liver and feces by a mixture of chloroform/methanol (2:1, vol/vol) (9). The neutral sterol in each total lipid obtained by saponification was acetylated (10) and analyzed by gas–liquid chromatography (GLC) using a Shimadzu 14A chromatograph (Kyoto, Japan) with a DB17 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) with nitrogen as the carrier gas. Acidic sterols in feces were measured by GLC following the method of Grundy *et al.* (11). A part of the cecum was taken out into desalting water in a vial without exposure to air, and suspended. The suspension of cecum was deproteinized with perchloric acid (final concentration 50 g/L) cooled in ice, and the supernatant was added to a NaOH solution to precipitate perchloric acid and to form sodium salts of the SCFA. Individual SCFA was measured by GLC with a glass column (2000 × 3 mm) packed with 80–100 mesh Chromosorb W-AW DMCS with $H_3PO_4$ (100 mL/L) as the liquid phase after adding $H_3PO_4$ by the procedure of Hara *et al.* (12).

*RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR), and Southern blot analysis.* Total RNA was isolated by the acid guanidium/phenol/chloroform method, using Isogen (Nippon Gene, Tokyo, Japan) from liver (13). mRNA encoding apolipoprotein (apo) B, LDL receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an invariant control) was analyzed by semiquantitative RT-PCR and subsequent Southern hybridization of the PCR products with each inner oligonucleotide probe. Total RNA samples were treated with DNase RQ1 (Promega, Madison, WI) to remove genomic DNA and subjected to RT-PCR by using Moloney murine leukemia virus reverse transcriptase (GIBCO, Gaithersburg, MD) and EX-Taq polymerase (Takara, Tokyo, Japan) with apo B primers of oligonucleotides (upstream primer, 5′-GAAAGCATGCTGAAAACAACC-3′; downstream primer, 5′-AGGCCTGACTCGTGGAAGAA-3′), LDL receptor primers of oligonucleotides (upstream primer, 5′-ATTTTGGAGGATGAGAAGCAG-3′; downstream primer, 5′-CAGGGCGGGGAGGTGTGAGAA-3′), and GAPDH primers of oligonucleotides (upstream primer, 5′-GCCATCAACGACCCCTTCATT-3′, downstream primer, 5′-CGCCTGCTTCACCACCTTCTT-3′). The reaction mixtures for the PCR contained 25 pmol of each primer, 1.25 U EX-Taq polymerase, 1 × PCR buffer (Takara), and 200 mM deoxynucleoside 5′-triphosphates in a 50 μL reaction volume. The expected sizes of DNA fragments amplified with these primers were 725 bp for apo B, 931 bp for the LDL receptor, and 702 bp for GAPDH. Temperature cycling was as follows: first cycle, denaturation at 94°C for 3 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. Subsequent cycles were denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. The thermal cycling was completed by terminal extension at 72°C for 10 min. In total, 25 cycles were performed for the apo B and the LDL receptor, and 20 cycles for GAPDH. Amplification products were electrophoresed on 2% agarose gel, and transferred to a nylon membrane (Biodyne B; Pall Bio-

Support, East Hills, NY). Blots were hybridized with an apo B probe of a 54-base oligonucleotide (5′-TCCTTGCTTAC-CAAAAAGAGCTTC CAGTGTTGGCTCAAAGCCCTTT-CCTTCTAA-3′), LDL receptor probe of a 54-base oligonucleotide (5′-GTGAAC-TTGGGTGAGTGGGCACTGATCT-GAGGGGCAGGCAG-GCACATGTACTGG-3′), and GAPDH probe of a 54-base oligonucleotide (5′-TGATGACCAGCTTC-CCATTCTCAGCCTTGACTGTGCCGTTGAACTTGC-CGTGGG-3′). The probe was 3′-tailing labeled with digoxigenin (DIG), using a DIG oligonucleotide tailing kit (Boehringer Mannheim, Mannheim, Germany). Prehybridization, hybridization, and detection were carried out with a DIG luminescent detection kit (Boehringer Mannheim) as recommended by the manufacturer. The relative quantity of mRNA was estimated by densitometry scanning with X-ray film.

*Statistical analysis.* Data are presented as means and standard deviations. The mean and standard deviation for serum total cholesterol, HDL-cholesterol, and VLDL + IDL + LDL-cholesterol for each time point were calculated. The significance of differences among treatment groups was determined by analysis of variance with Duncan's multiple-range test (SAS Institute, Cary, NC). Results were considered significant at $P < 0.05$.

## RESULTS

*Feed intake, rat growth, and liver weight.* The results are summarized in Table 2. There were no significant differences in body weight among groups through the experimental period. The liver weight in the CS group was 1.1–1.2 times lower than that in the AS, KS, and TS groups. The cecum weight in the TS group was 1.4 times higher than that in the CS group, and the cecal pH in the CS group was significantly higher than in the other groups.

*Tissue lipid concentration.* Table 3 shows the serum total cholesterol, VLDL + IDL + LDL-cholesterol, HDL-choles-

**TABLE 2**
**Body Weight, Food Intake, Liver Weight, Cecum Weight, and Cecal pH in Rats Fed Bean Starches for 4 wk[a]**

| Diet group | Body weight (g) | | Food intake | Liver weight | Cecum weight | |
|---|---|---|---|---|---|---|
| | Initial | Gain | (g/4 wk) | (g/100g body wt) | (g/100 g body wt) | Cecal pH |
| CS | 178 ± 11 | 61 ± 10 | 391 ± 31 | 3.8 ± 0.3[a] | 1.4 ± 0.2[b] | 7.4 ± 0.2[a] |
| AS | 177 ± 8 | 59 ± 6 | 394 ± 34 | 3.5 ± 0.2[b] | 1.6 ± 0.2[a,b] | 7.0 ± 0.1[b] |
| KS | 176 ± 11 | 59 ± 4 | 406 ± 28 | 3.2 ± 0.1[c] | 1.5 ± 0.2[a,b] | 6.9 ± 0.1[b] |
| TS | 178 ± 6 | 62 ± 5 | 418 ± 25 | 3.4 ± 0.1[b,c] | 1.9 ± 0.3[a] | 6.8 ± 0.1[b] |

[a]Values are expressed as means ± standard deviations for six rats. Means within the same columns bearing different superscript roman letters are significantly different ($P < 0.05$). See Table 1 for abbreviations.

**TABLE 3**
**Serum Total Cholesterol, VLDL + IDL + LDL-Cholesterol, HDL-Cholesterol, and Triglyceride Concentrations and Total Cholesterol/HDL-Cholesterol Ratio in Rats Fed Bean Starches for 4 wk[a]**

| Diet group | 0 wk | 1 wk | 2 wk | 4 wk |
|---|---|---|---|---|
| Total cholesterol (mmol/L) | | | | |
| CS | 1.80 ± 0.07 | 2.34 ± 0.31[a] | 2.43 ± 0.28[a] | 2.65 ± 0.22[a] |
| AS | 1.77 ± 0.09 | 1.93 ± 0.15[b] | 1.84 ± 0.15[b] | 2.19 ± 0.19[b] |
| KS | 1.76 ± 0.12 | 1.88 ± 0.26[b] | 1.79 ± 0.13[b] | 2.07 ± 0.23[b] |
| TS | 1.75 ± 0.10 | 1.80 ± 0.10[b] | 1.70 ± 0.07[b] | 2.06 ± 0.19[b] |
| VLDL + IDL + LDL-cholesterol (mmol/L) | | | | |
| CS | 0.62 ± 0.05 | 0.80 ± 0.16[a] | 0.79 ± 0.10[a] | 0.89 ± 0.12[a] |
| AS | 0.62 ± 0.04 | 0.56 ± 0.11[b] | 0.53 ± 0.05[b] | 0.61 ± 0.05[b] |
| KS | 0.59 ± 0.04 | 0.53 ± 0.07[b] | 0.53 ± 0.06[b] | 0.58 ± 0.10[b] |
| TS | 0.58 ± 0.05 | 0.48 ± 0.09[b] | 0.52 ± 0.04[b] | 0.60 ± 0.07[b] |
| HDL-cholesterol (mmol/L) | | | | |
| CS | 1.18 ± 0.06 | 1.55 ± 0.19[a] | 1.64 ± 0.22[a] | 1.77 ± 0.11[a] |
| AS | 1.15 ± 0.07 | 1.36 ± 0.07[b] | 1.31 ± 0.13[b] | 1.58 ± 0.15[b] |
| KS | 1.17 ± 0.10 | 1.35 ± 0.21[b] | 1.26 ± 0.08[b] | 1.49 ± 0.15[b] |
| TS | 1.17 ± 0.10 | 1.31 ± 0.08[b] | 1.18 ± 0.05[b] | 1.46 ± 0.13[b] |
| Triglyceride (mmol/L) | | | | |
| CS | 0.67 ± 0.13 | 1.42 ± 0.33[a] | 1.78 ± 0.32[a] | 1.69 ± 0.34[a] |
| AS | 0.63 ± 0.17 | 0.86 ± 0.21[b] | 0.95 ± 0.20[b] | 1.36 ± 0.29[a,b] |
| KS | 0.64 ± 0.10 | 0.85 ± 0.20[b] | 1.08 ± 0.27[b] | 1.04 ± 0.31[b] |
| TS | 0.77 ± 0.20 | 0.86 ± 0.26[b] | 0.90 ± 0.15[b] | 1.12 ± 0.24[b] |
| Total cholesterol/HDL-cholesterol ratio | | | | |
| CS | 1.53 ± 0.05 | 1.52 ± 0.09[a] | 1.49 ± 0.06[a] | 1.50 ± 0.04[a] |
| AS | 1.51 ± 0.03 | 1.41 ± 0.07[b] | 1.41 ± 0.04[b] | 1.39 ± 0.05[b] |
| KS | 1.49 ± 0.05 | 1.39 ± 0.05[b] | 1.42 ± 0.04[b] | 1.39 ± 0.04[b] |
| TS | 1.50 ± 0.04 | 1.37 ± 0.08[b] | 1.44 ± 0.04[a,b] | 1.41 ± 0.04[b] |

[a]Values are expressed as means ± standard deviations for six rats. Means within the same columns bearing different superscript roman letters are significantly different ($P < 0.05$). HDL, high density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; see Table 1 for other abbreviations.

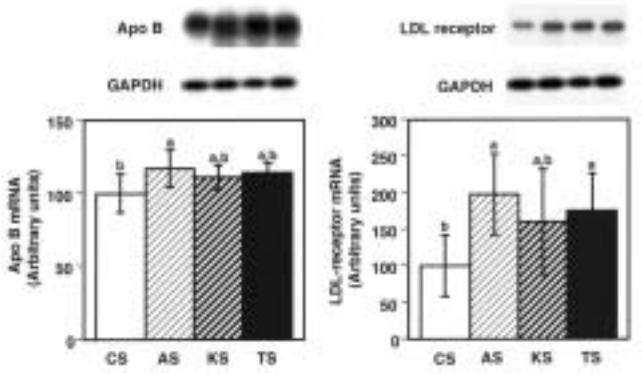


**FIG. 1.** Hepatic apolipoprotein (apo) B mRNA and low density lipoprotein (LDL) receptor mRNA concentrations in rats fed bean starches for 4 wk. Each value represents the mean ± standard deviations for data obtained from six animals. Means values were significantly different ($P < 0.05$), as determined by analysis of variance with Duncan's multiple-range test. The values of apo B mRNA and LDL receptor mRNA were normalized to the value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and values for the rats fed the adzuki starch (AS), kintoki starch (KS), and tebou starch (TS) diets are expressed relative to the average values for rats fed the corn starch (CS) diet, which was set to 100. Inset illustrates the representative Southern hybridization of polymerase chain reaction-amplified apo B cDNA and LDL receptor cDNA of hepatic RNA.

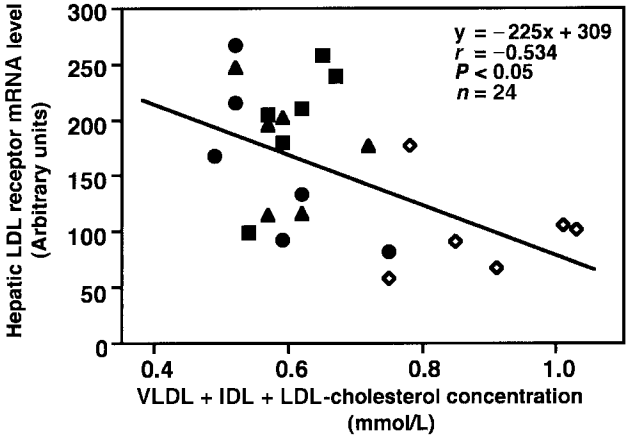


**FIG. 2.** Relationships between the hepatic LDL receptor mRNA level and serum very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) + LDL-cholesterol concentration in rats fed CS (◇), AS (■), KS (●), or TS (▲) for 4 wk. The values were significantly different ($P < 0.05$), as determined by simple correlation. For abbreviations see Figure 1.

terol and triglyceride concentrations, and total cholesterol/HDL-cholesterol ratio in rats. The serum total cholesterol, VLDL + IDL + LDL-cholesterol, and HDL-cholesterol concentrations in the bean starch groups were significantly lower than those in the CS group throughout the feeding period. The serum triglyceride concentrations in the KS and TS groups were significantly lower than those in the CS group at the end of the 4-wk feeding period. The total cholesterol/HDL-cholesterol ratio in the bean starch groups was also significantly lower than that in the CS group at the end of the 4-wk feeding period.

The effects of the bean starches on the liver cholesterol concentration in rats at the end of the experimental period were as follows (μmol/g wet liver): CS, 2.7 ± 1.1; AS, 4.0 ± 0.9; KS, 4.0 ± 1.0; and TS, 5.0 ± 2.0. (Values are expressed as means ± standard deviations for six rats.) The cholesterol concentration in the TS group was significantly higher than in the CS group.

*Hepatic apo B and LDL receptor mRNA.* The relative quantities of mRNA were determined by the Southern hybridization of PCR-amplified apo B cDNA and LDL receptor cDNA (Fig. 1) in the rat liver. The values of apo B and LDL receptor mRNA were normalized to the value of GAPDH. The values of the bean starch-fed rats were expressed relative to the average values of the CS-diet group, which were normalized to 100. The relative quantity of hepatic apo B mRNA in the AS group was significantly higher than that in the CS group ($P < 0.05$). Hepatic LDL receptor mRNA levels in the AS and TS groups were significantly higher than in the CS group ($P < 0.05$). Hepatic LDL receptor mRNA levels in the KS groups also tended to be elevated compared to the CS group. The hepatic LDL receptor mRNA level in relation to the serum VLDL + IDL + LDL-cholesterol concentration is shown in Figure 2. The hepatic LDL receptor mRNA level correlated negatively with the serum VLDL + IDL + LDL-cholesterol concentration, the correlation coefficient ($r$) being –0.534 ($P < 0.05$).

*Cecal and fecal lipid concentrations.* Table 4 shows the SCFA concentrations in the cecum in rats. The cecal propionic acid and *n*-butyric acid pool sizes were significantly higher in the resistant starch groups than those in the CS group. The cecal total SCFA pool size in the TS group was higher than in the CS, AS, and KS groups. The neutral and

**TABLE 4**
**Cecal Short-Chain Fatty Acid Pool Sizes in Rats Fed Bean Starches for 4 wk[a]**

| Diet group | Acetic acid | Propionic acid | *n*-Butyric acid | Total[b] |
|---|---|---|---|---|
| (mmol) | | | | |
| CS | 128 ± 45$^{a,b}$ | 8 ± 5$^{c}$ | 11 ± 7$^{c}$ | 146 ± 45$^{b}$ |
| AS | 105 ± 31$^{a,b}$ | 10 ± 5$^{a,b}$ | 23 ± 8$^{b}$ | 138 ± 35$^{b}$ |
| KS | 97 ± 19$^{b}$ | 10 ± 4$^{a,b}$ | 24 ± 9$^{b}$ | 131 ± 29$^{b}$ |
| TS | 151 ± 50$^{a}$ | 16 ± 8$^{a}$ | 39 ± 5$^{a}$ | 206 ± 55$^{a}$ |

[a]Values are expressed as means ± standard deviations for six rats. Means within the same columns bearing different superscript roman letters are significantly different ($P < 0.05$). See Table 1 for abbreviations.

**TABLE 5**
**Fecal Steroid Concentrations in Rats Fed Bean Starches for 4 wk[a]**

| Component | Diet group | | | |
|---|---|---|---|---|
| | CS | AS | KS | TS |
| | (μmol/100 g body wt/d) | | | |
| Cholesterol | 1.08 ± 0.16 | 0.87 ± 0.26 | 1.26 ± 0.16 | 1.21 ± 0.62 |
| Coprostanol | 0.12 ± 0.04 | 0.15 ± 0.06 | 0.15 ± 0.11 | 0.16 ± 0.11 |
| LCA | 0.12 ± 0.07[b] | 0.08 ± 0.03[b] | 0.20 ± 0.07[a] | 0.14 ± 0.05[a,b] |
| DCA | 0.25 ± 0.16[a,b] | 0.19 ± 0.13[b] | 0.46 ± 0.26[a] | 0.31 ± 0.17[a,b] |
| CDCA | 0.15 ± 0.10 | 0.08 ± 0.06 | 0.16 ± 0.06 | 0.10 ± 0.05 |
| CA | 0.11 ± 0.06 | 0.14 ± 0.10 | 0.15 ± 0.13 | 0.10 ± 0.06 |
| TBA | 0.63 ± 0.36[a,b] | 0.49 ± 0.29[b] | 0.95 ± 0.46[a] | 0.65 ± 0.25[a,b] |

[a]Values are expressed as means ± standard deviations for six rats. Means within the same rows bearing different superscript roman letters are significantly different ($P < 0.05$). LCA, lithocholic acid; DCA deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; TBA, total bile acid. See Table 1 for other abbreviations.

acidic steroid concentrations in feces at the end of the experimental period are shown in Table 5. The fecal total bile acid extraction in the KS group was higher than in the AS group, and there were no significant differences in fecal cholesterol concentrations among groups.

## DISCUSSION

In the present study we examined the effects of bean starches on serum cholesterol and hepatic LDL receptor mRNA levels in rats. The serum total cholesterol concentrations in the bean starch groups were significantly lower than in the CS group. The factor lowering the cholesterol concentrations in all resistant starch groups was the lowering of HDL- and VLDL + IDL + LDL-cholesterol. It has been suggested that sugar beet fiber reduces ileal concentrations of apo A-I and apo A-IV mRNA in rats (5). In the present experiment, the total cholesterol/HDL-cholesterol ratio in the bean starch groups was significantly lower than that in the CS group at the end of the 4-wk feeding period. It is suggested that lowering the LDL-cholesterol concentration may be also important factor in lowering serum total cholesterol concentration. In fact, the VLDL + IDL + LDL-cholesterol concentrations in the AS, KS, and TS groups were significantly lower than in the CS group. The LDL-receptor mRNA levels in the AS and TS groups were also significantly higher than in the CS group. One of the reasons for the lower serum VLDL + IDL + LDL-cholesterol concentrations in the AS and TS groups may have been elevation of the LDL receptor level, because the hepatic LDL receptor mRNA level correlated negatively with the serum VLDL + IDL + LDL-cholesterol concentration. It has been reported that dietary fish oil and dietary fiber elevate hepatic LDL receptor activity and hepatic LDL receptor mRNA level, respectively (6,14) and dietary high cholesterol and saturated fat suppress hepatic LDL receptor mRNA in African green monkeys (15). However, there are almost no reports of a relationship between dietary resistant starch and hepatic LDL receptor mRNA. In this experiment, we used the cholesterol-free diet to eliminate possible related diet effect on the cholesterol metabolism in rats. The elevation of the hepatic LDL receptor mRNA level observed in both the AS and TS fed rats appears interesting.

There was no significant difference in the liver cholesterol concentration among the groups, except for the TS group. It has been reported that dietary fiber and resistant starch decreased or had no effect on liver cholesterol concentration (3,6,16). However, the liver cholesterol concentration in the TS group was significantly higher than in the CS group in the present experiment. Although the reason for this result was unclear, it is possible that an increase of liver cholesterol concentration was due to an increase of hepatic LDL receptor mRNA level, and there were no significant differences in fecal cholesterol and total bile acid concentrations between the CS and TS groups. Hara *et al.* (17) reported that products of fermentation of sugar beet fiber by cecal bacteria lower the plasma cholesterol concentration in rats and that SCFA, as fermentation products, suppress cholesterol synthesis in the rat liver and intestine (18). The propionic acid and *n*-butyric acid pool sizes were elevated in the cecum in rats fed retrograded starch as compared with the control group in this experiment. Younes *et al.* (3) reported that amylase-resistant starch led to a marked rise in the cecal pool of SCFA and the activity of hydroxymethylglutaryl-CoA reductase was increased twofold by resistant starch compared with wheat starch. Furthermore, Evans *et al.* (19) reported that the chemical composition and structure of dietary galactomannans lowered plasma cholesterol and hepatic cholesterol synthesis. This may result from differences in the chemical composition and structure of the AS, KS, and TS, although these data were not considered here.

Trautwein *et al.* (20) reported that antihyperlipemia activities of resistant starch were due to the acceleration of bile acid excretion and that there was no effect on excretion of neutral steroids. Buhman *et al.* (21) also reported that feeding psyllium to rats enhanced fecal bile acid and total steroid excretion as well as cholesterol 7α-hydroxylase activity. However, there was no correlation between the fecal bile acid excretions and serum total cholesterol concentration, respectively ($r = -0.278$, $P > 0.05$). The bile acid may be reabsorbed abundantly in enterophepatic circulation.

In conclusion, the effects of the bean starches were most clearly seen when compared with rats fed CS. The resistant starch elevated hepatic LDL receptor mRNA level in the AS and TS groups, reduced the HDL-cholesterol concentration in the AS, TS, and KS groups, and lowered serum total and IDL + LDL-cholesterol concentrations in the resistant starch groups.

## ACKNOWLEDGMENT

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# Dietary Supplementation with Conjugated Linoleic Acid Does Not Alter the Resistance of Mice to *Listeria monocytogenes* Infection

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**ABSTRACT:** Conjugated linoleic acid (CLA) has been used experimentally as a dietary supplement to increase lean body weight and to modulate inflammation in a variety of animal species. In addition, human use of dietary CLA as a supplement to regulate body fat has received both scientific and public attention. No reports have been published regarding the effects of dietary CLA on antimicrobial resistance. In this study, we provide evidence that feeding CLA for up to 4 wk does not alter host defense against *Listeria monocytogenes* in mice. These findings suggest that the anti-inflammatory effects of CLA do not impair cellular immunity to this intracellular pathogen.

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Conjugated linoleic acid (CLA) has been studied as a dietary supplement. There is evidence that it increases resistance to tumor initiation and growth, increases lean body mass, and has other beneficial effects (1–5). At least some of these biological activities are thought to be mediated in part through the capacity of CLA to modulate immune and inflammatory responses. For example, there is evidence that CLA can decrease the biological activity of tumor necrosis factor (TNF) *in vivo* (5–7) and the release of IL-2 by lymphocytes (8) and IL-6 and TNF by macrophages *ex vivo* (9). Although these studies suggest that dietary CLA is a potent immunomodulator, there has been little or no investigation of the direct effects of dietary supplementation with CLA on resistance to bacterial infection.

Murine listeriosis is a valuable model for studying cellular immunity to an intracellular facultative bacterial pathogen. *Listeria monocytogenes* can invade and multiply in macrophages, hepatocytes, and various other cell types (10). Anti-*Listeria* resistance requires coordination of an effective cellular immune response. This involves both innate immune mechanisms (i.e., granulocytes) and an antigen-specific adaptive immune response (i.e., $CD4^+$ and $CD8^+$ T-cells) (10–12). In particular, rapid mobilization of an innate immune response, consisting of neutrophils and inflammatory cytokines, is essential for restricting the early growth of *L. monocytogenes* in the spleens and liver of experimentally infected mice (10–15). Because CLA is reported to dampen the inflammatory response to bacterial products (5,6), we hypothesized that it might impair anti-*Listeria* resistance. Therefore, in this study we used murine listeriosis as a model system to assess the possible effects of dietary supplementation with CLA on antibacterial resistance.

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Abbreviations: CLA, conjugated liinoleic acid; IL, interleukin; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor.

## MATERIALS AND METHODS

*Mice.* Female CD1 mice were obtained at six wk of age from Charles Rivers Laboratories (Indianapolis, IN) and housed in the AAALAC certified animal care facility of the UW-Madison School of Veterinary Medicine. Mice were used at either 2 or 6 mon of age. Individual mice were identified by ear punches. We received approval for these experiments from the University of Wisconsin School of Veterinary Medicine Animal Use and Care Committee.

*Diet.* Mice were fed *ad libitum* a semipurified diet (TD94060, 99% basal mix; Harlan-Teklad, Madison, WI) that contained 5% corn oil. To the diet was added 0.5% sucrose (w/w) and either 0.5% CLA or 0.5% corn oil. The CLA contained 95% conjugated dienes, primarily consisting of *cis*9,*trans*11- (43%), and *trans*10,*cis*12-isomers (44%) as described previously (1). Mice were individually weighed before they were put on their respective diets, immediately before inoculation with *L. monocytogenes*, and at the time they were euthanized.

*Listeria monocytogenes. Listeria monocytogenes* strain EGD was grown and prepared for inoculation in our laboratory as described previously (12). Briefly, the organism had been passaged through a mouse by intravenous infection. The mouse was euthanized and its spleen removed and homogenized in sterile saline. The spleen homogenate was diluted in saline and plated on blood agar. A single colony was picked from those that grew on blood agar, inoculated into brain heart infusion broth, and grown to log-phase at 37°C. Glycerol was then added (20% by volume), and the bacteria were stored as aliquots at −70°C. Immediately before an experiment, an aliquot was thawed and diluted to the indicated concentrations in sterile saline.

*Experimental infection.* In two separate experiments, we examined the effects of dietary supplementation with CLA on the subsequent resistance of mice to intraperitoneal (i.p.) infection with *L. monocytogenes*. Two- or six-month-old female CD1 mice were fed the diet mentioned above that contained

either 0.5% CLA, or corn oil as a control, *ad libitum*. Mice were given the diet for 14 d (Expt. 1) or 32 d (Expt. 2) before they were challenged i.p. with *L. monocytogenes* strain EGD ($2.5 \times 10^5$ and $1.5 \times 10^5$ in 0.2 mL sterile saline, respectively, in the two experiments). These challenge doses were chosen because they would result in a sublethal infection. At 4 and 8 d after bacterial inoculation, groups of 4 or 5 mice were euthanized by asphyxiation with $CO_2$, followed by cervical dislocation. These time points were chosen because they represent the peak of the bacterial burden during experimental infection (day 4), and a time point when the listeriae should be in the process of being cleared from these tissues (day 8). After weighing the mice, their abdomens were aseptically opened and one-half of the spleen and the front lobe of the liver were removed. These tissues were weighed and then placed in sterile tissue grinders that contained cold sterile saline. The tissues were homogenized with a pestle, and the homogenates serially diluted in sterile saline. Samples (0.1 mL) of appropriate dilutions were plated in duplicate on blood agar. The plates were incubated for 48 h at 37°C and the colonies enumerated. The results are expressed as the $\log_{10}$ CFU *L. monocytogenes* per g tissue (wet wt).

*Histopathology.* Portions of the spleen and liver were removed to buffered formalin at necropsy. Following fixation, these sections were cut, mounted on glass slides, and stained with hematoxylin and eosin, or with periodic acid Schiff stain to detect lipid deposits. These slides were then examined microscopically by an American College of Veterinary Pathologists board certified pathologist (H.S.), and representative slides photographed.

*Data analysis.* Data were analyzed by a two-way analysis of variance. If a significant $F$ value ($P < .05$) was obtained, then relevant comparisons were made using paired $t$ tests as performed by the Prism software package (GraphPad Software, San Diego, CA).

## RESULTS

Our results indicated that dietary supplementation with CLA had little ($P > .05$) effect on the body weight of mice in the experiment. The CLA-treated mice exhibited slightly decreased body weight before challenge, whereas the control mice exhibited a slight weight gain (Table 1). However, both CLA-treated and control mice lost weight during *L. monocytogenes* infection. Thus, CLA supplementation was unable to spare mice from the cachexia that occurs during experimental listeriosis.

In two separate experiments, CLA feeding had no significant effect ($P > .05$) on the numbers of *L. monocytogenes* recovered from the spleens and livers of experimentally infected mice (Table 2). Although we observed some variability between the two experiments, this was likely due to the differences in age and weight of the mice used in the two experiments (Table 2).

Although we did not perform a quantitative histopathologic analysis, the severity of damage to the spleen and liver did not differ substantially between the two groups of mice. In the control animals, scattered necrotic foci were observed in the liver. Most of these were small to moderate in size and contained inflammatory neutrophils and mononuclear cells. Scattered aggregates of inflammatory neutrophils and mononuclear cells were observed in CLA-treated mice, some of which were associated with necrotic hepatocytes. In Experiment 2, we observed a tendency for hepatic lipidosis in the livers of CLA-treated animals. As illustrated in Figure 1, histopathological examination revealed random hepatocytes with macrovesicular lipid vacuoles in the CLA-treated mice. Periodic acid Schiff staining confirmed that the vacuoles contained lipid (not shown). In contrast, control mice exhibited slightly swollen hepatocytes with lacy cytoplasm (Fig. 1). However, the presence or absence of lipidosis had no apparent relationship with the severity of histopathologic lesions caused by *L. monocytogenes* infection, or the numbers of viable *L. monocytogenes* recovered from the spleen and liver.

## DISCUSSION

The results of this study were somewhat surprising. Our prediction had been that treatment with CLA, which is known to diminish the catabolic response to endotoxin and the inflammatory cytokine TNFα (5,6,9), would dampen host defense against *L. monocytogenes*. This supposition was based on pre-

**TABLE 1**
**Effects of Conjugated Linoleic Acid (CLA) Supplementation on Body Weight Before and After *Listeria monocytogenes* Infection**

| | | Body weight (g), mean ± SEM | | | |
|---|---|---|---|---|---|
| Expt. # | Treatment group | Before diet | After diet | Day 4 of infection | Day 8 of infection |
| 1[a] | CLA | 29.1 ± 0.5 | 29.1 ± 0.7 | 25.8 ± 1.9 | 26.3 ± 2.2 |
| | Control | 28.6 ± 0.4 | 29.3 ± 0.7 | 25.7 ± 0.7 | 26.7 ± 1.8 |
| 2[b] | CLA | 39.0 ± 1.7 | 37.9 ± 1.2 | 36.0 ± 1.9 | ND[c] |
| | Control | 38.1 ± 2.4 | 42.9 ± 1.8 | 39.8 ± 1.6 | ND |

[a]In Experiment 1, two groups of 2-mon-old female CD1 mice (eight mice per group) were fed CLA-supplemented or control diet for 14 d before intraperitoneal (i.p.) inoculation of $2.5 \times 10^5$ *L. monocytogenes*. The mice were continued on the diet after infection, until groups of four mice were euthanized on days 4 and 8.
[b]In Experiment 2, 6-mon-old female CD1 mice were fed CLA-supplemented diet (five mice) or control diet (four mice) for 32 d before i.p inoculation with $1.5 \times 10^5$ *L. monocytogenes*. The mice were continued on the diet after infection until they were euthanized four days after bacterial inoculation.
[c]Not done.

**TABLE 2**
**Prior Feeding of a CLA-Supplemented Diet Does Not Alter Resistance to *L. monocytogenes* Infection**

| | | Mean ± SEM $Log_{10}$ *L. monocytogenes* | | | |
|---|---|---|---|---|---|
| | | Day 4 of infection | | Day 8 of infection | |
| Expt. # | Treatment group | Spleen | Liver | Spleen | Liver |
| 1[a] | CLA | 6.3 ± 0.9 | 5.6 ± 1.4 | 3.8 ± 1.5 | 3.1 ± 1.7 |
| | Control | 7.0 ± 0.3 | 5.6 ± 0.7 | 4.4 ± 1.5 | 3.6 ± 1.7 |
| 2[b] | CLA | 4.4 ± 0.3 | 2.5 ± 0.3 | ND[c] | ND |
| | Control | 4.4 ± 0.2 | 2.7 ± 0.5 | ND | ND |

[a]In Experiment 1, 2-mon-old female CD1 mice were fed CLA-supplemented or control diet for 14 d before i.p. inoculation with $2.5 \times 10^5$ *L. monocytogenes*. Mice were continued on their respective diets after infection. Groups of four mice per treatment group were euthanized 4 and 8 d later, and the number of viable *L. monocytogenes* per g wet weight spleen and liver, was determined.
[b]In Experiment 2, 6-mon-old female CD1 mice were fed CLA-supplemented or control diet for 32 d before i.p inoculation with $1.5 \times 10^5$ *L. monocytogenes*. Mice were continued on their respective diets until they were euthanized 4 d after bacterial inoculation. The numbers of viable *L. monocytogenes* per g (wet weight) in the spleen and liver were then determined as discussed earlier. For abbreviations see Table 1.
[c]Not done.

vious studies which demonstrated that early release of TNFα and other inflammatory cytokines (ie., IL-12 and interferon-γ), was essential for restricting the multiplication of *L. monocytogenes* and reducing the severity of listeriosis in mice (10,13,15). Unfortunately, we did not verify the effects of CLA treatment on TNFα expression in our experiments. However, previous studies conducted using diet-fed mice under similar circumstances to those reported here indicated a decreased response to TNFα (7). Likewise, work performed in one of our laboratories (M.E.C.) demonstrated that autoimmune disease-prone NZB/W mice fed the same diet, with a similar concentration of CLA, exhibited less severe endstage lupus-like disease (16).

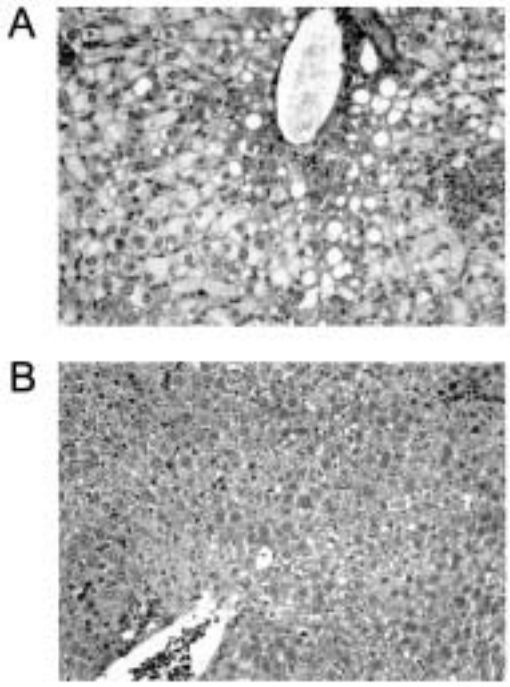


**FIG. 1.** Mice that received the conjugated linoleic acid (CLA)-supplemented diet for 32 d exhibited hepatic lipidosis during *Listeria monocytogenes* infection. (A), Mice on the CLA-supplemented diet exhibited midzonal swollen hepatocytes with lacy cytoplasm, and the presence of macrovesicular lipid vacuoles. (B), In contrast, mice on the control diet (0.5% corn oil) exhibited scattered random clusters of slightly swollen hepatocytes with lacy cytoplasm. Liver sections were stained with hematoxylin and eosin and photographed at 200× magnification.

One possible explanation for the absence of an adverse effect of CLA is that CLA treatment dampens the release of both protective inflammatory cytokines like TNFα and anti-inflammatory mediators (i.e., prostaglandin $E_2$, IL-4, and IL-10) that have been demonstrated to reduce resistance to listeriosis in mice (10,15,17,18). A different biological role of CLA also deserves consideration. It has been reported that CLA is potent high-affinity ligand and activator of peroxisome proliferator-activated receptor alpha (PPARα) in hepatocytes (4,19). Since *L. monocytogenes* multiplies to a large extent in hepatocytes (11–13), one can envision that an alteration in hepatocyte physiology might alter its ability to multiply. Although modulation of immune reactivity by PPAR receptors has been reported (20,21), their ability to alter antibacterial resistance *in vivo* has not been established. The absence of an effect of CLA on anti-*Listeria* resistance in the present study suggests that neither cytokines nor PPAR, were modulated in a manner that was adverse to host defense.

There have been other investigations of the effects of dietary lipid on anti-*Listeria* resistance. Mice fed a high-fat diet (20% corn oil) at 3 wk of age exhibited enhanced resistance to listeriosis (22). However, mice fed the same diet at 6 or 12 wk of age had impaired resistance (22). Mice fed a diet high in cholesterol, lard, and sucrose also exhibited improved anti-*Listeria* resistance (23). Mice fed a diet containing 17% fish oil and 3% corn oil exhibited less resistance to *L. monocytogenes* than mice fed lard or soybean oil (24). These same investigators later showed that mice fed n-3 polyunsaturated fatty acids from fish oil produced less IL 12, and more interferon gamma during *L. monocytogenes* infection than control mice (25). Recently, it was reported that mice fed a hydrogenated coconut oil diet exhibited increased resistance to *L. monocytogenes* infection (26).

In summary, these results suggest that dietary supplemen-

tation with CLA does not alter antibacterial resistance, as evaluated using murine listeriosis as a model. Although our results are limited to this one animal model, murine listeriosis is widely used to assess antibacterial resistance following modulation of the immune system, or exposure to toxicologic insults. Our findings are also consistent with the absence of any reported relationship between dietary CLA supplementation in humans and altered resistance to bacterial infection.

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# Effect of Conjugated Linoleic Acid on Fungal Δ6-Desaturase Activity in a Transformed Yeast System

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**ABSTRACT:** Conjugated linoleic acid (CLA; 18:2), a group of positional and geometric isomers of linoleic acid (LA; 18:2n-6), has been shown to modulate immune function through its effect on eicosanoid synthesis. This effect has been attributed to a reduced production of n-6 polyunsaturated fatty acid (PUFA), the precursor of eicosanoids. Since Δ6-desaturase is the rate-limiting enzyme of the n-6 PUFA production, it is our hypothesis that CLA, which has similar chemical structure to LA, interacts directly with Δ6-desaturase. A unique and simple model, i.e., baker's yeast *(Saccharomyces cerevisiae)* transformed with fungal Δ6-desaturase gene, previously established, was used to investigate the direct effect of CLA on Δ6-desaturase. This model allows LA to be converted to γ-linolenic acid (GLA; 18:3n-6) but not GLA to its metabolite(s). No metabolites of CLA were found in the lipids of the yeast transformed with Δ6-desaturase. The inability to convert CLA to conjugated GLA was not due to the failure of yeast cells to take up the CLA isomers. CLA mixture and individual isomers significantly inhibited the activity of Δ6-desaturase of the transformed yeast *in vivo*. Even though its uptake by the yeast was low, CLA *c*9,*t*11 isomer was found to be the most potent inhibitor of the four isomers tested, owing to its high inhibitory effect on Δ6-desaturase. Since CLA did not cause significant changes in the level of Δ6-desaturase mRNA, the inhibition of GLA production could not be attributed to suppression of Δ6-desaturase gene expression at the transcriptional level.

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Conjugated linoleic acid (CLA) refers to a group of geometric and positional dienoic isomers derived from linoleic acid (LA). CLA exhibits many beneficial biological effects in cell cultures and animal models. For example, CLA decreases breast cancer cell proliferation and inhibits tumorigenesis in mammary gland, skin, and stomach in experimental animals (1–5). CLA can also reduce the development of atherosclerosis in hamsters and rabbits, and it modulates immune function in rats (6–8).

The mechanism by which CLA exerts its biological function is still not clear. It has been shown that CLA modulates immune function through its effect on eicosanoid synthesis (9–11). This effect has been attributed to a reduced production of n-6 polyunsaturated fatty acid (PUFA), the precursor of eicosanoids. Since Δ6-desaturase is the rate-limiting enzyme of n-6 PUFA production, it is our hypothesis that CLA, which has similar chemical structure to LA, interacts directly with Δ6-desaturase. However, owing to the complexity of mammalian cell systems and to the unavailability of purified Δ6-desaturase, there is no evidence which demonstrates that CLA interacts directly with Δ6-desaturase.

We have previously established a transformed yeast (*Saccharomyces cerevisiae*) culture in which a fungal (*Mortierella alpina*) Δ6-desaturase gene has been introduced, and is expressed (12). This model provides a unique and simple system to investigate whether CLA interacts directly with Δ6-desaturase. The objectives of this study were (i) to determine if CLA can be metabolized by Δ6-desaturase and (ii) to examine whether CLA affects the activity of Δ6-desaturase.

## MATERIALS AND METHODS

*Chemicals.* LA and a mixture of CLA isomers (free fatty acid form, containing 41% *c*9,*t*11/*t*9,*c*11 isomers, 44% *t*10,*c*12 isomer, and others) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Four major isomers of CLA (*c*9,*t*11; *t*9,*t*11; *c*9,*c*11, and *t*10,*c*12) were obtained as individual free fatty acids from Matreya Inc. (Pleasant Gap, PA). Yeast minimal medium (YMM) composed of dropout base medium with 2% galactose plus a complete supplement mixture lacking uracil were from Bio 101, Inc. (Vista, CA). YPD medium containing yeast extract, peptone, and dextrose was from Difco Laboratories (Detroit, MI). Hexane was ultraviolet (UV) grade and other solvents were distilled-in-glass quality.

*Plasmids and yeast strain.* Two plasmids, pYES2 (vector only) and pCGR5 (with fungal Δ6-desaturase gene), were constructed and transformed into a host strain of baker's yeast (*S. cerevisiae*), SC334 (12). The transformation protocol and growth conditions followed the procedure described previously (13).

*Incubation conditions and experimental design.* Colonies of transformants were grown overnight in YPD media at 30°C. Cultures ($1 \times 10^8$ cells) were then used to inoculate 100 mL of YMM. Cell numbers were maintained at the same level in all studies. The culture was grown for 48 h at 15°C. This

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Abbreviations: CLA, conjugated linoleic acid; GC, gas chromatography; GLA, γ-linolenic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid; YMM, yeast minimal medium; YPD, yeast extract, peptone, and dextrose medium.

temperature (15°C) has previously been shown to be optimal for expression of the Δ6-desaturase activity (12). After harvest by centrifugation, cell pellets were washed once with sterile distilled/deionized $H_2O$ to remove the media. The host strain transformed with vector (pYES2) alone was used as a negative control as indicated.

To confirm the activity of Δ6-desaturase (conversion of LA to γ-linolenic acid, GLA) in the transformed yeast used in this study, LA (25 μM) was provided as the exogenous substrate in the minimal media. To examine whether CLA could be converted to conjugated GLA by Δ6-desaturase, 25 μM CLA was added to the medium. To study if addition of CLA could affect the conversion of LA to GLA by Δ6-desaturase, both LA and CLA were supplemented at a fixed concentration of 25 μM. To determine if CLA isomers could be taken up by transformed yeast, four CLA isomers were supplemented in media at different levels (10, 25, and 50 μM). To determine if CLA affects the expression of Δ6-desaturase, the levels of Δ6-desaturase mRNA in cultures incubated with 50 μM LA (control) and a mixture containing 50 μM CLA and 50 μM LA were compared.

*Lipid extraction and fatty acid analysis.* The extraction of yeast lipids followed the procedures described previously (13). Briefly, rinsed cell pellets were extracted with 6.5 mL of methanol and 13 mL of chloroform containing 16 μg of tritridecanoin (a synthetic triacylglycerol containing three molecules of tridecanoic acid, 13:0, used as the internal standard). After extraction, the yeast lipids were saponified and methylated. Fatty acid methyl esters were then analyzed by gas chromatography (GC) by using a Hewlett-Packard 5890 II Plus gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector and a fused-silica capillary column (Omegawax; 30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA). In the present study, the Δ6-desaturase activity was determined from the ratio of the product and substrate concentrations. The amount of CLA isomers taken up by the yeast was calculated from the percentage of CLA isomers in total yeast lipids.

*Isolation and analysis of RNA.* Total RNA amounts were isolated by hot phenol/chloroform extraction as described previously (14). Equal amounts of total RNA from various experimental cultures were separated by 1% formaldehyde/agarose gel electrophoresis, transferred to a nylon membrane (Roche, Indianapolis, IN), and auto cross-linked to the membrane with an UV Stratalinker 1800 (Stratagene, La Jolla, CA). The DNA probes used for hybridization overnight at 50°C were synthesized using the DIG High Prime Random Labeling kit (Roche, Indianapolis, IN). The *M. alpina* Δ6-desaturase gene from pCGR5 (12) was used for desaturase mRNA detection, and the 2.2 kb *Hind*III fragment of *CryI* (15) was used to show that equal amounts of RNA had been loaded. Following hybridization, detection of the signal was performed with reagents from Roche following the manufacturer's instructions. Finally, the hybridization signals were quantitated by densitometry (IS-1000 system; Alpha Innoteck, San Leandro, CA).

*Statistical analyses*. Data were analyzed by analysis of variance and Fisher's protected least significant difference to determine differences between means of the uptake rate of CLA isomers and between means of the decrease in Δ6-desaturase activity. Means differences were considered significant at the $P \leq 0.05$ level.

## RESULTS

Significant levels of GLA (18:3 $\Delta^{6,9,12}$ converted from 18:2 $\Delta^{9,12}$) were formed in yeast strain 334(pCGR5) when incubated for 48 h with 25 μmM LA (Fig. 1B). A small level of 16:2 $\Delta^{6,9}$ (converted from 16:1 $\Delta^{9}$) was also observed. Neither 16:2 nor GLA was formed in the 334(pYES2) yeast, which lacks the Δ6-desaturase gene (Fig. 1A). This confirms that Δ6-desaturase was synthesized and active in the transformed 334(pCGR5) yeast used in this study.

When the transformed yeast cells, with or without Δ6-desaturase, were incubated with 25 μM CLA for 48 h, CLA was incorporated mostly into the phospholipid fraction (53.4% of total CLA in yeast lipid extract), followed by the free fatty acid fraction (26.6%). However, there were no metabolites of CLA in total lipid extract (Fig. 2) (data not shown). Thus, CLA under the current condition described in this study could not be metabolized by the Δ6-desaturase.

Figure 3 shows that conversion of LA to GLA was significantly inhibited (33%) by the addition of mixed CLA into the growth medium. When four CLA isomers were examined individually, the extent of inhibition of Δ6-desaturase activity was greatest (40%) when *c*9,*t*11 was added to the medium. Although *t*10,*c*12, *c*9,*c*11, and *t*9,*t*11 isomers significantly inhibited the conversion of LA to GLA, there was no signifi-

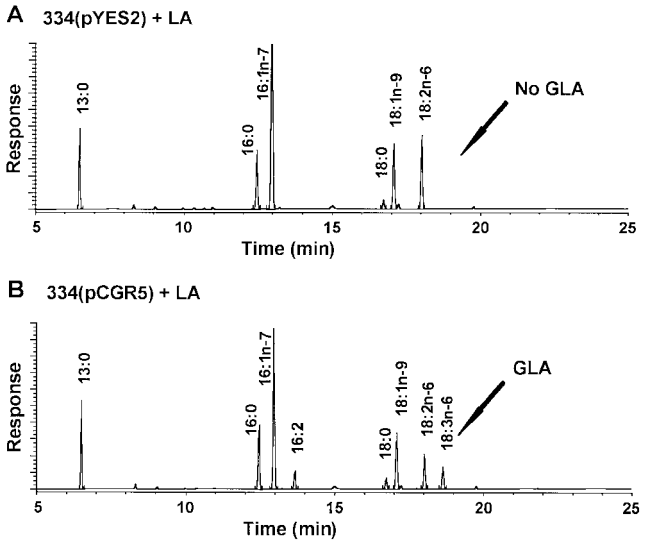


**FIG. 1.** Gas chromatographic analysis of fatty acid methyl esters of total lipids in *Saccharomyces cerevisiae* 334 harboring (A) the vector pYES2, and (B) the Δ6-desaturase gene-bearing plasmid pCGR5. All yeast cells were incubated in the medium containing linoleic acid (LA). Solid arrows indicate the appearance of γ-linolenic acid (GLA).

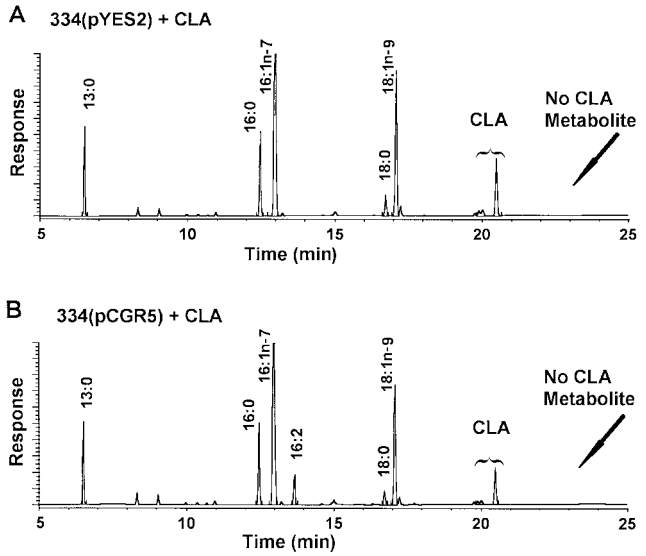


**FIG. 2.** Gas chromatographic analysis of fatty acid methyl esters of total lipids in the transformed yeast with vector only [334(pYES2)] (A) and those with the Δ6-desaturase gene [334(pCGR5)] (B). Cells had been incubated in medium containing conjugated linoleic acid (CLA). Solid arrows indicate the appearance of CLA metabolites.

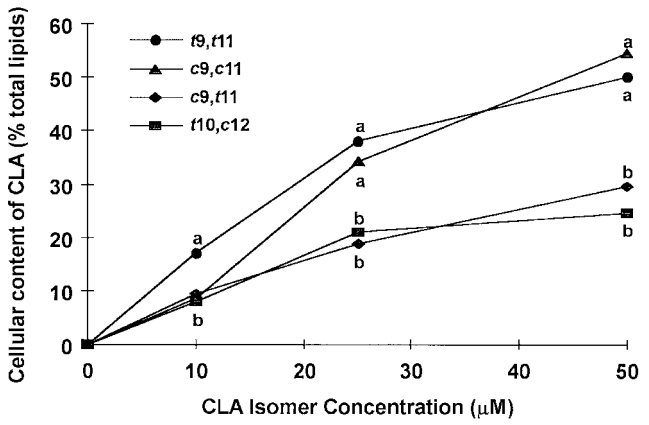


**FIG. 4.** Uptake of different CLA isomers by transformed yeast with Δ6-desaturase gene. The uptake of CLA isomers is indicated by the presence of CLA isomers (% total lipids) in yeast lipids. Each value point represents the mean of four incubations. Different letters indicate a significant difference ($P < 0.05$) within the same concentration point. CLA *c*9,*t*11 isomer (◆), CLA *t*10,*c*12 isomer (■), CLA *c*9,*c*11 isomer (▲), and CLA *t*9,*t*11 isomer (●).

cant difference among them. The inhibitory effects of individual isomers were dose-dependent (data not shown).

The uptake rate of CLA isomers by the transformed yeasts is shown in Figure 4. The level of uptake was directly proportional to the concentration of CLA isomers in the medium. However, the rate of uptake varied significantly among the four CLA isomers. The uptakes of *t*9,*t*11 and *c*9,*c*11 isomers were greater than those of *c*9,*t*11 and *t*10,*c*12.

To determine whether the inhibitory effect of CLA on Δ6-desaturase activity was regulated through the Δ6-desaturase gene expression, the transformed yeast were cultured in YMM containing 50 μM of LA with or without the addition of 50 μM of CLA. Figure 5 shows that there were no significant differences in the relative abundance of Δ6-desaturase mRNA between the transformed yeasts treated or not treated with CLA.

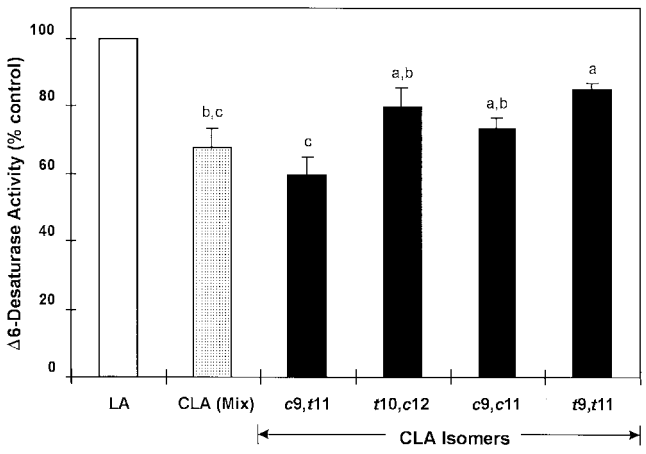


**FIG. 3.** Effect of CLA isomers on the Δ6-desaturase activity in transformed yeast cultures. All results are mean ± SE of four incubations. Values with different letters are significantly different from each other at $P < 0.05$. For abbreviations see Figures 1 and 2.

## DISCUSSION

In the present study, a unique model, in which baker's yeast was transformed with fungal Δ6-desaturase gene, was used to provide a direct assay for studying the first regulating step of n-6 PUFA production. Since no PUFA is found in baker's yeast cells owing to the lack of enzymes for PUFA synthesis, the insertion of Δ6-desaturase gene enables these yeast cells to convert exogenous LA to GLA. Based on our unpublished observation (Chuang, L.-T., J.M. Thurmond, J.-W. Liu, and Y.-S. Huang), the newly formed GLA was not further metabolized. Therefore, the ratio of GLA to LA could serve an index of Δ6-desaturase activity.

In using this model, we clearly demonstrated that none of the CLA isomers could be metabolized by the recombinant Δ6-desaturase to conjugated GLA, despite the similarity in chemical structure between LA and CLA. In addition, the inability to convert CLA to conjugated GLA was not due to the failure of yeast cell to take up the CLA isomers. The competitive inhibition of Δ6-desaturase between CLA and LA was also demonstrated. The simplicity of our model allows us to define the interaction between CLA and Δ6-desaturase. Our finding differs from previous studies which found CLA metabolites in animals fed CLA (10,16). Multiple steps or alternative metabolic pathways could have been involved in these studies.

Our results showed that all CLA isomers inhibited significantly the activity of Δ6-desaturase. The *c*9,*t*11 isomer was found to be the most potent inhibitor (40% inhibition) (Fig. 3), despite its lower uptake by the yeast cells when compared with other isomers (Fig. 4). The CLA mixture also exhibited a significant inhibition (33%) on the activity of Δ6-

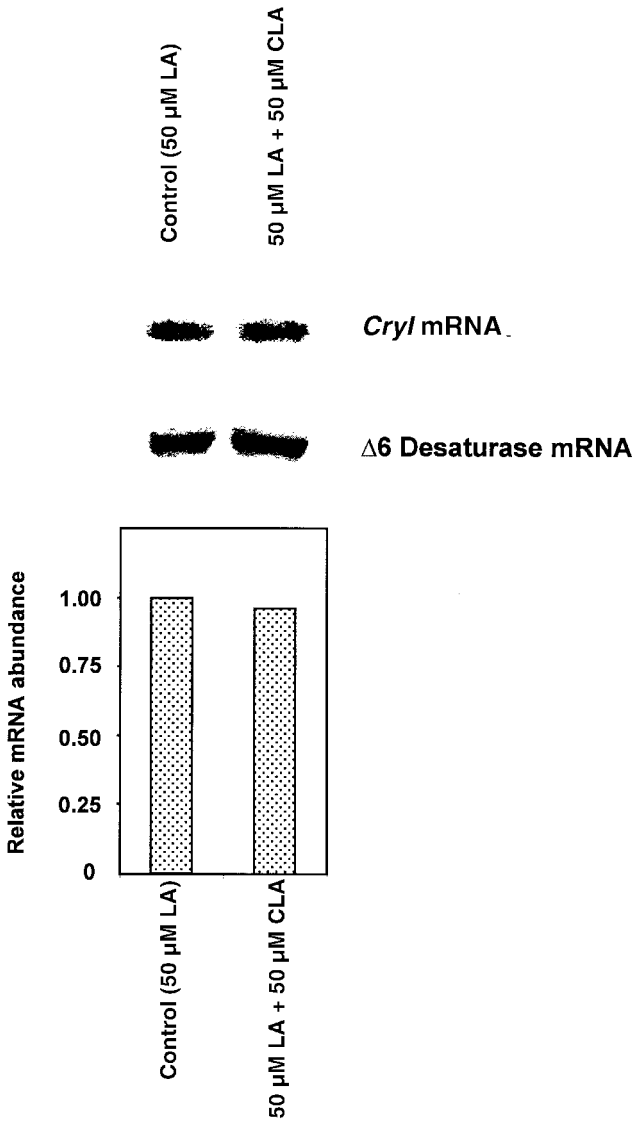


**FIG. 5.** Effect of CLA on Δ6-desaturase mRNA expression in transformed yeast 334(PCGR5). Data are expressed as the ratio of Δ6-desaturase/*Cryl* mRNA. Similar results were observed in three separate experiments. For abbreviations see Figure 1.

desaturase. This could be due to the presence of a high percentage (41%) of *c*9,*t*11/*t*9,*c*11 isomers in the mixture. This finding indicates that *c*9,*t*11 may be the most active CLA isomer. Based on their studies in mice supplemented with CLA, Ha *et al*. (5) and Ip *et al*. (2) have also suggested that *c*9,*t*11 is the active form of CLA. This conclusion was supported by an early observation reported by Bretillon *et al*. (17) in which hepatic microsomes were used as the source of Δ6-desaturase.

Inhibition of CLA on Δ6-desaturase activity could be due to its ability to modulate cellular signal transduction by upregulating gene expression and protein production as suggested by Lee *et al*. (18). The suggestion was based on the finding that CLA reduced stearoyl-CoA desaturase activity by decreasing the stearoyl-CoA desaturase gene mRNA in mouse liver and H2.35 cells. Results in the present study showed that there were no changes in the level of Δ6-desaturase mRNA in the transformed yeast cells treated with CLA. This suggests that the inhibitory effect of CLA on Δ6-desaturase activity was not exerted through the suppression of Δ6-desaturase gene transcription. Alternatively, CLA may inhibit the binding of LA to the catalytic sites of Δ6-desaturase by simply occupying the same sites due to the similarity in their chemical structures. Further study is needed to verify this hypothesis.

In summary, results in the present study clearly demonstrated the interaction between CLA and Δ6-desaturase using a simple but unique transformed yeast model. We have shown that CLA could not be metabolized by Δ6-desaturase directly. Nevertheless, CLA, particularly the *c*9,*t*11 isomer, could inhibit Δ6-desaturase activity. The *c*9,*t*11 isomer may be the most potent isomer of CLA owing to its high inhibitory effect on Δ6-desaturase activity even though its uptake by yeast was low. Since CLA did not result in significant changes in the level of Δ6-desaturase mRNA, their inhibition on GLA production could not be attributed to the suppression of CLA on gene expression at the transcription level.

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# Cultured Fish Cells Metabolize Octadecapentaenoic Acid (all-*cis* Δ3,6,9,12,15-18:5) to Octadecatetraenoic Acid (all-*cis* Δ6,9,12,15-18:4) *via* Its 2-*trans* Intermediate (*trans* Δ2, all-*cis* Δ6,9,12,15-18:5)

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**ABSTRACT:** Octadecapentaenoic acid (all-*cis* Δ3,6,9,12,15-18:5; 18:5n-3) is an unusual fatty acid found in marine dinophytes, haptophytes, and prasinophytes. It is not present at higher trophic levels in the marine food web, but its metabolism by animals ingesting algae is unknown. Here we studied the metabolism of 18:5n-3 in cell lines derived from turbot *(Scophthalmus maximus),* gilthead sea bream *(Sparus aurata),* and Atlantic salmon *(Salmo salar)*. Cells were incubated in the presence of approximately 1 μM [U-$^{14}$C]18:5n-3 methyl ester or [U-$^{14}$C]18:4n-3 (octadecatetraenoic acid; all-*cis* Δ6,9,12,15-18:4) methyl ester, both derived from the alga *Isochrysis galbana* grown in $H^{14}CO_3^-$, and also with 25 μM unlabeled 18:5n-3 or 18:4n-3. Cells were also incubated with 25 μM *trans* Δ2, all-*cis* Δ6,9,12,15-18:5 (2-*trans* 18:5n-3) produced by alkaline isomerization of 18:5n-3 chemically synthesized from docosahexaenoic acid (all-*cis* Δ4,7,10,13,16,19-22:6). Radioisotope and mass analyses of total fatty acids extracted from cells incubated with 18:5n-3 were consistent with this fatty acid being rapidly metabolized to 18:4n-3 which was then elongated and further desaturated to eicosatetraenoic acid (all-*cis* Δ8,11,14,17,19-20:4) and eicosapentaenoic acid (all-*cis* Δ5,8,11,14,17-20:5). Similar mass increases of 18:4n-3 and its elongation and further desaturation products occurred in cells incubated with 18:5n-3 or 2-*trans* 18:5n-3. We conclude that 18:5n-3 is readily converted biochemically to 18:4n-3 *via* a 2-*trans* 18:5n-3 intermediate generated by a $\Delta^3$,$\Delta^2$-enoyl-CoA-isomerase acting on 18:5n-3. Thus, 2-*trans* 18:5n-3 is implicated as a common intermediate in the β-oxidation of both 18:5n-3 and 18:4n-3.

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Abbreviations: 18:4n-3, octadecatetraenoic acid (all-*cis* Δ6,9,12,15-18:4); 18:5n-3, octadecapentaenoic acid (all-*cis* Δ3,6,9,12,15-18:5); 2-*trans* 18:5n-3, 2-*trans* octadecapentaenoic acid (*trans* Δ2, *cis* Δ6,9,12,15-18:5); 20:4n-3, eicosatetraenoic acid; 20:5n-3, eicosapentaenoic acid; 22:6n-3, docosahexaenoic acid; AS, Atlantic salmon cell line; FBS, fetal bovine serum; GC, gas chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acid; SAF-1, gilthead sea bream cell line; TF, turbot fin cell line; TLC, thin-layer chromatography.

Octadecapentaenoic acid (all-*cis* 18:5n-3) is a fatty acid characteristically present in certain algal groups in marine phytoplankton (1), including dinoflagellates (2), haptophytes (3,4), and prasinophytes (5), all of which have important roles in the marine ecosystem. The acid 18:5n-3 is usually co-associated in these organisms with docosahexaenoic acid (22:6n-3). Given that biosynthesis of 22:6n-3 involves peroxisomal chain shortening of its precursor 24:6n-3, it is possible that 18:5n-3 is biosynthesized by chain shortening of eicosapentaenoic acid (20:5n-3) (see Ref. 6). Marine zooplankton and fish ingesting phytoplankton contain little or no 18:5n-3, demonstrating that this fatty acid is readily metabolized by marine animals. Clearly it could be completely catabolized by marine animals by β-oxidation, but it may also be directly chain-elongated to 20:5n-3. We decided to test the latter possibility in fish cell cultures because marine fish in general have a very limited ability to convert 18:3n-3 to 20:5n-3 and thence to 22:6n-3 (7,8). In some species of marine fish, e.g., turbot, this appears to be due to a deficiency of $C_{18}$ to $C_{20}$ fatty acid elongase (9), whereas in others, e.g., sea bream, it appears to be due to a deficiency of Δ5 fatty acid desaturase (10). The availability of 18:5n-3 can help distinguish between these two possibilities and, in the event of it being a substrate for $C_{18}$ to $C_{20}$ fatty acid elongase, algae containing this fatty acid could be useful dietary supplements in marine fish larval culture.

In this study we prepared 18:5n-3 from the haptophycean alga *Isochrysis galbana* and also by chemical synthesis from 22:6n-3, and studied its metabolism in cultured cells from turbot, seabream, and Atlantic salmon that differ in their abilities to perform $C_{18}$ to $C_{20}$ elongation and Δ5 fatty acid desaturation reactions. The results show that 18:5n-3 is very readily converted by cells from all three species to octadecatetraenoic acid (18:4n-3) *via* a 2-*trans* 18:5n-3 intermediate.

## MATERIALS AND METHODS

*Fatty acid substrates.* The methyl esters of [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3 were prepared from cultures of *I. galbana* (Parke) (S.M.B.A. strain No. 58 C.C.A.P. strain 927/1) grown in $H^{14}CO_3^-$ as described by Ghioni *et al.* (9). In brief, total lipid was extracted from the radioactive algal cells and transmethylated by incubation with 1% sulfuric acid

in methanol at 50°C for 16 h to generate fatty acid methyl esters; the methyl esters of 18:4n-3 and 18:5n-3 were then isolated by silver nitrate thin-layer chromatography ($AgNO_3$-TLC) (11). A total of 5 μCi of methyl esters of both [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3 were obtained, with a specific activity of approximately 12 and 19 mCi/mmol, respectively. The identity of the 18:4n-3 and 18:5n-3 methyl esters was confirmed, and their purity (>99%) and specific activity were determined by radio-gas chromatography (GC) as described by Buzzi *et al*. (12).

Unlabeled methyl esters of 18:5n-3 and 18:4n-3 were prepared by extracting total lipid from *I. galbana* cultures that were not incubated with labeled bicarbonate, transmethylating as described above, and recovering the fatty acid methyl ester fraction enriched in polyunsaturated fatty acids (PUFA) by TLC in hexane/diethyl ether/acetic acid (90:10:1, by vol). Methyl esters of 18:5n-3 and 18:4n-3 were then separated from the fatty acid methyl ester fraction on an ODS C18 high-performance liquid chromatography (HPLC) column (diameter 5 mm) by eluting with acetonitrile at 1.5 mL/min, using ultraviolet (UV) detection at 215 nm. Under these conditions, 18:5n-3 was the first fatty acid eluted. The purity of the methyl esters of both 18:5n-3 and 18:4n-3 was >99% as determined by GC and by GC–mass spectrometry as detailed previously (9,13).

Unlabeled 18:5n-3 was also prepared in greater quantities as the free acid by chemical synthesis according to the method of Kuklev *et al*. (14), which involved a γ-iodo-lactonization of 22:6n-3. The 22:6n-3 used in the synthesis was a concentrate (>95%) kindly supplied by Croda Universal Ltd. (Hull, United Kingdom).

The methyl ester of unlabeled 2-*trans* 18:5n-3 was prepared by first saponifying up to 10 mg of all-*cis* 18:5n-3 methyl ester in 1 M KOH in ethanol/water (95:5, vol/vol) at 78°C for 1 h. The solution was then acidified with HCl, extracted with isohexane/diethyl ether (1:1, vol/vol), evaporated to dryness, and transmethylated and extracted as for the other fatty acid samples. The procedure yielded four compounds in constant relative proportions, all of which were confirmed as methyl esters of 18:5n-3 by electron impact GC–mass spectrometry (13). The four isomers of 18:5n-3 methyl ester were separated by isocratic HPLC on an ODS column using acetonitrile as eluting solvent as described above. Isomer 1 (25% of the total) had a retention time on HPLC corresponding to the original all-*cis* Δ3,6,9,12,15-18:5 (18:5n-3) prepared from *I. galbana* or by chemical synthesis, and its chemical structure was confirmed by $^1$H nuclear magnetic resonance (NMR) spectroscopy at 600 MHz: δ 0.97 (*t*, 3H, *J* = 7.5 Hz, H-18), 2.07 (quintet, 2H, *J* = 7.5 Hz, H-17), 2.60–2.83 (four overlapping *t*, 8H, H-5, H-8, H-11, H-14), 3.13 (*d*, 2H, *J* = 5.8 Hz, H-2), 3.68 (*s*, 3H, –$OCH_3$), 5.34–5.44 (8H, overlapping *m*, alkene-H), 5.53–5.62 (2H, overlapping *m*, alkene-H). Isomer 3 (62% of the total) was obtained as a pure compound by the ODS-HPLC and identified as *trans* Δ2, all-*cis* Δ6,9,12,15-18:5 (2-*trans* 18:5n-3) by $^1$H NMR spectroscopy at 600 MHz: δ 0.96 (*t*, 3H, *J* = 7.5 Hz, H-18), 2.07 (quintet, 2H, *J* = 7.5 Hz, H-17), 2.20–2.29 (*m*, 4H, H-4, H-5), 2.78–2.84 (*m*, 6H, H-8, H-11, H-14), 3.72 (*s*, 3H, –$OCH_3$), 5.27–5.43 (8H, overlapping *m*, *cis*-alkene-H), 5.85 (*dt*, 1H, *J* = 15.6, 1.6 Hz, H-2), 6.96 (*dt*, 1H, *J* = 15.6, 6.6 Hz, H-3). The remaining two isomers, 2 (4% of the total) and 4 (9% of the total), could not be obtained in sufficient amounts for $^1$H NMR identification. Complete epoxidation of the isomeric methyl ester mixture with peracetic acid (15) yielded two distinct epoxide species: (i), 89.4% of the total, which accounts for the sum of isomers 1 and 3; (ii), 10.6% of the total, which accounts for the sum of isomers 2 and 4.

*Cell cultures.* The Atlantic salmon (*Salmo salar*) cell line (AS) (16) was originally obtained from Dr. N. Frerichs (Virology Unit, Institute of Aquaculture, University of Stirling, United Kingdom). The turbot (*Scophthalmus maximus*) cell line (TF) was supplied by Dr. B. Hill (Ministry of Agriculture, Food and Fisheries, Fish Diseases Laboratory, Weymouth, United Kingdom). The gilthead seabream (*Sparus aurata* L.) cell line, SAF-1, developed from fin tissue without immortalization, was provided by Dr. M.C. Alvarez (Department of Cell Biology and Genetics, University of Malaga, Spain) (17).

Cell cultures were grown in 75 cm$^2$ flasks at 22°C in Leibovitz L-15 medium containing 10 mM HEPES and supplemented with 2mM glutamine, 50 IU/mL penicillin, 50 mg/mL streptomycin, and 10% fetal bovine serum (FBS). Approximately 24 h prior to experimentation the cells were subcultured into fresh medium as above except containing only 2% FBS. The cells were then incubated with fatty acid substrates for 6 d as follows. Labeled substrates were added in 50 μL of ethanol at a radioactive concentration of 0.25 μCi per flask containing 15 mL of medium, equivalent to a total mass of 0.021 μmol (1.4 μM) and 0.013 μmol (0.87 μM) for the methyl esters of [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3, respectively. Unlabeled fatty acid methyl esters were added as above at a level of approximately 0.375 μmol/flask providing a concentration of 25 μM. All incubations/experiments were conducted in triplicate.

Preliminary experiments incubating AS cells with 25 μM unlabeled 18:4n-3, added as the fatty acid salt complexed to bovine serum albumin, as the methyl ester complexed to bovine serum albumin, or as the methyl ester in ethanol, showed no differences in the metabolism of these substrates *via* desaturation and elongation to 20:4n-3 and 20:5n-3 (see Ref. 9). No methyl esters were detectable in total lipid extracted from cells incubated with methyl esters. Therefore, the methyl esters of both 18:4n-3 and 18:5n-3 (and 2-*trans* 18:5n-3) were used in order to avoid having to saponify the methyl ester of 18:5n-3 to its free fatty acid (see the Results section).

*Lipid extraction and analysis.* Methods for the extraction of total lipids from cells, preparing fatty acid methyl esters from total lipid, determining radioactivity in fatty acid methyl esters separated on $AgNO_3$, and analyzing fatty acids by GC and radio-GC were as described in detail previously (9,10, 12). The identities of individual radioactive fatty acid methyl esters separated by $AgNO_3$ for radioassay were confirmed by direct radio-GC analyses (12).

**TABLE 1**
**Incorporation of Radioactivity into Total Lipid and Fatty Acids in Fish Cell Cultures After Supplementation with [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5-3[a]**

| | TF | | SAF-1 | | AS | |
|---|---|---|---|---|---|---|
| | $^{14}$C-18:4 | $^{14}$C-18:5 | $^{14}$C-18:4 | $^{14}$C-18:5 | $^{14}$C-18:4 | $^{14}$C-18:5 |
| Total lipid | | | | | | |
| Total μCi | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.08 ± 0.02 | 0.06 ± 0.01 | 0.09 ± 0.01 | 0.05 ± 0.01* |
| pmol/mg total lipid | 2.6 ± 0.3 | 2.7 ± 0.3 | 3.4 ± 0.9 | 2.7 ± 0.3 | 3.0 ± 0.3 | 2.5 ± 0.1 |
| Fatty acid | | | | | | |
| 18:4n-3 | 74.1 ± 0.8 | 76.7 ± 0.5* | 81.0 ± 2.0 | 82.6 ± 0.6 | 18.8 ± 1.0 | 24.0 ± 2.5* |
| 18:5n-3 | ND | ND | ND | ND | ND | ND |
| 20:4n-3 | 4.4 ± 0.0 | 4.5 ± 0.3 | 13.2 ± 1.4 | 10.3 ± 1.2 | 23.6 ± 1.3 | 23.2 ± 1.7 |
| 20:5n-3 | 16.4 ± 0.5 | 14.8 ± 0.8 | 0.7 ± 0.5 | 1.1 ± 0.6 | 48.4 ± 1.7 | 46.1 ± 2.2 |
| 22:4n-3 | 0.8 ± 0.1 | 1.1 ± 0.3 | 5.1 ± 0.2 | 6.0 ± 0.5 | 1.2 ± 0.3 | 1.1 ± 0.3 |
| 22:5n-3 | 1.6 ± 0.3 | 1.2 ± 0.1 | ND | ND | 4.5 ± 0.5 | 3.3 ± 0.7 |
| 22:6n-3 | ND | ND | ND | ND | 1.7 ± 0.3 | ND* |

[a]The [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3 had specific activities of approximately 12 and 19 mCi/mmol, respectively, and were added to cell cultures as methyl esters in 50 μL ethanol at an isotopic concentration of 0.25 μCi per flask containing 15 mL of medium, equivalent to 0.021 μmol (1.4 μM) and 0.013 μmol (0.87 μM) for [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3, respectively. Data for incorporation into fatty acid fractions are percentages of total radioactivity recovered in fatty acids. All results are presented as means ±SD of triplicate experiments. The statistical significance of differences between mean values obtained for each cell line was analyzed by the Student's *t*-test with differences reported as significant as indicated by an asterisk if $P < 0.05$. ND, not detected; AS, Atlantic salmon cell line; SAF-1, gilthead sea bream cell line; TF, turbot fin cell line.

*Materials.* Sodium [$^{14}$C]bicarbonate (~50 mCi/mmol) was purchased from ICN Biomedicals Ltd. (Thame, United Kingdom). TLC (20 cm × 20 cm × 0.25 mm) and high-performance TLC (10 cm × 10 cm × 0.15 mm) plates, precoated with silica gel 60, were obtained from Merck (Darmstadt, Germany). All solvents were of HPLC grade (Rathburn Chemicals, Walkerburn, Peebleshire, Scotland). Ecoscint A was purchased from National Diagnostic (Atlanta, GA). Leibovitz L-15 medium, Hanks' balanced salt solution, FBS, glutamine/penicillin/streptomycin (200 mM L-glutamate, 10,000 U penicillin, and 10 mg streptomycin per mL of 0.9% NaCl), HEPES buffer, fatty acid-free bovine serum albumin, trypsin/EDTA, and standard octadecatetraenoic acid (all-*cis* 18:4n-3) were obtained from Sigma Chemical Co. Ltd. (Poole, United Kingdom).

*Statistical analysis.* All results are presented as means ± SD of three experiments. The statistical significance of differences between mean values in Table 1 obtained for each cell line were analyzed by the Student's *t*-test with differences reported as significant if $P < 0.05$ (18).

## RESULTS

The initial experiments in this study were performed with radiolabeled 18:4n-3 and 18:5n-3 isolated from the alga *I. galbana* grown in the presence of radioactive bicarbonate. The fatty acids were prepared as methyl esters following transmethylation of total lipid extracted from the alga. In preparing the free acid by saponifying the methyl ester of 18:5n-3, we noted by TLC analyses of the reaction products that all-*cis* 18:5n-3 was present in low yield and accompanied by other unknown compounds. We compared the incorporation of 18:5n-3 free acid and its methyl ester into lipids of the three cultured cell lines chosen for study, adding the substrates to the cells in ethanol. No differences were found in the incorporation patterns for the free acid and its methyl ester. Therefore, to maximize the use of the limited amounts of 18:5n-3 methyl ester prepared from *I. galbana*, we routinely used the methyl ester of 18:5n-3 in subsequent experiments.

The results of the incorporation of the methyl esters of [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3 into the three cell lines are shown in Table 1. In these experiments fatty acid methyl esters were prepared from total lipid isolated from the cell cultures and then separated by $AgNO_3$-TLC for radioassay, with the results being confirmed by radio-GC. In no case was radioactive 18:5n-3 recovered from the cells. Rather, 18:4n-3, 20:4n-3, and 20:5n-3 were the major fatty acids labeled after incubation with either [U-$^{14}$C]18:4n-3 or [U-$^{14}$C]18:5n-3 in all three cell lines. The percentage distribution of radioactivity between 18:4n-3, 20:4n-3, and 20:5n-3 differed in the different cell lines depending upon the relative activities of $C_{18}$–$C_{20}$ elongase and Δ5 desaturase. In TF, the percentages of radioactivity recovered in 18:4n-3, 20:4n-3, and 20:5n-3 were the same in cells incubated with [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3, with 18:4n-3 containing a high percentage of radioactivity, 20:5n-3 a modest percentage, and 20:4n-3 a minor percentage. A similar result was obtained for SAF-1 except that the percentages of radioactivity in 20:4n-3 and 20:5n-3 were essentially reversed compared to the turbot cell line. AS gave a different result in that the highest percentage of radioactivity was recovered in 20:5n-3, with both 18:4n-3 and 20:4n-3 having moderate percentages of radioactivity. The species (cell line) differences were emphasized by presenting the data as the summed products for each enzymic step in the pathway, with the marine fish cells clearly show-

**TABLE 2**
**Products of Desaturase and Elongase Activities in Cell Cultures Incubated with [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3[a]**

| | TF | | SAF-1 | | AS | |
|---|---|---|---|---|---|---|
| Enzyme activity | $^{14}$C-18:4 | $^{14}$C-18:5 | $^{14}$C-18:4 | $^{14}$C-18:5 | $^{14}$C-18:4 | $^{14}$C-18:5 |
| C18-20 elongase | 25.9 ± 0.8 | 21.6 ± 0.4* | 19.0 ± 2.1 | 17.4 ± 0.5 | 81.2 ± 1.0 | 73.8 ± 2.4 |
| Δ-5 desaturase | 19.5 ± 0.5 | 17.7 ± 0.9 | 0.7 ± 0.4 | 1.1 ± 0.6 | 56.4 ± 2.6 | 51.7 ± 2.8 |
| C20-22 elongase | 5.1 ± 0.5 | 2.3 ± 0.4* | 5.1 ± 0.3 | 6.0 ± 0.5* | 9.2 ± 0.7 | 4.5 ± 0.9* |
| Δ-6′ desaturase | ND | ND | ND | ND | 1.7 ± 0.3 | ND* |

[a]All results (percentage of total radioactivity recovered) are presented as means ± SD of triplicate experiments. The statistical significance of differences between mean values obtained for each cell line was analyzed by the Student's *t*-test with differences reported as significant if $P < 0.05$ (denoted by an asterisk). Δ-6′ desaturase, formerly termed Δ-4 desaturase; for other abbreviations see Table 1.

ing considerably lower $C_{18}$–$C_{20}$ elongase activity and, in the case of sea bream cells, lower Δ5 desaturase activity compared to the salmon cells (Table 2). However, as with the marine fish cell lines, the percentage distribution patterns in AS were the same for cells incubated with [U-$^{14}$C]18:4n-3 and cells incubated with [U-$^{14}$C]18:5n-3. Thus, the two radioactive substrates yielded the same result in a given cell line irrespective of the pattern of desaturation/elongation expressed by the cell line. Table 1 also shows that of the 0.25 μCi of radioactive fatty acid substrate added, averaged values of 32 and 23% of radioactivity were recovered as fatty acids from cells incubated with [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3, respectively, from the three cell lines. However, the difference between the two substrates was significant only for AS.

An obvious explanation for the findings in Table 1 is that 18:5n-3 is rapidly converted to 18:4n-3 in the cells. This possibility, together with the apparent lability of 18:5n-3 during alkaline saponification of its methyl ester, prompted us to examine the stability of 18:5n-3 in more detail. Therefore, we synthesized 18:5n-3 as the free acid from 22:6n-3 using the γ-iodo-lactonization of 22:6n-3 method as described by Kuklev *et al*. (14). Treating the 18:5n-3 product with aqueous alkali generated four compounds, two major and two minor, as shown by HPLC analysis (see the Materials and Methods section). The two major products were identified by $^1$H NMR and GC as all-*cis* 18:5n-3 (25% of the total products) and 2-*trans* 18:5n-3 (62% of the total products). We then studied the metabolism of the methyl esters of the two isomers of 18:5n-3 (and 18:4n-3) in AS. As there was insufficient isotopically labeled 18:5n-3 available to produce labeled 2-*trans* 18:5n-3 using the method above, the experiment was performed with unlabeled fatty acid substrates at higher concentrations (25 μM) to enable analysis by conventional GC methods. The results showed that cells incubated with 25 μM of all three fatty acid substrates showed increased proportions of 18:4n-3, 20:4n-3 and, to a lesser extent, 22:4n-3 and 20:5n-3 in their lipids over time up to 24 h after supplementation (Table 3). There was no change in the proportions of 22:6n-3 or 22:5n-3 in cell cultures incubated with any of the three fatty acid substrates. The increased concentration of 18:4n-3 in cellular lipids was somewhat less with 2-*trans* 18:5n-3 than all-*cis* 18:5n-3, but the patterns were similar for all three substrates and broadly the same as those observed with the methyl esters of [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3 in Table 1. The results in Table 3 also show that no 18:5n-3 was detected in lipids recovered from the cells incubated with any of the fatty acid substrates.

## DISCUSSION

Octadecapentaenoic acid (all-*cis* 18:5n-3; Fig. 1) is present in classes of algae that have major roles in the marine environ-

**TABLE 3**
**Selected Fatty Acids in Composition of Total Lipid of AS Cells Harvested at Different Times After Addition of 18:4n-3, all-*cis* 18:5n-3, and 2-*trans* 18:5n-3[a]**

| Fatty | 18:4n-3 | | | | | all-*cis* 18:5n-3 | | | | | 2-*trans* 18:5n-3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| acid | 1 h | 3 h | 5 h | 24 h | 48 h | 1 h | 3 h | 5 h | 24 h | 48 h | 5 h | 24 h | 48 h |
| 18:4n-3 | 1.0 ± 0.2 | 2.3 ± 0.4 | 5.3 ± 0.8 | 8.9 ± 1.1 | 3.8 ± 1.4 | 1.0 ± 0.2 | 1.7 ± 0.4 | 6.9 ± 1.6 | 11.8 ± 0.4 | 4.3 ± 1.2 | 1.4 ± 0.1 | 3.9 ± 0.6 | 3.7 ± 2.5 |
| 18:5n-3 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 20:4n-3 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.6 ± 0.1 | 1.3 ± 0.4 | 1.1 ± 0.4 | ND | ND | 0.5 ± 0.2 | 1.4 ± 0.1 | 0.8 ± 0.5 | 0.4 ± 0.1 | 1.2 ± 0.1 | 1.5 ± 1.1 |
| 20:5n-3 | 1.3 ± 0.3 | 1.5 ± 0.2 | 0.6 ± 0.2 | 2.0 ± 0.5 | 1.4 ± 0.6 | 1.5 ± 0.1 | 1.2 ± 0.3 | 1.6 ± 0.5 | 2.0 ± 0.1 | 1.0 ± 0.4 | 1.5 ± 0.1 | 1.5 ± 0.2 | 1.1 ± 0.8 |
| 22:4n-3 | 0.5 ± 0.4 | 0.6 ± 0.3 | 0.8 ± 0.1 | 1.0 ± 0.5 | 0.7 ± 0.3 | 1.0 ± 0.2 | 1.1 ± 0.3 | 0.7 ± 0.2 | 1.1 ± 0.4 | 0.5 ± 0.2 | 0.5 ± 0.1 | 0.9 ± 0.6 | 0.8 ± 0.6 |
| 22:5n-3 | 3.3 ± 0.5 | 3.1 ± 0.4 | 3.2 ± 0.2 | 3.4 ± 0.4 | 3.1 ± 0.4 | 3.1 ± 0.1 | 3.6 ± 0.3 | 3.5 ± 0.4 | 3.5 ± 0.6 | 2.8 ± 0.5 | 3.4 ± 0.2 | 3.4 ± 0.4 | 2.8 ± 0.6 |
| 22:6n-3 | 5.1 ± 0.6 | 4.5 ± 0.4 | 4.9 ± 0.3 | 5.0 ± 0.6 | 4.9 ± 0.5 | 4.8 ± 0.3 | 5.1 ± 0.4 | 5.4 ± 0.7 | 4.9 ± 0.5 | 4.8 ± 0.7 | 4.8 ± 0..5 | 5.0 ± 0.6 | 4.6 ± 0.7 |

[a]All supplemented fatty acids were unlabeled and added as methyl esters in 50 μL ethanol at a fatty acid concentration of 25 μM as described in detail in the Materials and Methods section. Results are expressed as percentage of weight of total fatty acids and are mean values ±SD of three determinations. For abbreviations see Table 1.

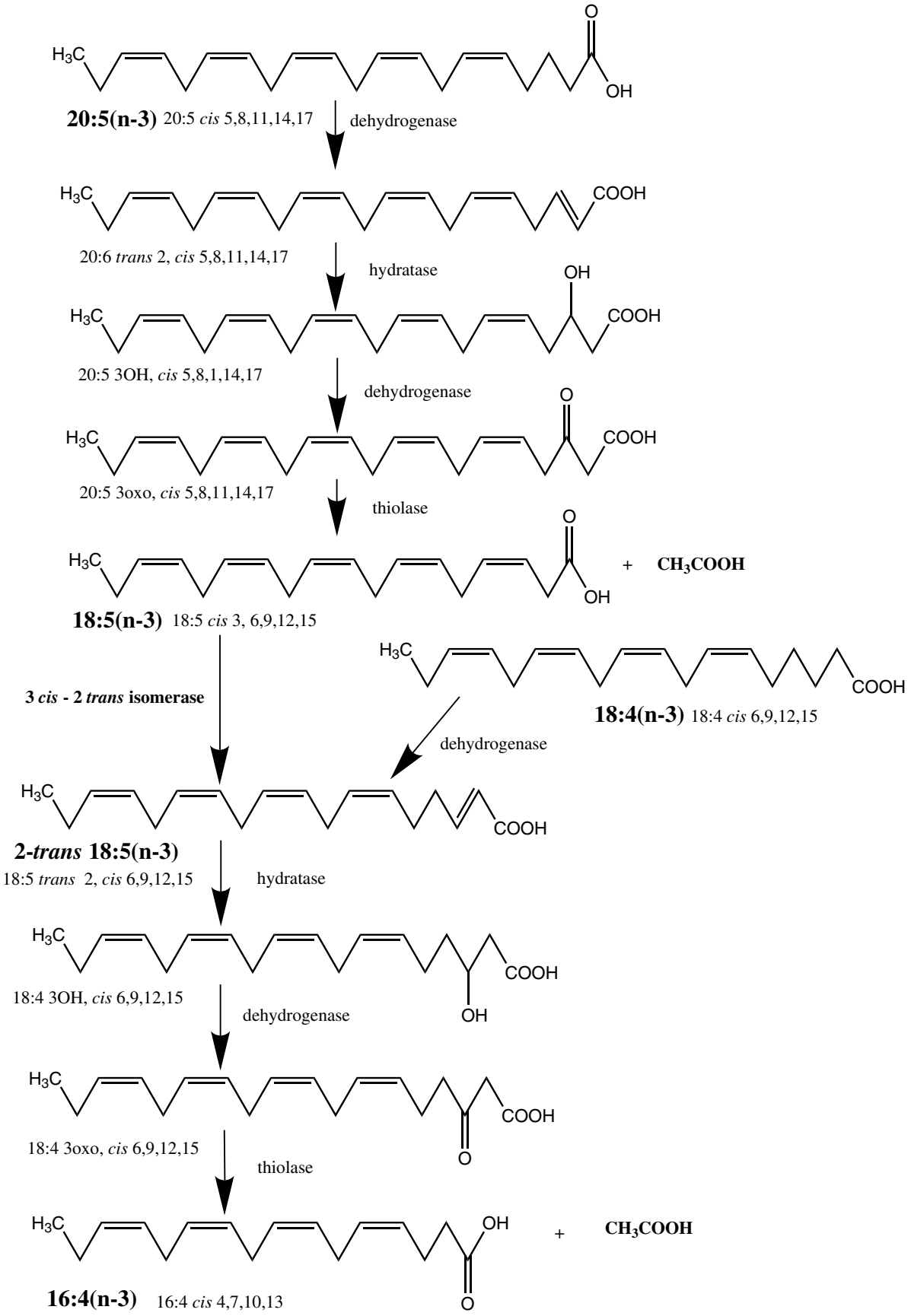


**FIG. 1.** Section of the β-oxidation pathway for n-3 polyunsaturated fatty acids showing the position of 2-*trans* 18:5n-3 as a common intermediate in the β-oxidation of 18:5n-3 and 18:4n-3.

ment (6). Thus, dinoflagellates can form large toxic blooms; the haptophycean *Phaeocystis pouchetti* is a major source of scum polluting beaches and inshore waters of mainland European coasts; the haptophycean coccolithophore *Emiliania huxleyi* is a major source of marine $CaCO_3$ deposits; the latter two organisms are major sources of dimethyl sulfide implicated as a source of "acid rain." The acid 18:5n-3 can account for more than 15% of the total fatty acids in these organisms, being present in glycolipids where it can account for 66% of the total fatty acids (6). However, it is seldom if ever detected in significant amounts in phytoplankton-consuming animals (1), i.e., in zooplankton and some fish, consistent with its being rapidly metabolized by marine animals.

The acid 18:5n-3 coexists in the aforementioned organisms with 22:6n-3, and it is unusual in sharing with 22:6n-3 the property of having the maximum number of double bonds possible in a carbon chain. However, unlike 22:6n-3 whose terminal double bond is four carbons from the carboxyl terminus, the terminal double bond in 18:5n-3 is three carbons from the carboxyl terminus. The insertion of the last (Δ4) double bond in 22:6n-3 occurs by a mechanism whereby 24:5n-3 is Δ6-desaturated to 24:6n-3, which is then chain-shortened in peroxisomes to 22:6n-3 (19). Similarly, the biosynthesis of 18:5n-3 is not thought to be possible solely through the action of desaturases and elongases with the conventional pathway from 18:3n-3 to 20:5n-3 proceeding *via* 18:4n-3 then 20:4n-3. However, by analogy with the biosynthesis of 22:6n-3, the formation of 18:5n-3 is probably straightforward, at least in principle, in that it can be formed directly from 20:5n-3 by chain-shortening as postulated previously (6). The precise subcellular location(s) of these putative chain shortening steps in the biosynthesis of 22:6n-3 and 18:5n-3 in these marine phytoplankton are not known (20).

As its biosynthesis requires a special mechanism, so the catabolism of 22:6n-3 requires a special mechanism in that the 2-*trans*,4-*cis* intermediate formed by the initial direct dehydrogenation of 22:6n-3 is converted by a 2,4-dienoyl reductase to a 3-*trans* intermediate, which is then converted by a $\Delta^3$,$\Delta^2$-enoyl isomerase to a 2-*trans* intermediate (21,22). The latter two enzymes are auxiliary enzymes to the multifunctional enzymes of β-oxidation required for the β-oxidation of PUFA in both mitochondria and peroxisomes (23,24). The 2-*trans* 22:6n-3 intermediate can then continue in the conventional β-oxidation pathway through hydration across the 2,3-*trans* double bond to yield the 3-hydroxy and then the 3-keto intermediate. Similarly, catabolism of 18:5n-3 requires, in principle, the operation of a $\Delta^3$,$\Delta^2$-enoyl isomerase to generate the 2-*trans* intermediate required for entry into the β-oxidation pathway though, to our knowledge, this reaction has never been studied with 18:5n-3 (Fig. 1).

The results here establish the ease with which all-*cis* 18:5n-3 can be converted to its 2-*trans* isomer. Thus, all-*cis* 18:5n-3, chemically synthesized from 22:6n-3, was readily converted in high yield to its 2-*trans* isomer by treatment with alkali in aqueous ethanol. The protons at C-2 of all-*cis* 18:5n-3 are both allylic to the *cis*-3,4-double bond and α to the terminal carboxyl group, and hence should experience a reduction in $pK_a$ (relative to, for example, the corresponding protons in 18:4n-3). These combined effects would be expected to result in an increased tendency of the 18:5n-3 to undergo enolization, particularly in protic solvents. Enolization results in the conjugation of the 3,4-double bond with the enol and hence the configurational stability of the 3,4-double bond may be compromised. Furthermore, when reprotonation of the intermediate enol occurs, it can do so either at C-2 (regenerating 18:5n-3 with either *cis*- or *trans*-stereochemistry at C-3), or at C-4, which would result in the conjugation of the double bond with the carboxylic acid. The latter process is expected to be thermodynamically preferred and would be expected to result in the preferential formation of the *trans* Δ2, all-*cis* Δ6,9,12,15-18:5n-3 acid. The chemical isomerization of all-*cis* 18:5n-3 to *trans* Δ2, all-*cis* Δ6,9,12,15-18:5n-3 has not, to our knowledge, been reported before. However, it may have been observed but misinterpreted in that, in reducing all-*cis* 18:5n-3 with hydrazine to determine double bonds, Napolitano *et al.* (25) noted a loss of 18:5n-3 and mentioned the occurrence of an extra peak of 18:4n-3 in gas chromatograms of the reaction products. It is possible that the extra peak was a 2-*trans* 18:5n-3 isomer whose formation was prompted by availability of protons in the reaction.

In contrast to 18:5n-3, which is an intermediate in the conventional β-oxidation pathway of 22:6n-3 and 20:5n-3, 18:4n-3 is not, as one cycle of β-oxidation of 18:5n-3 results in the formation of 16:4n-3 *via* 2-*trans* 18:5n-3, 3-hydroxy 18:4n-3 and 3-oxo 18:4n-3 (26) (Fig.1). However, the first step in the β-oxidation of 18:4n-3 is the action of dehydrogenase to yield 2-*trans* 18:5n-3. Therefore, 2-*trans* 18:5n-3 is a common intermediate in the β-oxidation of both 18:5n-3 and 18:4n-3 (Fig. 1).

When the various cell lines were incubated with [U-$^{14}$C]-18:5n-3 methyl ester, no radioactive 18:5n-3 was detected in total lipid extracted from the cells. However, radioactivity was readily detected in 18:4n-3 and its elongated and further desaturated products, 20:4n-3 and 20:5n-3. Moreover, for a given cell line, the distributions of radioactivity in 18:4n-3, 20:4n-3, and 20:5n-3 from cells incubated with [U-$^{14}$C]-18:5n-3 were essentially the same as those generated from the same cells incubated with [U-$^{14}$C]18:4n-3. Similarly, the increases in the mass percentage of 18:4n-3, 20:4n-3, and 20:5n-3 in Atlantic salmon cells incubated with 25 μM 18:4n-3 were similar in cells incubated with 25 μM all-*cis* 18:5n-3 and also with 25 μM 2-*trans* 18:5n-3. The observed patterns are in accord with our previous findings and conclusions that Atlantic salmon cells convert 18:4n-3 to 20:5n-3 more readily than either turbot or sea bream cells, owing to a relative deficiency in the $C_{18}$–$C_{20}$ fatty acid elongase in turbot cells and a relative deficiency in the Δ5 fatty acid desaturase in the sea bream cells (9,10). Consequently, production of radiolabeled 22:5n-3 was higher in the salmon cells incubated with labeled 18:4n-3 and 18:5n-3 than in turbot and sea bream cells, but although 22:5n-3 is a poor substrate for retroconversion to 20:5n-3 and serves as a substrate for 22:6n-3

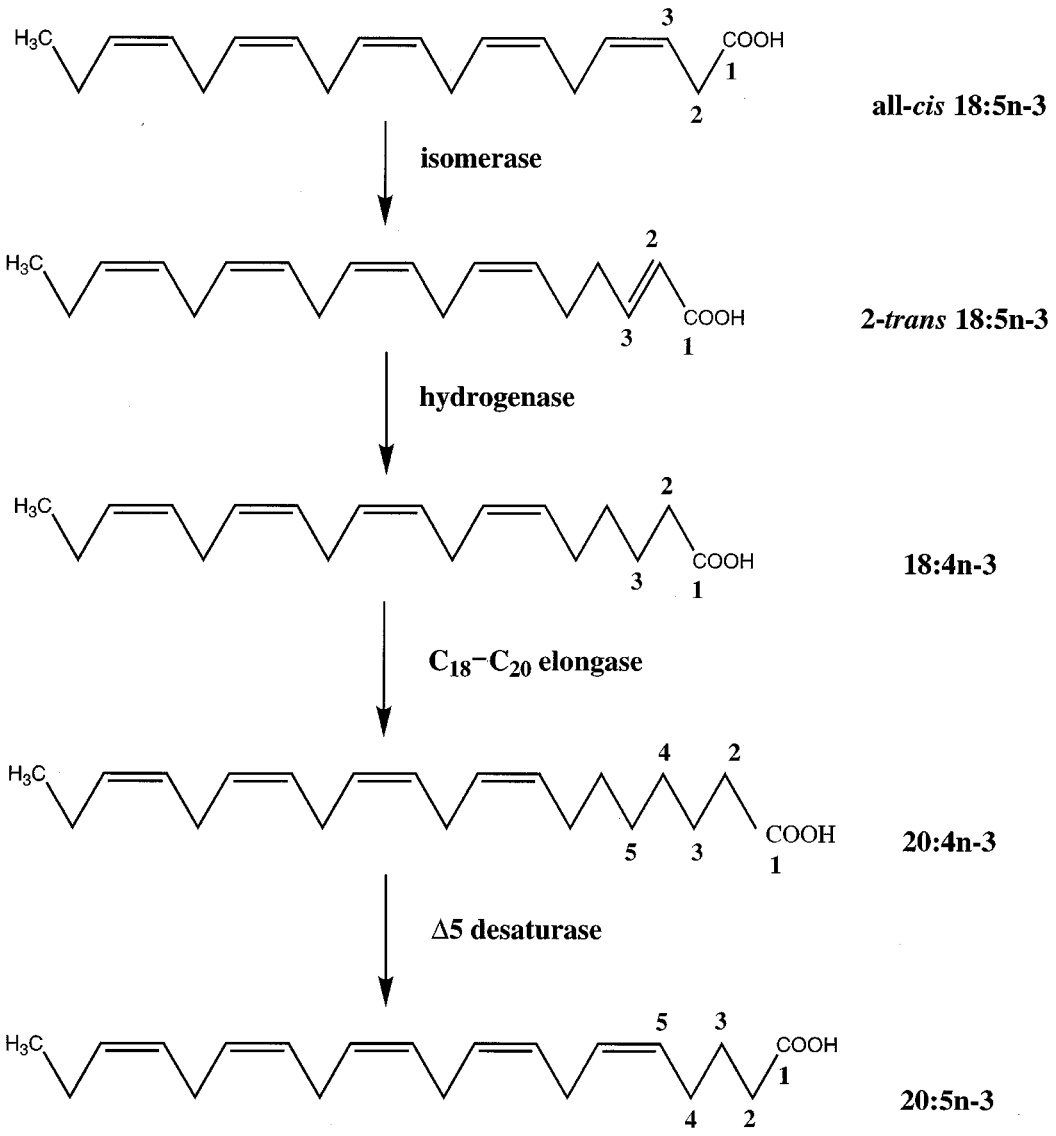


**FIG. 2.** Reaction scheme whereby 18:5n-3 is converted to 18:4n-3 and thence to 20:5n-3 in cultured fish cells.

formation (27), there was virtually no production of 22:6n-3 in salmon cells similar to the marine fish cell lines. This is not unexpected as most established cells in culture lack the ability to produce substantial amounts of 22:6n-3 although the precise reason in enzymic terms is unknown (20). The results are also consistent with all-*cis* 18:5n-3 being readily converted to 18:4n-3 in all the cell lines, *via* a $\Delta^3$,$\Delta^2$-enoyl isomerase generating a 2-*trans* 18:5n-3 intermediate followed by a hydrogenase (reversed dehydrogenase activity) operating on the 2-*trans* 18:5n-3 intermediate to generate 18:4n-3 (Fig. 2). The 18:4n-3 so produced is then available for elongation and desaturation reactions to generate 20:4n-3 and 20:5n-3 (Fig. 2).

As noted earlier, some 32 and 23% of the radioactivity added as [U-$^{14}$C]18:4n-3 and [U-$^{14}$C]18:5n-3, respectively, was recovered from cellular total lipid as 18:4n-3, 20:4n-3, and 20:5n-3, the remainder presumably being converted to β-oxidation products (Fig. 1). That 18:5n-3 is more readily β-oxidized than 18:4n-3 by the cells implies that the isomerization of added all-*cis* 18:5n-3 to 2-*trans* 18:5n-3 proceeds more readily in the cells than the 2,3-dehydrogenation of added 18:4n-3 to 2-*trans* 18:5n-3. This may reflect the fact that the cells studied here were actively growing and dividing and therefore as much concerned with directing exogenous PUFA into biosynthetic pathways directed toward membrane lipid formation as into β-oxidation.

Irrespective of how the relevant anabolic and catabolic pathways are controlled, the ease of conversion of all-*cis* 18:5n-3 to 2-*trans* 18:5n-3 and thence to 18:4n-3, demonstrated here in fish cells, readily accounts for the absence of 18:5n-3 from marine trophic levels higher than the algae.

## ACKNOWLEDGMENTS

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# Characterization of *N*-Acylphosphatidylethanolamine and Acylphosphatidylglycerol in Oats

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**ABSTRACT:** Two polar lipid classes, both with three acyl groups, were isolated from an extract of oats and characterized by nuclear magnetic resonance spectroscopy, electrospray mass spectrometry (MS), and electron ionization MS (EIMS). Distortionless enhancement by polarization transfer (DEPT) and the two-dimensional correlation experiments $^1$H-detected heteronuclear multiple quantum coherence spectroscopy, heteronuclear multiple bond correlation spectroscopy, double quantum filtered correlation spectroscopy, and total correlation spectroscopy provided sufficient information for determination of the structure of the two lipid classes. The polar lipid classes were found to be *N*-acylphosphatidylethanolamine [1,2-diacyl-*sn*-glycero-3-phospho-(*N*-acyl)-1′-ethanolamine; *N*-acyl-PE] and acylphosphatidylglycerol [1,2-diacyl-*sn*-glycero-3-phospho-(3′-acyl)-1′-*sn*-glycerol]. High-performance liquid chromatography with electrospray ionization MS (HPLC-ESMS) and with electrospray ionization tandem MS (HPLC-MS/MS) were utilized for the separation and subsequent determination of molecular species. With HPLC-ESMS, ions of deprotonated molecules were obtained and with HPLC-MS/MS carboxylate ions (representing acyl groups) were obtained as well as other structurally significant ions. Fifty molecular species of *N*-acylphosphatidylethanolamine and 24 molecular species of acylphosphatidylglycerol were found, with a molecular mass range of 924–1032 Da and 959–1035 Da, respectively. Identification of the fatty acid isomers, as picolinyl ester derivatives, was done with gas chromatography with EIMS. Three isomers of 16:1 fatty acids were found in *N*-acyl-PE, and their double bond positions were determined to 6, 9, and 11 with a relative abundance of 4:10:1.

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Cereals such as oats, wheat, and rye have traditionally not been considered as potential sources for lipids due to their low lipid content. However, oats (*Avena sativa* L.) has been reported to have higher lipid content, with a higher nutritional value, than other cereals (1–3). Oat oil and fractions thereof have interesting physical, nutritional, and dermatological properties, and can find many uses in the food, cosmetics, and pharmaceutical industries (4–6).

Polar lipid fractions from cereals show a high content of galactolipids as well as wide variety of different phospholipid classes, such as phosphatidylcholine, phosphatidylethanolamine (PE; 1,2-diacyl-*sn*-glycero-3-phospho-1′-ethanolamine) and phosphatidylglycerol (PG; 1,2-diacyl-*sn*-glycero-3-phospho-1′-*sn*-glycerol) (1). However, several of the minor components have not yet been identified.

Among the minor components in cereals that have been described, N-acylated glycerophospholipids are perhaps the most interesting, since they have been reported in only a handful of biological systems. The properties of N-acylated glycerophospholipids have been thoroughly reviewed (7), and their presence in microorganisms, plants, fish, and mammalian tissues are reported. It has been suggested that the occurrence of these compounds is an artifact owing to conditions of extensive cellular degeneration (8) or to incorrect identification (9), but later evidence showed that they play a metabolic role in the tissues where they appear (7,10,11). Recent reports indicate the importance of *N*-acylphosphatidylethanolamines (*N*-acyl-PE) as precursors for *N*-acylethanolamines, which in turn play a physiological role during germination of seeds and in defense systems in plants (12,13). The levels and decrease of *N*-acyl-PE during early germination of various seeds have been reported (14). The content of *N*-acyl-PE in oats was found to be significantly higher (up to 13% of total seed phospholipids) than in the other species studied.

Several aspects motivate the characterization of these components in cereals. Firstly, since the lipid class composition differs from other plant tissues, it is of interest to study the significance of the components for the membrane structure of the seed tissues. Secondly, it is important to investigate the chemical composition in order to understand the physical behavior of cereal lipid extracts (15,16). Lastly, as already indicated, some of them may have biological functions apart from being constituents of the biological membranes, and it is therefore important to be able to detect them.

Nuclear magnetic resonance (NMR) spectroscopy is an ideal technique for the study of polar lipids and especially

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Abbreviations: 1D, one-dimensional; 2D, two-dimensional; *N*-acyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-(*N*-acyl)-1′-ethanolamine; acyl-PG, 1,2-diacyl-*sn*-glycero-3-phospho-(3′-acyl)-1′-*sn*-glycerol; CID, collision-induced dissociation; DEPT, distortionless enhancement by polarization transfer; DQF-COSY, double quantum filtered correlation spectroscopy; EIMS, electron ionization mass spectrometry; ESMS, electrospray ionization mass spectrometry; GC, gas chromatography; HMBC, heteronuclear multiple-bond correlation spectroscopy; HMQC, $^1$H-detected heteronuclear multiple quantum coherence spectroscopy; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; PE, 1,2-diacyl-*sn*-glycero-3-phospho-1′-ethanolamine; PG, 1,2-diacyl-*sn*-glycero-3-phospho-1′-*sn*-glycerol; TIC, total ion current; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy.

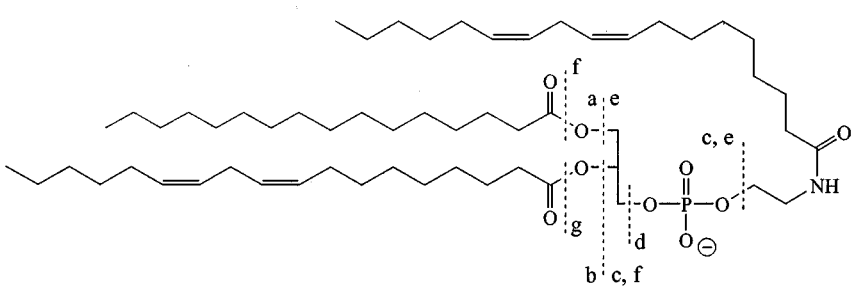


**FIG. 1.** Structure of *N*-acylphosphatidylethanolamine [1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phospho-(*N*-linoleoyl)-1′-ethanolamine; *N*-acyl-PE]. Suggested cleavage sites using negative ion electrospray with collision-induced dissociation are indicated by dashed lines.

glycerophospholipids (17,18). $^{1}H$, $^{13}C$, and $^{31}P$ spectra and correlation experiments of the combinations of these nuclei can be achieved. The diversity of functional groups within the molecules yields a good spread of signals in the $^{1}H$ and $^{13}C$ spectra, thus providing significant structural information.

Another powerful technique used today in the analysis of intact polar lipids is high-performance liquid chromatography (HPLC) on-line with mass spectrometry (MS) and/or tandem mass spectrometry (MS/MS). The process of first separating the polar lipid extract by HPLC into either different lipid classes or molecular species within a class and then detecting them with MS makes simultaneous determination of molecular ions and identification of several structural elements possible. Several papers have recently reported the use of electrospray ionization mass spectrometry (ESMS), with or without HPLC separation, in the structural determination of many different intact polar lipids (19–24).

Important features of lipid molecules are the chain length and position of double bonds and other structural elements in the fatty acid. A convenient analytical technique for obtaining this information is gas chromatography–mass spectrometry (GC–MS) of certain fatty acid derivatives (25). The derivative should be able to retain the charge when the molecule is ionized in the mass spectrometer, to minimize double-bond migration and to give diagnostic ions for structural elements. Nitrogen-containing derivatives meet these requirements, and the picolinyl ester is commonly used for the determination of double-bond positions and other structural features (26,27).

In this paper we have characterized two components in oats, *N*-acyl-PE (Fig. 1) and 3′-*O*-acylphosphatidylglycerol (acyl-PG, Fig. 2) by one- and two-dimensional $^{1}H$- and $^{13}C$-NMR, HPLC–ESMS, HPLC–MS/MS and GC–electron ionization mass spectrometry (EIMS) of fatty acid picolinyl esters. *N*-Acyl-PE has previously been reported as a component in oats and other plant seeds (1,14,28). To our knowledge, acyl-PG has been found in microorganisms such as mold (29), bacteria (30–32), trichomonads (33), and in mammalian tissues (34–37), but not in plants.

## EXPERIMENTAL PROCEDURES

*Polar lipids from oat*. Fractionated oat oil extracts (Galactolec™; Ref. 38), obtained from Scotia LipidTeknik AB (Stockholm, Sweden), were used as a starting material for most experiments. For quantitative determinations fresh oat flakes, obtained by rolling oat grains (*A. sativa* L., cv. Freia) under mild conditions, were extracted by ethanol.

*Chemicals*. Solvents used for liquid chromatography (LC) were of HPLC quality. All other solvents and chemicals were of analytical grade. Glacial acetic acid, aqueous ammonia 25%, potassium hydroxide, isohexane, diethyl ether, methanol, chloroform, and dichloromethane were purchased from

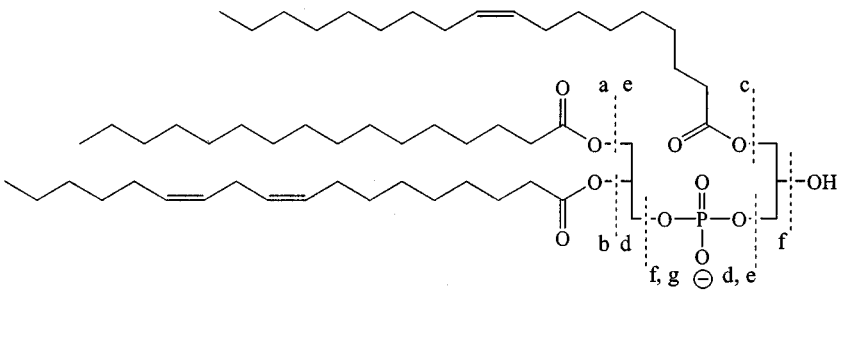


**FIG. 2.** Structure of acylphosphatidylglycerol [1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phospho-(3′-lineoloyl)-1′-glycerol; acyl-PG]. Suggested cleavage sites using negative ion electrospray with collision-induced dissociation are indicated by dashed lines.

Merck (Darmstadt, Germany), absolute ethanol from Kemetyl (Haninge, Sweden), and oxalyl chloride and 3-(hydroxymethyl)pyridine from Sigma-Aldrich (Steinheim, Germany). Hexane and isooctane were purchased from CertiFi (Fisher Scientific, London, United Kingdom), tetrahydrofuran and water from LabScan (Dublin, Ireland), isopropanol from Fisons (Loughborough, United Kingdom), and ammonium acetate from BDH Chemicals Ltd. (Poole, United Kingdom). Chloroform-$d_1$ and methanol-$d_4$ were purchased from Aldrich (Milwaukee, WI).

Nano-Platten® SIL-20 $UV_{254}$ (Macherey-Nagel, Düren, Germany) were used for analytical thin-layer chromatography (TLC) and PSC-Fertigplatten Kieselgel 60, 2 mm (Merck) for preparative TLC.

*Isolation*. Galactolec™ (100 mg) was dissolved in 0.5 mL of hexane and applied to two Sep-Pac Plus Silica cartridges (Waters, Milford, MA) coupled in series. The cartridges were subsequently washed with 10 mL of hexane, 10 mL of hexane/2-propanol (9:1, vol/vol), 40 mL of hexane/2-propanol (8:2), and 60 mL of acetone. A fraction was then eluted by 10 mL of chloroform/methanol (2:1) yielding 11 mg. TLC analysis on silica, with chloroform/methanol/concentrated ammonia (40:10:2, by vol) as eluant, indicated two major components in this fraction, **1** ($R_F$, 0.62) and **2** ($R_F$, 0.42). The procedure was repeated several times, and the obtained fractions were pooled. Components **1** and **2** were then separated by running preparative TLC with chloroform/methanol/aqueous ammonia 25% (40:10:2, by vol). The contents of **1** and **2** in the different fractions were roughly quantified by the use of a previously described HPLC method (39).

*NMR analysis*. Sample (10–20 mg) was dissolved in a mixture of 0.6 mL chloroform-$d_1$ and 0.3 mL methanol-$d_4$ and transferred to an NMR tube (5 mm o.d.).

The $^1$H NMR spectra were acquired at 500 MHz and the $^{13}$C spectra at 125.8 MHz.

To facilitate structural elucidation, distortionless enhancement by polarization transfer (DEPT) and the two-dimensional (2D) correlation experiments $^1$H-detected heteronuclear multiple quantum coherence spectroscopy (HMQC), heteronuclear multiple-bond correlation spectroscopy (HMBC), double quantum filtered correlation spectroscopy (DQF-COSY), and total correlation spectroscopy (TOCSY) were performed.

*HPLC–MS and HPLC–MS/MS analysis*. A Varian model 9012 gradient pump (Varian Chromatography Systems, Walnut Creek, CA) with a Rheodyne injector (Rohnert Park, CA) fitted with a 10-µL loop was used for the sample introduction. The LC separations were performed on a PVA-Sil column, 250 × 3.0 mm i.d. (5 µm; YMC, Inc., Wilmington, NC). Solvent mixture A consisted of hexane/isopropanol/tetrahydrofuran/isooctane/ammonium acetate (2.3 mM)/water (64:26:4.5:4.5:0.4:1, by vol) and solvent mixture B of isopropanol/tetrahydrofuran/isooctane/ammonium acetate (2.3 mM)/water (81:4.5:4.5:0.4:10, by vol). The flow rate was 0.4 mL/min and the column temperature 80°C. The solvent composition followed a linear gradient profile: at 0 min, 100% A; at 25 min, 0% A; at 26 min, 0% A; at 26.5 min, 100% A; and at 40 min, 100% A. Prior to use of the column, a blank gradient was run.

MS was performed on a Micromass QUATTRO II mass spectrometer (Micromass Instruments, Altrincham, United Kingdom) equipped with a pneumatically assisted electrospray ion source. Data handling was performed on a VG Masslynx NT32 data handling system (Manchester, United Kingdom). Full scan spectra, between *m/z* 150 and 2,000, were obtained at a scan speed of 250 Da/s with a mass resolution of 1 Da at half peak height. The LC effluent entered the mass spectrometer through an electrospray (ES) capillary set at 2.4 kV at a source temperature of 160°C and a cone voltage of 60 V. Nitrogen was used as the drying gas as well as the nebulizing gas at a flow rate of 250–300 and 20 L/h, respectively. The collision-induced dissociation (CID) spectra, at a cone voltage of 60 V, were recorded at a collision energy of 25 eV, with a mixture of xenon + argon (1:3, vol/vol) as collision gas at a pressure of $1 \times 10^{-3}$ mbar. Approximately 5–10 µg of sample was injected in all of the analyses.

*Preparation of picolinyl esters for GC–MS analysis*. Alkaline hydrolysis of the sample was performed on 5 mg of lipid dissolved in 1 mL of absolute ethanol using 0.1 mL of 1 M potassium hydroxide solution. The reaction was carried out in a stoppered tube at 50°C during 3 h. After cooling, 2 mL of water was added and the sample was acidified with glacial acetic acid. The free fatty acids were extracted twice with 6 mL of hexane/diethyl ether (1:1, vol/vol). The combined extracts were dried with sodium sulfate before being taken to dryness in a gentle stream of nitrogen.

Derivatization of the free fatty acids to picolinyl esters was accomplished *via* the acid chloride (40). Oxalyl chloride (0.5 mL) was added to the free fatty acids and kept in a stoppered tube at room temperature overnight. The excess oxalyl chloride was evaporated, and the acid chloride was taken immediately to derivatization. A volume of 0.5 mL of a solution of 3-(hydroxymethyl)pyridine, dissolved in dichloromethane (20 mg/mL) was added to the acid chloride. The sample was cooled in an ice bath for 5 min and then held at room temperature for an hour, after which the solvent was evaporated. Water (5 mL) was added, and the picolinyl esters were recovered by extraction with 2 mL hexane. The extract was dried with sodium sulfate, evaporated, and the picolinyl esters were redissolved in hexane to an appropriate concentration for GC–MS analysis.

*GC–MS analysis*. GC–MS analysis was performed using a Varian GC 3400 connected to a Varian Saturn GC MS (Varian, Walnut Creek, CA). The GC separations were done using a Supelcowax 10 fused-silica column, 30 m × 0.25 mm, 0.25 µm film thickness (Supelco Inc., Bellefonte, PA). The oven was programmed as follows: 130°C for 2 min, thereafter the temperature was raised first to 180°C at a rate of 20°C/min and next to 280°C at 2°C/min and held there for 60 min. The injector was programmed from 180 to 260°C in 0.6 min and held there for 2 min. The ion trap was held at 220°C and the transfer line at 240°C. The scan rate was 1 scan/s from 50 to 500 amu.

## RESULTS AND DISCUSSION

*Isolation*. Two fractions, containing **1** and **2**, respectively, were obtained from the preparative TLC run as described above. These two preparations were used for NMR analysis. For the HPLC–ESMS and GC–EIMS investigations, the mixture was used. HPLC analysis of fractions from fresh oat flakes indicates a content in dry oats of 0.8–1.1 mg/g for **1** and *ca.* 0.3 mg/g for **2**. This corresponds to 3–4% (by weight) and 1.0–1.3% (by weight) of total polar lipids, or 10–12% (by weight) and 3–4% (by weight) of the total phospholipids in oats for **1** and **2**, respectively.

*NMR results*. One-dimensional (1D) and 2D experiments were utilized to determine the structures of **1** and **2** as outlined below. The reasoning was partly based on comparison with spectral data from PE and PG.

The $^{1}$H NMR spectra of **1** and **2** (summarized in Tables 1 and 2) display a pattern of signals in the regions typical for polar glycerolipids, e.g., signals from the acyl groups at δ 0.8–2.8, from olefinic protons at δ 5.3, and from protons next to nitrogen or oxygen at δ 3.4–5.2 ppm.

In the $^{13}$C NMR spectrum signals from the acyl moiety appear in the δ 14–36 ppm region, with olefinic carbons at δ 128–131 and carbonyls at δ 173–175 ppm. Three distinct carbonyl signals are visible for both **1** and **2**, showing that three acyl groups are present in both compounds. This is also confirmed by the integral ratio between the methyl group (9H) and the signal at δ 5.18–5.19 ppm (1H) in the proton spectrum.

Signals from carbons attached to hydroxyl or carboxylate groups appear at δ 60–75 ppm in the $^{13}$C spectrum. By running DEPT experiments these carbons can be classified as methylene ($CH_2$) groups (δ 64.6, 63.9, and 63.0 ppm in **1** and δ 67.1, 65.2, 63.9, and 62.9 ppm in **2**) or methine (CH) groups (δ 70.9 ppm in **1** and δ 70.8 and 69.2 ppm in **2**).

The TOCSY experiment of **1** shows the different $^{1}$H spin systems in the molecule. It is possible to identify one spin system (1A) consisting of the signals at δ 5.18, 4.37, 4.13, and 3.93 ppm and another one (1B) with signals at δ 3.89 and 3.40 ppm.

In the 2D spectrum obtained from the HMQC experiment, each one-bond $^{1}$H-$^{13}$C coupling is represented by a cross peak (see Fig. 3). Thus, all the proton signals in the 1A spin system and the signal at δ 3.89 ppm can be paired with $^{13}$C signals of methylene and methine groups in the δ 60–75 ppm range.

The chemical shifts and geminal and vicinal coupling patterns of protons in the 1A spin system, as seen in DQF-COSY (Fig. 4), as well as the chemical shifts of the corresponding $^{13}$C signals, are almost identical to the data for the diacylglycerol moiety in PE and PG. This implies that **1** contains a phosphatidyl (diacylglycerophosphate) moiety.

The HMQC experiment shows that the $^{1}$H signal at δ 3.40 ppm has a cross peak at δ 40.6 ppm, a chemical shift typical for carbons attached to an amine or amide group, e.g., C-2 in PE at δ 40.9 ppm. The chemical shift for the protons at C-2 in PE is δ 3.09 ppm. With an acyl group attached to the nitrogen by an amide bond, the signal is shifted downfield, which ex-

**TABLE 1**
**NMR Chemical Shifts of *N*-Acyl-PE and PE in Chloroform-$d_1$/Methanol-$d_4$ (2:1, vol/vol)**

| | $^{1}$H chemical shift[a] (ppm) | | $^{13}$C chemical shift[b] (ppm) | |
|---|---|---|---|---|
| Compound | *N*-Acyl-PE | PE from egg | *N*-Acyl-PE | PE from egg |
| Acyl moieties | | | | |
| C(n) | 0.85 | 0.83 | 14.29, 14.26 | 14.24 |
| C(n-1) | 1.27 | 1.24 | 23.07, 22.96 | 22.99 |
| C(n-2) | 1.27 | 1.24 | 32.34, 31.93 | 32.25 |
| C, methylene | 1.27 | 1.24 | 29.5–30.2 | 29.5–30.0 |
| C, allylic | 2.00 | 2.00 | 27.58 | 27.51 |
| C, doubly allylic | 2.72 | 2.78 | 26.29, 26.01 | 25.94 |
| C, olefinic | 5.29 | 5.32 | 128.3–130.5 | 127.4–132.3 |
| C3 | 1.57 | 1.55 | 25.30, 25.27 | 25.20 |
| C2, ester | 2.27 | 2.27 | 34.62, 34,47 | 34.39 |
| C2, amide | 2.15 | | 36.71 | |
| C1, ester | | | 174.42, 174.05 | 173.7, 173.9, 174.3 |
| C1, amide | | | 175.42 | |
| Glycerol moiety | | | | |
| *sn*-1 | 4.37, 4.13 | 4.37, 4.13 | 63.06 | 62.89 |
| *sn*-2 | 5.18 | 5.17 | 70.93 | 70.74 |
| *sn*-3 | 3.93 | 3.94 | 63.91 | 64.13 |
| Ethanolamine moiety | | | | |
| C1 ($CH_2$-OP) | 3.86 | 4.01 | 64.59 | 61.94 |
| C2 ($CH_2$-N) | 3.37 | 3.09 | 40.63 | 40.89 |

[a]The signal at δ 3.30 ppm from residual $CD_2HOD$ was used as shift reference.
[b]The signal at δ 49.0 ppm from $CD_3OD$ was used as shift reference. NMR, nuclear magnetic resonance; *N*-acyl-PE, 1,2-diacyl-*sn*-glycero-3-phospho-(*N*-acyl)-1′-ethanolamine; PE, phosphatidylethanolamine.

**TABLE 2**
**NMR Chemical Shifts of Acyl-PG and PG in Chloroform-$d_1$/Methanol-$d_4$ (2:1, vol/vol)**

| | $^1$H chemical shift[a] (ppm) | | $^{13}$C chemical shift[b] (ppm) | |
|---|---|---|---|---|
| Compound | Acyl-PG | Distearoyl-PG[c] | Acyl-PG | Distearoyl-PG[c] |
| Acyl moieties | | | | |
| C(n) | 0.83 | 0.84 | 14.25 | 14.18 |
| C(n-1) | 1.22, 1.28 | 1.25 | 23.01 | 23.02 |
| C(n-2) | 1.22, 1.28 | 1.25 | 32.28 | 32.31 |
| C, methylene | 1.22, 1.28 | 1.25 | 29.5–30.1 | 29.5–30.1 |
| C, allylic | 1.98 | | 27.53 | |
| C, doubly allylic | 2.83 | | 25.95 | |
| C, olefinic | 5.28 | | 128.27, 128.42, 130.33, 130.50 | |
| C3 | 1.55 | 1.57 | 25.23 | 25.32 |
| C2 | 2.28 | 2.27 | 34.43 | 34.52, 34.68 |
| C1 | | | 173.96, 174.39, 174.75 | 174.10, 174.45 |
| Glycerol moiety (backbone) | | | | |
| *sn*-1 | 4.37, 4.13 | 4.38, 4.16 | 62.91 | 63.10 |
| *sn*-2 | 5.19 | 5.20 | 70.83 | 71.10 |
| *sn*-3 | 3.94 | 3.95 | 63.87 | 64.03 |
| Glycerol moiety (second) | | | | |
| *sn*-1′ | 3.87, 3.81 | 3.88 | 67.08 | 66.97 |
| *sn*-2′ | 3.92 | 3.75 | 69.20 | 71.66 |
| sn-3′ | 4.09 | 3.59, 3.56 | 65.25 | 63.02 |

[a]The signal at δ 3.30 ppm from residual $CD_2HOD$ was used as shift reference.
[b]The signal at δ 49.0 ppm from $CD_3OD$ was used as shift reference.
[c]In chloroform-$d_1$/methanol-$d_4$ (1:1, vol/vol). Acyl-PG, 1,2-diacyl-*sn*-glycero-3-phospho-(3′-acyl)-1′-*sn*-glycerol; PG, 1,2-diacyl-*sn*-glycero-3-phospho-1′-*sn*-glycerol. For other abbreviation see Table 1.

plains the observed difference (approximately 0.3 ppm) in the proton shifts.

The effect on the proton shift at C-1 in the ethanolamine moiety is less pronounced (*ca*. 0.1 ppm upfield) whereas the $^{13}$C signal at C-1 is shifted *ca*. 2.6 ppm downfield, which is in accordance with empirical calculations.

Protons at the C-2 position in acyl groups are generally slightly more shielded in amides as compared to esters, which

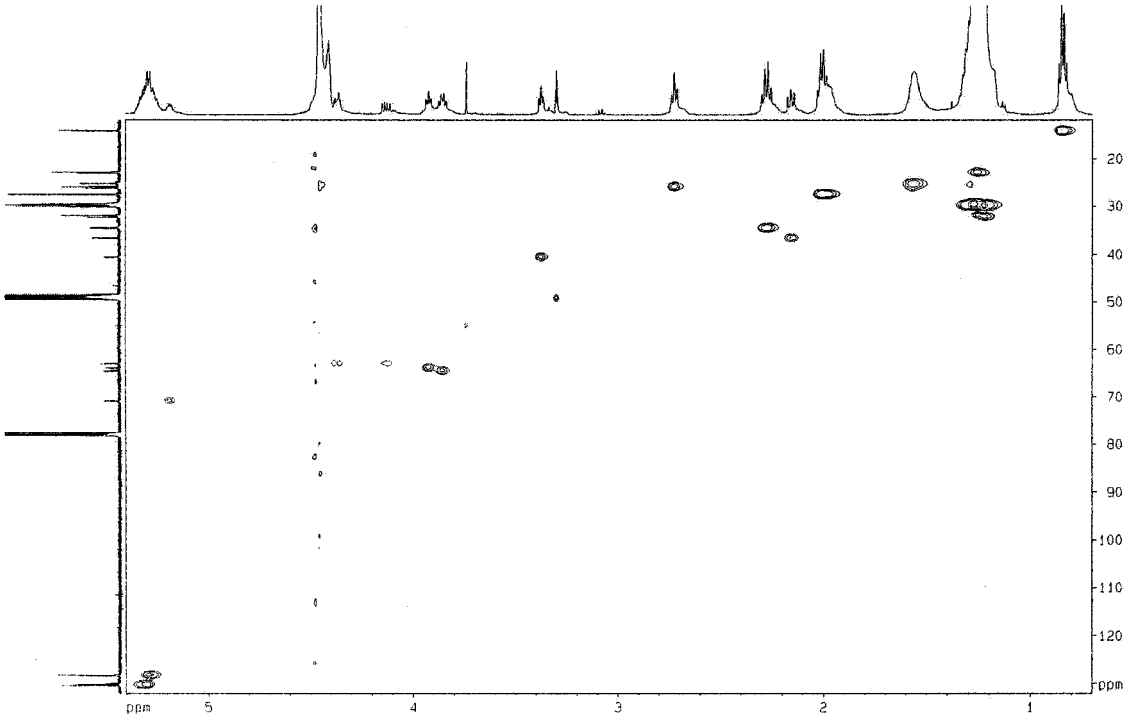


**FIG. 3.** $^1$H-Detected heteronuclear multiple quantum coherence spectroscopy spectrum of *N*-acyl-PE, showing cross peaks due to $^1J_{CH}$-couplings. For abbreviation see Figure 1.

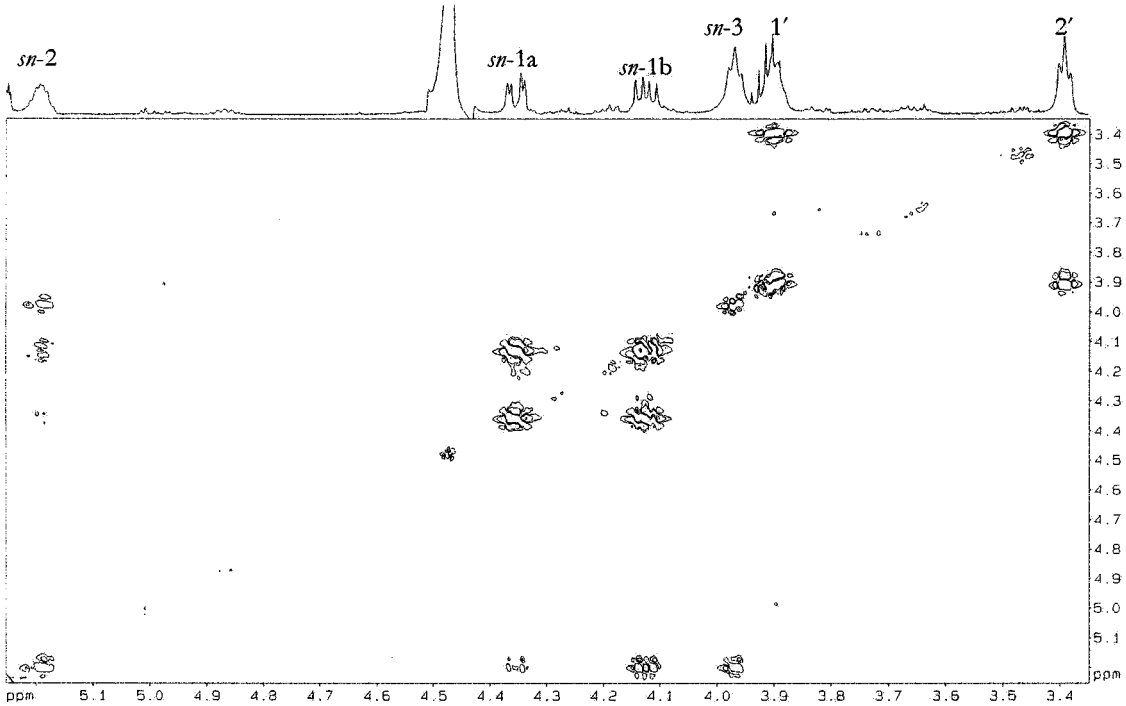


**FIG. 4.** Detail of the double quantum filtered correlation spectroscopy spectrum of *N*-acyl-PE, showing cross peaks due to $^{2}J_{HH}$ and $^{3}J_{HH}$ couplings in the glycerol moiety (*sn*-1a, *sn*-1b, *sn*-2, and *sn*-3) and the ethanolamine moiety (1′ and 2′). For abbreviation see Figure 1.

explains the signal at δ 2.17 ppm in the spectrum of **1** that does not appear in the spectrum of PE. The integral ratio between this signal and the C-2 signal from ester-bound acyl groups (δ 2.27 ppm) is also, as expected, approximately 1:2.

This evidence allows us to conclude that compound **1** is 1,2-diacyl-*sn*-glycero-3-phospho-(*N*-acyl)-1′-ethanolamine (*N*-acylphosphatidylethanolamine; *N*-acyl-PE).

For compound **2**, the TOCSY experiment allows the identification of one spin system for the proton signals at δ 5.19, 4.37, 4.13, and 3.94 ppm (2A) and another one at δ 4.09, 3.92, 3.87, and 3.81 ppm (2B). For the 2A spin system, similar reasoning as above implies that we have a diacylglycerophosphate moiety present.

The 2B spin system also resembles the glycerol moieties in the reference compounds with the exception of the signal at the *sn*-2 position, which has been shifted upfield. This effect could be explained by the substitution of a carboxylate for a hydroxyl group in this position, which leads to the conclusion that **2** is an acylphosphatidylglycerol (acyl-PG).

From these experiments it is not possible to determine the absolute stereochemistry of acyl-PG. However, it can be assumed that this compound is derived from the form of PG that is frequently found in nature, and has the phosphate ester bond at the *sn*-1′ position of the second glycerol. The extra acyl group is attached to the other primary hydroxyl group, which with the mentioned assumption implies the *sn*-3′ position. This leads to the conclusion that the compound is 1,2-diacyl-*sn*-glycero-3-phospho-(3′-acyl)-1′-*sn*-glycerol.

The conclusions regarding the structures of **1** and **2**, outlined above, can be confirmed by studying $^{13}C$-$^{31}P$ coupling constants and long-range $^{13}C$-$^{1}H$ couplings.

The signals from carbons situated close to the phosphate in the molecule appear as doublets due to the $^{13}C$-$^{31}P$ spin-spin coupling. For glycerophospholipids $^{3}J_{CP}$ is generally larger than $^{2}J_{CP}$, owing to the preferred *trans* conformation of the *sn*-3 C–O bond which gives a torsion angle of approximately 180° (41,42). In *N*-acyl-PE this is demonstrated by comparing the coupling constants for the *sn*-2 carbon in the glycerol (8 Hz) and C-2 in the ethanolamine moiety (7 Hz) with the coupling constants for the *sn*-3 carbon (5 Hz) and C-1 in the ethanolamine moiety (5 Hz).

For acyl-PG, doublets due to the $^{3}J_{CP}$ coupling can be seen in the signals for the *sn*-2 carbons in both glycerol moieties (8 and 7 Hz, respectively). These can be compared to the $^{2}J_{CP}$ coupling constants for the *sn*-3 carbon in the diacylglycerol moiety (5 Hz) and the *sn*-1′ carbon in the monoacylglycerol moiety (5 Hz).

Carbons without directly bonded protons can be assigned by utilizing an HMBC experiment in which $^{2}J_{CH}$ and $^{3}J_{CH}$ couplings give rise to cross peaks, provided that the experimental settings match the long-range coupling constants. This also holds true for couplings through ester bonds. In Figure 5, for instance, $^{3}J_{COCH}$ cross peaks can be observed for the three carbonyl carbons.

*HPLC–MS results*. The sample was first analyzed with both positive and negative electrospray (ES, ES–) MS. The total ion current (TIC) at the retention time of both acyl-PG and *N*-acyl-PE was one order of magnitude higher in ES– than in ES. In ES–, deprotonated molecules were obtained

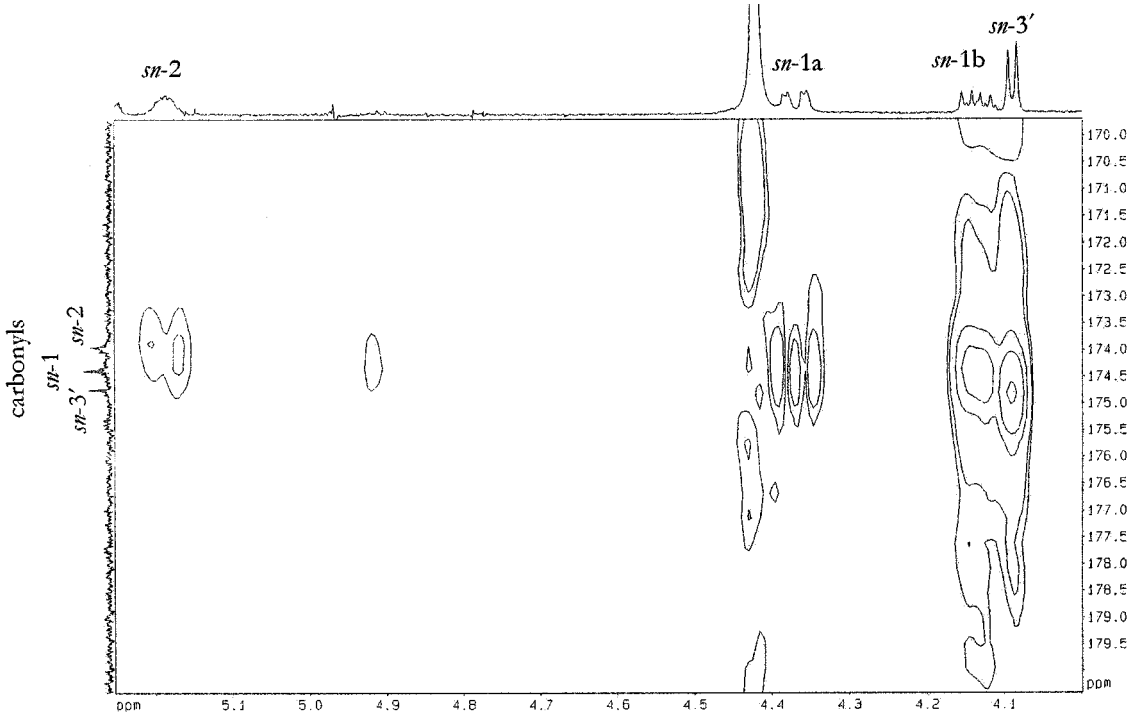


**FIG. 5.** Detail of the heteronuclear multiple-bond correlation spectroscopy spectrum of acyl-PG, showing cross peaks due to $^{3}J_{COCH}$ couplings between $^{13}C$ in carbonyls, over the ester bonds, to $^{1}H$ in the glycerol moieties. For abbreviation see Figure 2.

whereas in ES both sodium adducts ions and protonated molecules were obtained. Thus, since the TIC was higher in ES– and distinct deprotonated molecules (without adduct ions) were obtained, ES– was chosen for the MS-analysis of acyl-PG and *N*-acyl-PE in this work.

The detected ions of deprotonated *N*-acyl-PE (even-numbered integers) and acyl-PG (odd-numbered integers) in Figures 6 and 7 are summarized in Table 3 and 4. Some chromatographical overlapping is observed in the mass spectra in Figures 6 and 7, in that relatively polar molecular species of

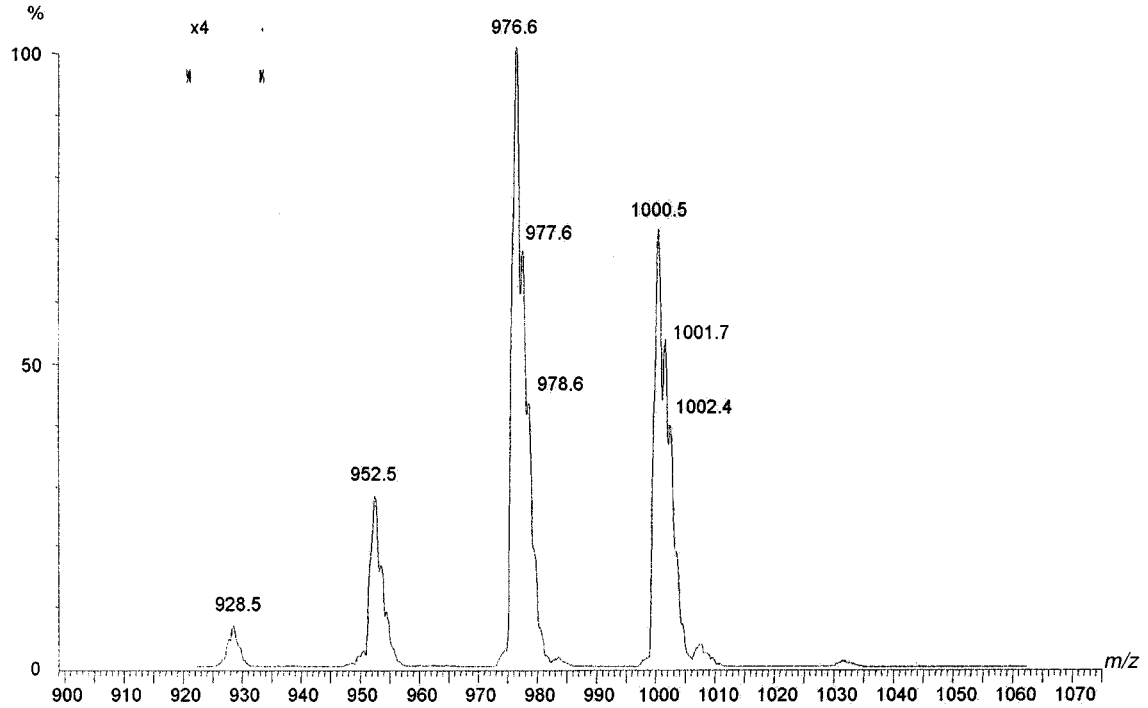


**FIG. 6.** Negative ion electrospray mass spectrum of deprotonated molecular ions of N-acyl-PE. *Note:* The interval 920–934 is amplified ×4.

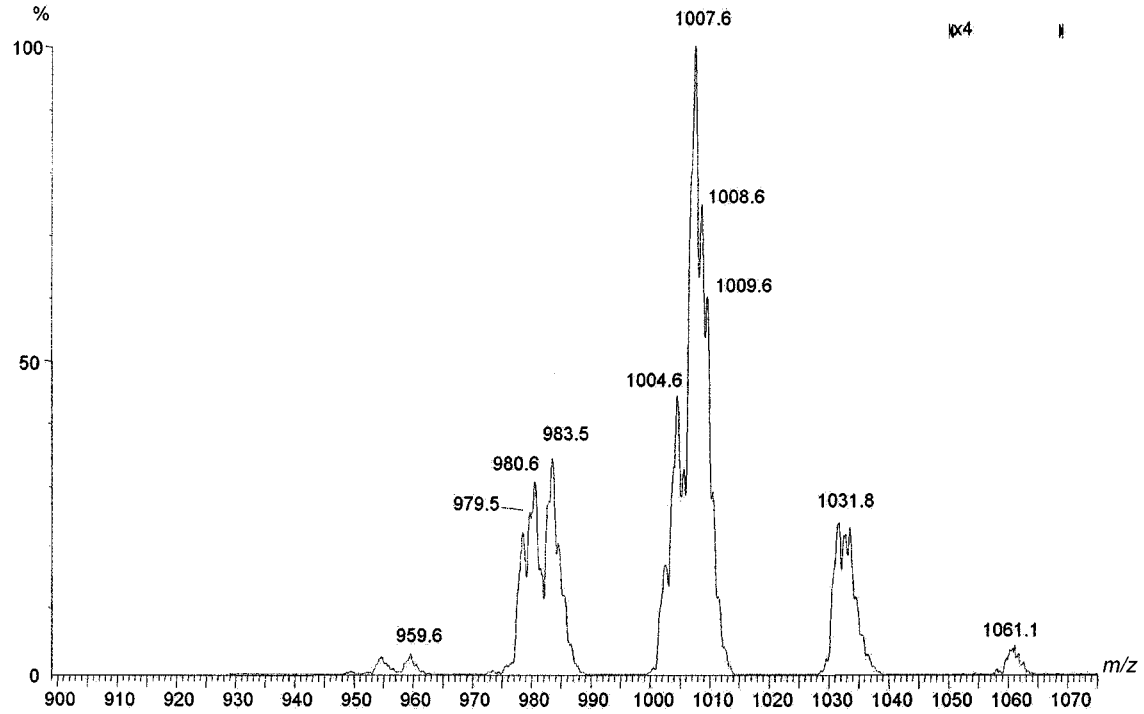


**FIG. 7.** Negative ion electrospray mass spectrum of deprotonated molecular ions of acyl-PG. *Note:* The interval 1050–1070 is amplified ×4. For abbreviation see Figure 2.

acyl-PG (e.g., ions with *m/z* 1001.7) elute with *N*-acyl-PE. Also, relatively nonpolar molecular species of *N*-acyl-PE (e.g., ions with *m/z* 980.6) elute with acyl-PG. Other deprotonated molecules and/or fatty acids than those described in Tables 3 and 4 may exist but were then present in amounts below the detection limit.

*HPLC–MS/MS results*. The deprotonated molecules in Table 3 and 4 were first detected by negative ion electrospray. Then, in product ion analysis (ES–, CID) with the deprotonated molecules as precursor ions, product ions representative of the fatty acids (i.e., carboxylate ions) of the deprotonated molecules were obtained (20) (Figs. 8,9). Also, as a second confirmation as to the origin of the product ions obtained, precursor ion analysis of the selected product ions was performed (Fig. 10). In this way, the fatty acid compositions of the chosen precursor ions (deprotonated molecular ions) were determined. For acyl-PG, the two major peaks in the product ion spectrum of precursor ions with *m/z* 1009.5 are carboxylate ions with *m/z* 255, 279, and 281 representing 16:0, 18:2, and 18:1 fatty acids, respectively (Fig. 9). This is in accor-

**TABLE 3**
**Assignments of Fatty Acid Residues in the Molecular Ions Obtained by Negative Ion Electrospray Mass Spectrometry of Deprotonated *N*-acyl-PE**

| Deprotonated molecule | Fatty acid | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 14:0 | 16:1 | 16:0 | 18:3 | 18:2 | 18:1 | 18:0 | 20:1 |
| 924 | X | | X | | X | | | |
| 948 | X | | | | X | | | |
| 950 | X | X | X | X | X | X | | |
| 952 | X | | X | | X | | | |
| 974 | | X | X | X | X | | | |
| 976 | | | X | | X | | | |
| 978 | | | X | | X | X | | |
| 980 | | | X | | X | X | X | |
| 1000 | | | | | X | | | |
| 1002 | | | | | X | X | | |
| 1004 | | | | X | X | X | X | |
| 1006[a] | | | X | X | X | | X | X |
| 1008 | | | X | | X | X | X | X |
| 1010[a] | | | | | | X | X | |
| 1030[a] | | | | | X | | | X |
| 1032 | | | | | X | X | | X |

[a]Not confirmed *via* product ion analysis (ES–, CID) [electrospray (ES–); collision-induced dissociation (CID)]. For other abbreviation see Table 1.

**TABLE 4**
**Assignments of Fatty Acid Residues in Molecular Ions Obtained by Negative Ion Electrospray Mass Spectrometry of Deprotonated Acyl-PG**

| Deprotonated molecule | Fatty acid | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 14:0 | 16:1 | 16:0 | 18:3 | 18:2 | 18:1 | 18:0 | 20:1 |
| 959 | | | X | | | | | |
| 983 | | | X | | X | | X | |
| 1005 | | | X | X | X | X | X | |
| 1007 | | | X | X | X | | X | |
| 1009 | | | X | | X | X | | |
| 1011[a] | | | X | | X | X | X | |
| 1031 | | | | | X | | | |
| 1033 | | | | | X | X | | |
| 1035[a] | | | | | X | X | X | |

[a]Not confirmed *via* product ion analysis (ES–, CID). For other abbreviations see Tables 2 and 3.

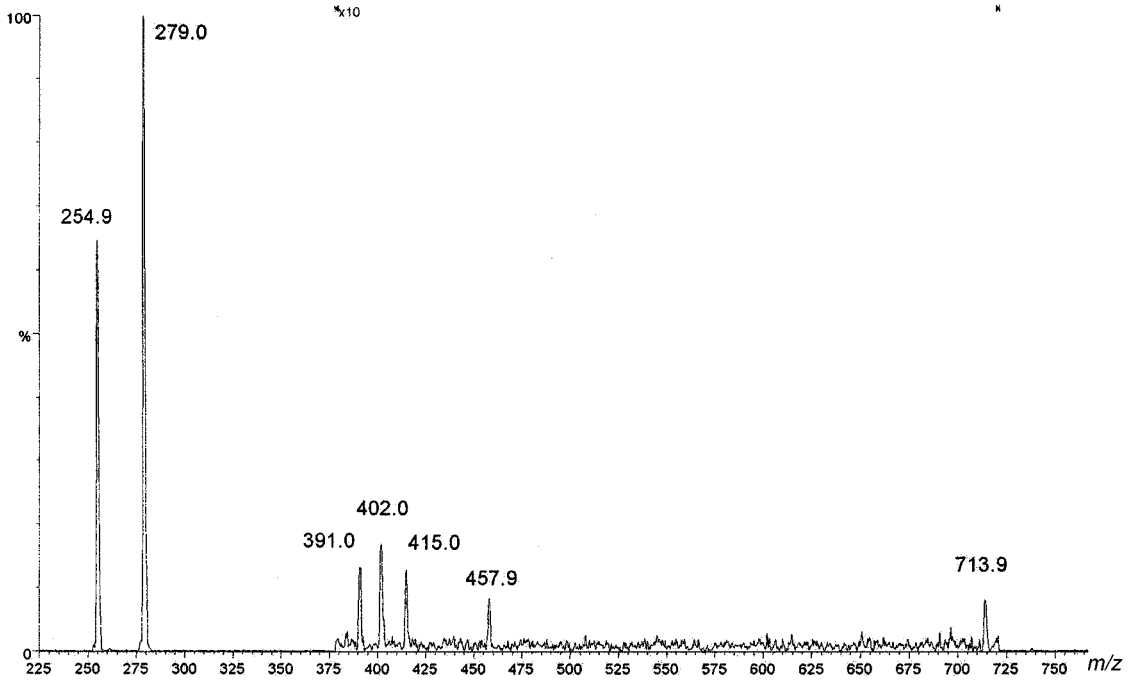


**FIG. 8.** Product ion mass spectra (ES–, CID) of deprotonated *N*-acyl-PE with *m/z* 976.5. *Note:* The interval 378–722 is amplified ×10. ES–, electrospray; CID, collision-induced dissociation; for other abbreviation see Figure 1.

dance with an acyl-PG molecular species containing 16:0/18:2/18:1 fatty acids.

Other fragments appear following two tentative cleavage routes (see Fig. 2). One route involves the loss of the monoacylglycerol part together with loss of an acyl part in either the *sn*-1 or *sn*-2 position of the diacylglycerol part, i.e., $(M - H–R^3COOCH_2CHOHCH_2–R^1COOH)^-$ or $(M - H–R^3COOCH_2CHOHCH_2–R^2COOH)^-$, (19). $R^1$, $R^2$, and $R^3$ refer to the hydrocarbon chains of the acyl groups at *sn*-1, *sn*-2, and *sn*-3′, respectively. For $R^1COO–$ = 16:0, $R^2COO–$ = 18:2, and $R^3COO–$ = 18:1, this gives product ions with *m/z* 391 and 415. With 16:0 at the *sn*-3′ position, ions with *m/z*

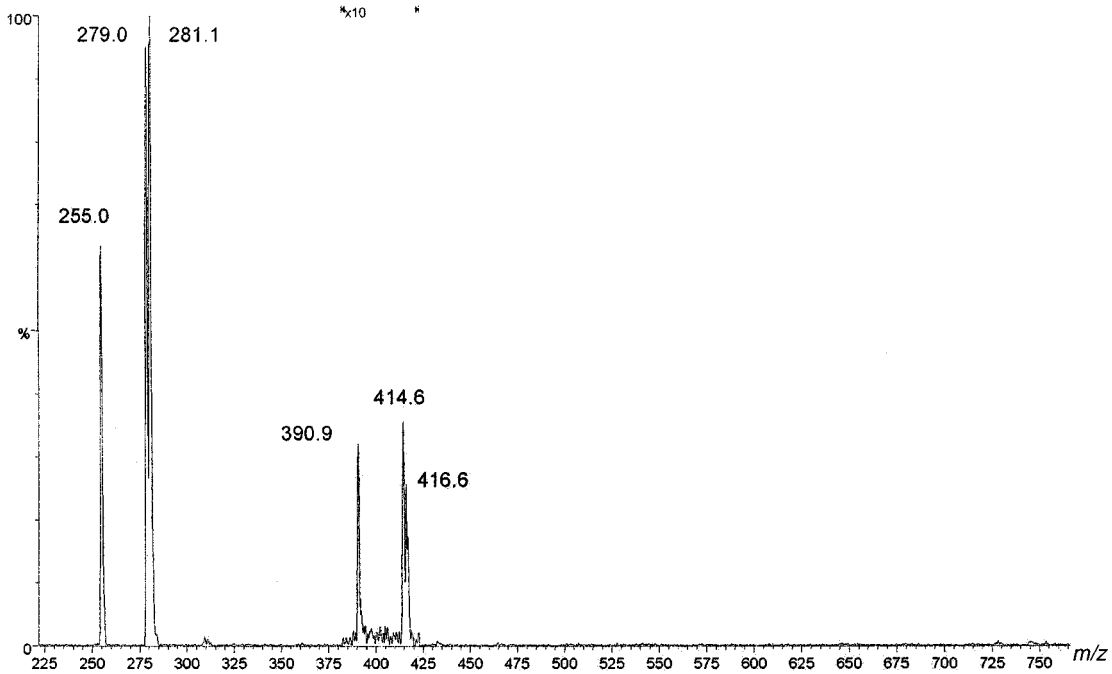


**FIG. 9.** Product ion mass spectra (ES–, CID) of deprotonated acyl-PG with *m/z* 1009.5. *Note:* The interval 380–425 is amplified ×10. For abbreviations see Figures 2 and 8.

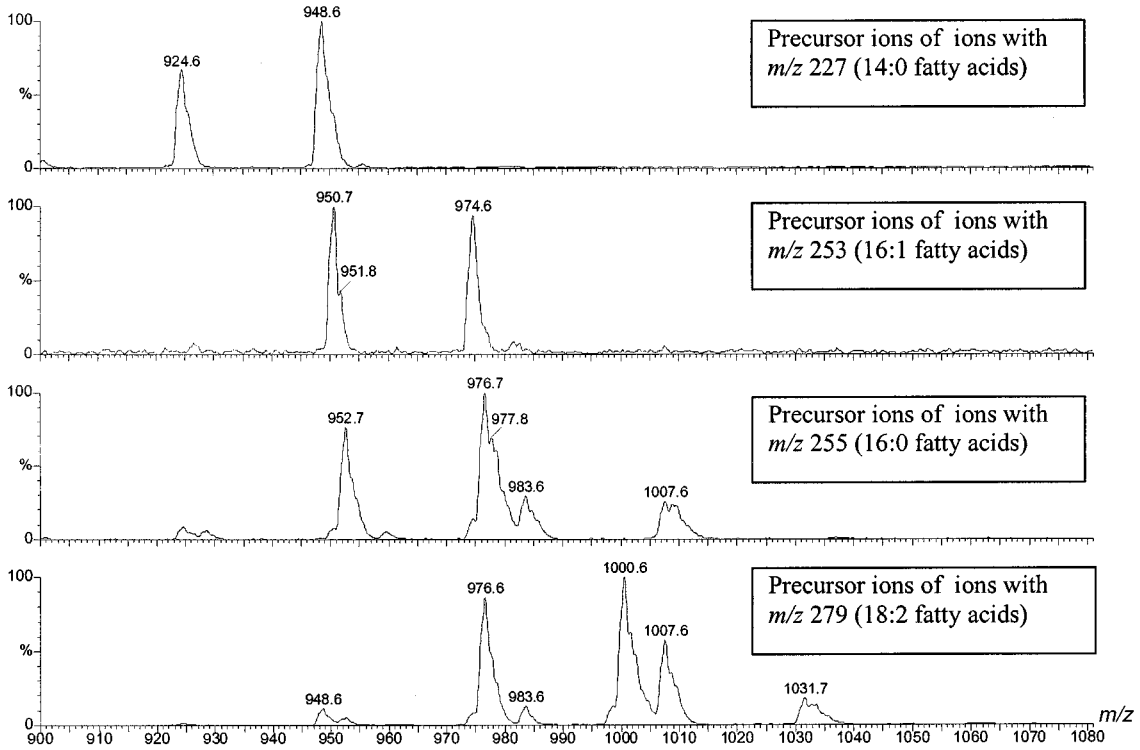


**FIG. 10.** Precursor ion mass spectra (ES–, CID) of ions with *m/z* 227, 253, 255, and 279, representing the fatty acids 14:0, 16:1, 16:0, and 18:2, respectively. For abbreviations see Figure 8.

415 and 417 are observed. Rearrangement of the fatty acids at different positions also give the above-mentioned ions. Another cleavage route involves the loss of the diacylglycerol part and, together with the loss of one molecule of water, the product ions $R^3COOCH_2CHOHCH_2O_3POH–H_2O^-$ are formed that, for $R^3COO–$ = 18:1, 18:2, or 16:0, give product ions with *m/z* 417, 415, or 391, respectively. In some spectra, ions with *m/z* 433 were observed, indicating the presence of *m/z* 415 without loss of water. It may thus be noted that ions with a certain *m/z* value may then be composed of either one or several of the fragment ions described above.

For *N*-acyl-PE, the base peaks in the product ion mass spectrum of precursor ions with *m/z* 976.6 are the carboxylate ions with *m/z* 255 and 279 (Fig. 8). This gives a theoretical fatty acid composition of 16:0/18:2/18:2-*N*-acyl-PE. The fragmentation pattern of *N*-acyl-PE follows similar routes (Fig. 1) as compared to acyl-PG. The cleavage involving loss of the $R^3CONHCH_2CH_2$ (in this case $R^3$ represents the hydrocarbon chain of the N-acyl group) part and one acyl group leads to analogous product ions as for acyl-PG, i.e., ions with *m/z* 415 and 391.

Similar fragments as for acyl-PG are also obtained *via* another route, i.e., involving loss of the diacylglycerol part giving fragment ions with *m/z* 402, $(R^3CONHCH_2CH_2O_3POH)^-$ with $R^3CONH–$ = 18:2. With $R^3CONH–$ = 16:0 ions with *m/z* 378 would be produced. However, these were not observed in the product ion spectrum, indicating the absence of 16:0 fatty acids in the amide group. Ions with *m/z* 458 corresponding to deprotonated molecules with neutral loss of one carboxylic acid and one alkyl ketene from the *sn*-1 and *sn*-2 positions, respectively $[M - H–R^1COOH)^-$ and $(M - H–R^2C=O)^-$, where $R^1COO–$ = 16:0 and $R^2COO–$ = 18:2 were also observed (19). No ions of this type corresponding to $R^1COO–$ = $R^2COO–$ = 18:2 were observed. In addition to these ions, high-mass ions with *m/z* 714 are observed, corresponding to deprotonated molecules with neutral loss of one 18:2-ketene from the *sn*-2 position. Saturated fatty acids are generally observed at the *sn*-1 position whereas unsaturated fatty acids are found at the *sn*-2 position (43). Also, several authors have described that *sn*-2 carboxylate ions appear at higher intensities than the *sn*-1 carboxylate ions following CID of deprotonated molecules (19). Thus, for the *N*-acyl-PE molecular species in Figure 8, 16:0 may assumed to be found at the *sn*-1 position with 18:2 fatty acids at *sn*-2 and at the ethanolamine part. For acyl-PG the same reasoning indicates that 16:0 fatty acids are generally found at the *sn*-1 position since the corresponding ions appear with lower intensity than those of 18:2 and 18:1 fatty acids. The fact that the 18:2/16:0 ratio is higher in the *N*-acyl-PE spectrum than in a spectrum of 16:0/18:2/18:2-acyl-PG (mass spectrum not shown) may be explained by the fact that, for acyl-PG, 18:2 fatty acids from the monoacylglycerol part also contribute to the intensity of the 18:2 peak. For *N*-acyl-PE, the 18:2 fatty acid in the ethanolamine part is amide-bound and does not form carboxylate ions. Thus, for the ions described above, in case of several different carboxylate ions in a product ion mass spectrum, the base peak may be assumed to represent the fatty acid at the *sn*-2 position.

**TABLE 5**
**Fragments for Identification of Double-Bond Positions in Gas Chromatography—Mass Spectrometry Spectra of Picolinyl Esters of *N*-Acyl-PE**

| | Double-bond position between carbon $k$ and $k + 1$ | |
|---|---|---|
| $k$-16:1 $[M^+] = 345$ | Double bond fragments $k - 1$ and $k + 1$ ($m/z$) | Allylic fragments $k + 2$ and $k + 3$ ($m/z$) |
| $k = 6$ | 206[a], 218 | —[b], 246 |
| $k = 9$ | 234, 260 | 274, 288 |
| $k = 11$ | 262, 288 | 302, 316 |

[a]This fragment represents cleavage of the double bond.
[b]No $k + 2$ fragment was found in this spectrum. For abbreviation see Table 1.

*GC–MS results*. GC–MS analysis of fatty acids as picolinyl ester derivatives showed several fatty acids containing double bonds in one or more positions. Of special interest were three isomers of 16:1 which previously had been found in fractions of total polar lipids from oats and identified as 11-16:1, 9-16:1, and 7-16:1 (Arnoldsson, K.C., unpublished data). LC–MS analysis of *N*-acyl-PE showed several molecular species containing 16:1 fatty acids, whereas acyl-PG had practically no 16:1-containing species.

Three 16:1 fatty acid isomers were identified from the mass spectrum of their picolinyl ester derivatives and shown to be 11-16:1, 9-16:1, and 6-16:1 (data in Table 5, sample spectrum in Fig. 11). As a result of the charge-retaining properties of the picolinyl ester derivative, the fragmentation occurs along the carbon chain from the methyl end giving fragments 14 Da apart. The position of the double bond was determined by the specific gap of 26 Da ($C_2H_2$) between carbon chain fragments, which represents cleavage on each side of the double bond. In the spectrum of 9-16:1 this gap occurs between $m/z$ 234 and 260 (Fig. 11). Often, there are also prominent losses due to abstraction of allylic hydrogens, resulting in formation of conjugated species (44), which gives fragments containing the double bond plus one or two carbons, represented by $m/z$ 274 and 288 in Figure 11. Deviations from this occur mostly when the double bond is near the terminal end of the carbon chain or near the carboxyl group as in 6-16:1 where the $k + 2$ fragment, $m/z$ 232, could not be found (see Table 5).

Two 16:1 isomers (11-16:1 and 9-16:1) found in total polar lipid fractions from oats were thus also found in the *N*-acyl-PE/acyl-PG fraction together with 6-16:1, which could not be seen in the total polar lipid fraction. The relative abundance of 16:1 isomers in the *N*-acyl-PE/acyl-PG fraction was estimated to 4:10:1 for the 6-, 9-, and 11-double bond position respectively.

In conclusion, the following types of experiments were run: (i) 1D and 2D NMR experiments, which provided the general structure features of the compounds, (ii) HPLC–ESMS, which gives the molecular weight of the species present, (iii) the subsequent HPLC–MS/MS analysis which, by giving fragmentation information on both product ions and their precursors, revealed the molecular species composition, and (iv) GC–EIMS of picolinyl esters, which was used to determine the double-bond positions in the acyl groups.

The combination of these techniques proved to be a versatile tool for structural elucidation. Thus, the structures of *N*-acyl-PE and acyl-PG were confirmed, and assignments of all $^1H$ and $^{13}C$ NMR signals could be done. From HPLC–MS/MS, 50 different molecular species of *N*-acyl-PE were determined with a molecular mass range from 924 to 1032 Da. For acyl-

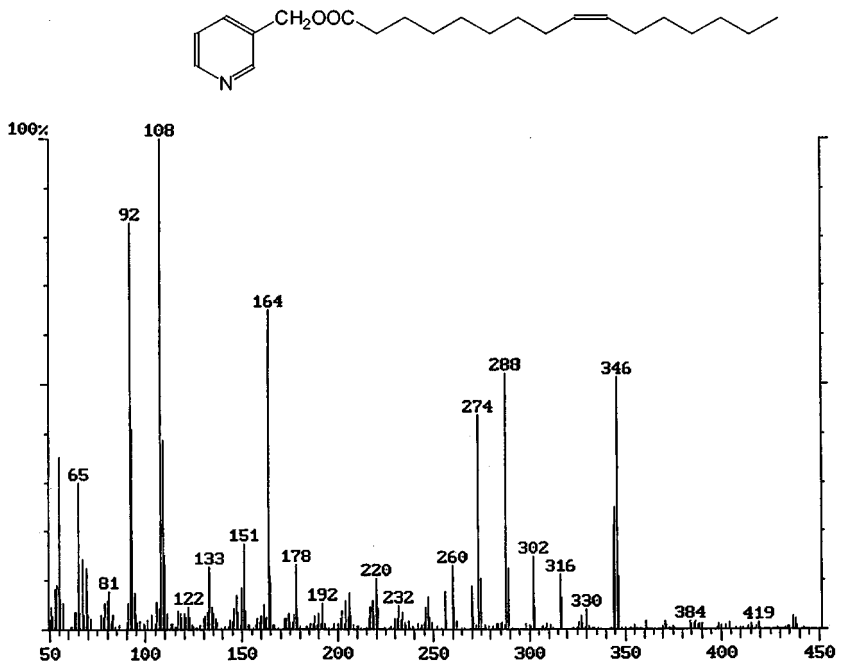


**FIG. 11.** Electron ionization mass spectrum of 9-16:1 picolinyl ester from N-acyl-PE. For abbreviation see Figure 1.

PG, 24 different molecular species were determined with a molecular mass range from 959 to 1035 Da. Three isomers of 16:1 fatty acids were found in *N*-acyl-PE, and the double-bond positions were determined by GC–EIMS to be 6, 9, and 11.

It was found that *N*-acyl-PE and acyl-PG together constitute as much as *ca.* 15% of the total phospholipids in oats. This is in agreement with early studies of the content of *N*-acyl-PE in oats (14).

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# Fatty Acid Composition of Lipids Present in Selected Lichenized Fungi: A Chemotyping Study

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**ABSTRACT:** The total-lipid composition of 21 lichens of the ascomycetous genera *Cladonia* (11) and *Cladina* (1) of the family Cladoniacea, *Cladia* (1), *Parmotrema* (3), *Ramalina* (2), *Leptogium* (1), *Cetraria* (1), and the basidiomycetous genus *Dictyonema* (1) was determined. Analyses of those of *Dictyonema glabratum* were carried out with a total extract and those obtained after successive extractions with various solvents. Each extract was partitioned between *n*-heptane/isopropanol and 1 M sulfuric acid, giving triglycerides (TG) in the upper phase. Extracts were methanolyzed and the resulting methyl esters were analyzed by gas chromatography–mass spectrometry. Methanolyzates of TG unexpectedly contained esters of 9-oxo-decanoic, 9-methyl-tetradecanoic, 6-methyl-tetradecanoic, 3-hydroxy-decanoic, nonanedioic, and decanedioic acids, as well as common fatty acids. Fatty acid methyl ester profiles from the lichens were submitted to cluster analysis, and the resulting dendogram showed a cluster consistent with *Cladonia* spp., suggesting an efficient aid to lichen taxonomy. The total carbohydrate content of each lipid extract was determined by a modified phenol-sulfuric acid method, which compensated for the presence of pigments.

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Many different types of lipids occur in lichens, including fatty acids, phospholipids, and glycolipids (1–6). A β-galactosyl ceramide and digalactosylglyceride were found in *Ramalina celastri* (5,6), the latter being isolated from a *Sticta* sp. after deacylation (7). Each component was analyzed in terms of carbohydrates and lipids. More recently, a family of galactolipids was obtained from the basidiomycetous lichen *Dictyonema glabratum* and completely characterized (8). The presence of fatty acids in fungi has long been observed (9–11) and summarized in a review by Wassef (12), who described the composition of lipids in many fungal species and their utilization for taxonomic purposes. This review proposed a separation into phycomycetes (>$C_{18:3}$, $C_{20:0}$, and $C_{22:0}$), ascomycetes, and deuteromycetes (fungi imperfecti), which contained little or no polyenoic acids and >$C_{16:1}$, and basidiomycetes with α- rather than γ-linoleic acid: all groups contained $C_{16:0}$, $C_{18:1}$, and $C_{18:2}$ as major fatty acids.

Investigations that involve cladistic analysis of fatty acids as an aid in the taxonomy of fungi gave rise to positive results for correlation of fungal species (13–15). The most important class of fungal lipids is the triglycerides (TG), which represent the major component found in the lipid extracts of fungi. These are mainly esterified by unsaturated fatty acids such as oleic and linoleic at the HO-2 position of the glycerol moiety and at HO-1 and HO-3 by saturated fatty acids. The presence of TG in terms of fatty acid composition in lichens has not been described to date.

There is a complex relationship between lichen phytobionts and mycobionts, but as the latter comprise ~90% of the total biomass (16), the fatty acids of total lipid might be expected to be those of the mycobiont. We now study the composition of the lipid extracts of 21 selected lichens from various genera, having ascomycetes and a basidiomycete as mycobionts, in terms of carbohydrates, fatty acids that arose from TG, and total fatty acid, which could serve as taxonomic aid, including a modification of the phenol-sulfuric acid method (17), modified to compensate for the presence of pigments.

## EXPERIMENTAL PROCEDURES

*Lichens.* Lichens of the genus *Cladonia (C. clathrata*, *C. imperialis, C. signata, C. furcata*) and *Cladia aggregata* were collected during May 1993 in the Serra da Mantiqueira, Itamonte, State of Minas Gerais, Brazil, and *C. connexa, C. crispatula*, *C. ibitipocae*, *C. miniata*, *C. penicillata*, *C. salmonea*, and *C. substellata* in the Serra da Ibitipoca, Lima Duarte, State of Minas Gerais, 1994. *Cladina rangiferina* was collected in Uusimaa, Finland, August 1998. *Dictyonema glabratum* was harvested September 1998 from an embankment close to the 47-km sign of the National Highway (BR) 277, at an altitude of 900 m, in the proximity of Curitiba, State of Paraná, Brazil. *Ramalina usnea, R. celastri,* and *Leptogium phyllocarpum* were collected in 1998, in the Serra da Graciosa, PR, Brazil. *Cetraria islandica* (Iceland moss) was obtained from S.S. Penick and Co. (New York, NY; material obtained in 1984). *Parmotrema delicatum, P. mantiqueirense,* and *P. shindler* were collected in 1998, Lapa, State of Paraná, Brazil. Brazilian lichens were identified by Prof. Marcelo Marcelli and the Finnish one by Dr. Teuvo

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Abbreviations: B, blank; EI, electron ionization; FAME, fatty acid methyl esters; GC–MS, gas chromatography–mass spectroscopy; OD, optical density; S, standard; TFA, trifluoroacetic acid; TG, triglycerides; TLC, thin-layer chromatography.

Ahti. All lichens were each cleaned, dried, and powdered prior to the lipid extraction procedures.

*General experimental procedures.* Lipid extracts obtained from each sample were obtained by three successive extractions with 10 vol/wt of refluxing $CHCl_3$/MeOH (2:1, vol/vol) and (1:1, vol/vol), for 2 h. *Dictyonema glabratum* (15 g) was similarly extracted in order to compare yields with: $Me_2CO$, $CHCl_3$/MeOH (2:1, vol/vol), $CHCl_3$/MeOH (1:1, vol/vol), EtOH/$H_2O$ (9:1, vol/vol), and $CHCl_3$/MeOH/$H_2O$ (7:10:3, by vol), except that the $Me_2CO$ extraction was carried out at room temperature. All extracts were evaporated at <40°C under reduced pressure, dried, and stored in sealed tubes maintained below −10°C. Thin-layer chromatography (TLC) was performed on silica gel G plates from Merck (Darmstadt, Germany); solvent: $CHCl_3$/MeOH/$H_2O$ (65:25:4, by vol) and isopropyl ether/HOAc (96:4, vol/vol).

In order to obtain TG, each total lipid extract was partitioned between *n*-heptane/isopropanol/1 M $H_2SO_4$ (33.6:59.1:7.3, by vol) following vortex homogenization for 1 min. The upper organic phase, which contained TG, was evaporated at <40°C under reduced pressure and stored in a sealed tube below −10°C: the lower one was discarded. TG were developed with one-dimensional, two-step TLC using (i) isopropyl ether/HOAc, 96:4, vol/vol) and (ii) *n*-heptane/$Et_2O$/HOAc, 90:10:1, by vol) and detected with a 40% aqueous $H_2SO_4$ spray with heating at 120°C (18). The TG samples were estimated by colorimetric determination of the glycerol component. They were dissolved in $CHCl_3$ (5 mL), and an aliquot (0.1 mL) was mixed with of 0.1 mL of 7 M KOH in EtOH/$H_2O$ (3:1, vol/vol). The mixture was maintained at 56°C for 15 min, after which was added 0.5 mL of water, followed by periodic acid/0.35 M $H_2SO_4$ (0.35 mL), which resulted in the formation of formaldehyde. For estimation of liberated formaldehyde, the solution was treated with 3.0 mL of the acetylacetone/$NH_4OAc$/sodium arsenate/water reagent (4 mL), and the mixture was maintained at 56°C for 5 min, resulting in an yellow complex that absorbed at 410 nm.

In order to analyze the fatty acid composition of the lichens, each total lipid extract and TG (5 mg) was methanolyzed by refluxing in 3% HCl in MeOH for 2 h (19). The resulting fatty acid methyl esters (FAME) were extracted from water with $CHCl_3$, and these were analyzed by their gas chromatography–mass spectrometry (GC–MS), $R_t$ values, and electron ionization–mass spectrometry (EI–MS) profiles and compared with those of standards (Sigma products for lipids) on a DB-23 capillary column (30 m × 0.25 mm i.d. and 60 m × 0.25 mm i.d.), programmed from 50 to 180°C and 200°C (40°C·min$^{-1}$), then held. The sugar components were examined by GC–MS as alditol acetates, on a DB-225 capillary column (30 m × 0.25 mm i.d), programmed from 50 to 220°C (40°C·min$^{-1}$), then held.

*Colorimetric estimation of sugars in the pigment-containing lipid extracts using a modified phenol-sulfuric acid method.* Lipid extracts were added to a tube (1 to 3 mg) containing M trifluoroacetic acid (TFA) (1 mL) and hydrolyzed at 100°C for 8 h, and to these mixtures were added $CHCl_3$ (1 mL) and the extracted lipids discarded. An aliquot (0.5 mL) of components soluble in the acid solution was added to a tube containing 0.5 mL of 5% aqueous PhOH-$H_2SO_4$ (2.5 mL). The optical density (OD) at 490 nm was determined against a blank (B) containing 0.5 m; of M TFA, plus reagent. Another tube (S) containing 24 µg of galactose and 2 mg of lipid extract (from *C. islandica*) was submitted to the above procedure as a control and gave an OD of 0.702. For calibration, standards used were of 10, 30, 50, and 70 µg of galactose; 35 µg of galactose gave an OD of 0.608.

*Aqueous acid hydrolysis of lipid extracts.* Hydrolyses were performed with 1 M TFA at 100°C for 8 h (8), followed by evaporation to dryness, and the residue was partitioned between $CHCl_3$ and water. The aqueous phase was reduced with $NaBH_4$ and the product acetylated with $Ac_2O$/NaOAc at 120°C for 1 h. The $Ac_2O$ was destroyed with ice water, and the resulting alditol acetates were extracted with $CHCl_3$ (20) and analyzed by GC–MS, as described above.

*Numerical analysis of FAME data.* Total lipids were extracted, and the FAME data from 21 lichen species (GC–MS) were submitted to cluster analysis. Dendograms were constructed using the unweighted pair group with mean average from the pairwise Euclidean distance units. The analysis was performed using an NTSys program (Exeter Software, Setauket, NY).

## RESULTS

*Relationship between organic solvent extracts with yields and fatty acid composition of the lichen species after successive extractions*. *Dictyonema glabratum* was extracted separately with the following solvents in order to compare the yields of extracts and the fatty acid composition of each one: $Me_2CO$ (ext. A), $CHCl_3$/MeOH (2:1, vol/vol, ext. B), $CHCl_3$/MeOH (1:1, vol/vol, ext. C), EtOH/$H_2O$ (9:1, vol/vol, ext. D), and $CHCl_3$/MeOH/$H_2O$ (7:10:3, by vol, ext. E), as shown in Table 1. The principal methyl esters obtained after methanolysis of the total extracts were from the saturated fatty acids, 14:0, 16:0, and 18:0, but the longer-chain 20:0, 22:0, and 24:0 derivatives were also observed. Odd-number esters were observed in smaller proportions, 17:0 being found in 13 lichens. Unsaturated fatty acids were present, the most abundant and common being 18:1 (Table 2).

Eleven species of the genus *Cladonia* showed similarities in their fatty acid composition in terms of the chain length, but with some percentage variation. Observed were 16:0, 18:0, 17:0, 23:0, and 24:0 in six lichens, 22:0 in eight, 14:0 and 18:1 in nine, and 20:0 in ten. The presence of other fatty acids is shown in Table 2. *Cladina rangiferina* components were compared with those of *Cladonia* spp., since it also belongs to the family Cladoniaceae. The fatty acid composition was quite similar, showing 14:0, 16:0, 17:0, 18:0, 18:1, 20:0, 22:0, and 23:0, the only difference being the proportion of each one. The fatty acid composition agrees with the data obtained by Dembitsky *et al*. (3). *Parmotrema delicatum, P. mantiqueirense,* and *P. shindler* did not give rise to long-chain

**TABLE 1**
**Fatty Acid Composition Arising from Different Types of Extraction (Ext) of *Dictyonema glabratum***

| Fatty acid | $R_t$[a] | Ext (A) | Ext (B) | Ext (C) | Ext (D) | Ext (E) |
|---|---|---|---|---|---|---|
| | | Percentage yields of the different types of extraction | | | | |
| | | 0.6 | 2.7 | 4.9 | 4.3 | 6.3 |
| 10:0 (caproic) | 5.49 | 5.3 | 1.6 | — | — | — |
| 12:0 (lauric) | 7.00 | 9.4 | 3.0 | 6.3 | 5.0 | 5.1 |
| 14:0 (myristic) | 8.32 | 8.45 | 3.75 | 6.3 | 4.7 | 5.8 |
| 16:0 (palmitic) | 10.47 | 61.5 | 55.0 | 74.0 | 50.2 | 56.0 |
| 16:2 (hexadecadienoic) | 11.13 | — | — | — | 2.7 | — |
| 17:0 (heptadecanoic) | 12.16 | — | 1.2 | — | — | — |
| 18:0 (stearic) | 14.01 | 15.5 | 8.2 | 9.4 | 9.0 | 7.9 |
| 18:1 (oleic) | 14.29 | — | 8.0 | — | 10 | — |
| 18:2 (linoleic) | 15.29 | — | 19.1 | 4.0 | 18.1 | 19.2 |
| 18:3 (linolenic) | 18.13 | — | 0.3 | — | — | — |
| 22:0 (behenic) | 25.20 | — | — | — | — | 6.0 |
| 24:0 (lignoceric) | 28.27 | — | — | — | 0.3 | — |

[a]Retention time ($R_t$) in minutes. Ext (A) $Me_2CO$, Ext (B) $CHCl_3$/MeOH (2:1), Ext (C) $CHCl_3$/MeOH (1:1, vol/vol), Ext (D) EtOH/$H_2O$ (9:1, vol/vol), and Ext (E) $CHCl_3$/MeOH/$H_2O$ (7:10:3, by vol).

or odd-numbered fatty acid esters. The acids 16:0 and 18:0 were observed in all of the lichens examined as were unsaturated fatty acids 18:1 and 18:3. The fatty acid composition of the basidiolichen, *D. glabratum* (21), showed the presence of short-chain fatty acids such as 10:0, 12:0, and 16:2.

*Fatty acid composition of TAG extracts.* TG were extracted with a solution of *n*-heptane/isopropanol/1 M sulfuric acid (33.6:59.1:7.3, by vol) as described above, and the resulting upper phase contained TG, whose yields ranged from 11.4 to 37.3%: its respective fatty acid composition is shown in Table 3. The fatty acids were obtained *via* methanolysis and analyzed by GC–MS. The main saturated FAME were: 14:0, 16:0, and 18:0, along with smaller proportions of 20:0, 22:0, 22:1, 22:2, 23:0, and 24:0. Detected in lower quantities were the odd-number fatty acids, 15:0 and 17:0, which were found in 13 of 15 lichens. The unsaturated fatty acids 16:1, 18:1, 18:2, 18:3, and 22:2 were also observed (Table 3).

Unusual lipids such as dicarboxylic fatty acids, a long-chain alcohol, branched- and hydroxy-fatty acids, and keto-fatty acids were unexpectedly detected (Table 4). The resulting GC–MS profile of each sample was analyzed on the basis of a EI–MS library, incorporating the NIST/NBS Library of Masslinks Library Service, to determine chemical structures. The main mass fragments are shown in Table 4. The most frequently encountered unusual FAME was nonanedioic acid methyl ester, this compound being present in 15 species. Its main fragments (*m/z*) and intensities (%) were, respectively, 185 (27), 152 (70), 143 (30), 111 (45), 83 (63), 74 (100), 69 (47), 55 (81), 43 and 41 (40), and 40 (2), although its ion peak did not appear. Also present were esters of 9-oxo-decanoic and 9-oxo-nonanoic. The latter showed fragments with *m/z*: 155 (16), 143 (35), 115 (7), 111 (33), 87 (53), 74 (100), 55 (47), 43 and 41 (35), and 40 (2).

*Dendograms of fatty acid profiles: evaluation of lichen relationships.* The FAME profiles of the 21 lichen species were submitted to cluster analysis. Twenty different FAME, varying in chain length from 10 to 24 carbons (saturated or unsaturated), were identified (Table 2). The resulting dendogram resolved species most consistent with their taxonomic position. Ten *Cladonia* species are grouped in two related subclusters (I and II, Fig. 1). However, one *Cladonia* sp., *C. miniata*, was distantly related, clustering with *P. mantiqueirense. Cladina rangiferina* clustered within subcluster II and, surprisingly *P. delicatum* was clustered in this same group. The dendrogram was reinforced by a cophenetic coefficient of 0.89. This value gives an idea of the consistency of the dendrogram, when related to the distance matrix.

*Modification of the PhOH-$H_2SO_4$ method to estimate carbohydrates in the presence of pigments and monosaccharide composition.* The total carbohydrate content of the lipid extracts, obtained from 21 lichen species, was initially determined using a conventional PhOH-$H_2SO_4$ procedure (17), using 1000 µg to 50 µg of each extract. All the values obtained for the samples obeyed the Lambert-Beer's law over a range of 0–80 µg hexose. Good results were obtained with total hydrolysis using 1 M TFA at 100°C. This procedure produced free sugars, the lipids being removed by partition between chloroform and an aqueous solution. Another control of the method (S) was carried out using 24 µg of galactose (OD of 0.417) and 2 mg of the lipid extract from *C. islandica*; this control containing 40.4 µg of total sugar, giving an OD of 0.702. The total carbohydrate content of 2 mg for *C. islandica* was 16.92 µg corresponding to an OD of 0.294, such controls confirming the validity of the modified method. The standard curve and the total sugar yields are represented in Figure 2 and Table 5, respectively.

The yields in terms of total carbohydrates varied from 0.2 to 3.3% for all lichens of the genus *Cladonia*, whereas those of the genus *Parmotrema* varied from 0.9 to 1.9% and those of *Ramalina* from 0.8 to 1.3%. Values for other species are presented in Table 5.

The sugar compositions were determined as derived alditol

**TABLE 2**
**Fatty Acid Composition of Total Lipid Extracts, Obtained from 21 Lichens**

| | | Fatty acid | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lichen | Yield (%) | 10:0 | 12:0 | 14:0 | 15:0 | 16:0 | 16:1 | 16:2 | 17:0 | 17:1 | 17:2 | 18:0 | 18:1 | 18:2 | 18:3 | 19:0 | 20:0 | 22:0 | 22:1 | 23:0 | 24:0 |
| *Cetraria islandica* | 6.5 | — | — | 3.15 | 2.5 | 67.6 | — | — | 4.4 | — | — | 17.7 | — | — | — | — | 3.6 | — | — | — | 1.1 |
| *Cladonia clathrata* | 3.5 | — | — | — | — | 23.3 | — | — | — | — | — | 45.0 | 16.5 | 2.4 | — | — | 1.3 | 5.4 | 1.4 | — | 4.8 |
| *C. connexa* | 3.6 | — | — | 1.5 | — | 50.6 | — | — | — | 0.9 | — | 30.1 | 4.8 | 3.5 | — | — | 3.2 | — | — | 4.0 | 2.2 |
| *C. crispatula* | 3.9 | — | — | 3.1 | — | 46.1 | — | — | — | — | 2.2 | 19.1 | 20.8 | — | — | — | 2.8 | 3.6 | — | — | 2.0 |
| *C. furcata* | 4.2 | — | 1.2 | 1.2 | — | 43.5 | — | — | 2.8 | — | — | 39.8 | 7.2 | — | — | — | 2.3 | — | — | 1.1 | 1.0 |
| *C. ibitipocae* | 3.4 | — | — | 1.2 | — | 43.8 | — | — | — | — | — | 26.6 | 14.0 | — | — | — | 5,6 | 4.6 | — | 4.1 | — |
| *C. imperialis* | 2.9 | — | — | 0.8 | — | 31.8 | — | — | 2.6 | — | — | 29.4 | 16.9 | — | — | 11.3 | 1.1 | 0.7 | — | 2.5 | 2.4 |
| *C. miniata* | 4.6 | — | — | 48.7 | — | 31.6 | — | — | — | — | — | 19.6 | — | — | — | — | — | — | — | — | — |
| *C. penicillata* | 3.2 | — | — | 2.4 | — | 42.5 | — | — | 4.2 | — | — | 45.4 | 0.9 | — | — | — | 1.4 | 1.9 | — | — | 1.2 |
| *C. salmonea* | 7.9 | — | — | — | — | 40.7 | — | — | 2.3 | — | — | 26.2 | 22.3 | — | — | — | 3.6 | 1.7 | — | 3.2 | — |
| *C. signata* | 2.7 | — | — | 0.5 | — | 43.6 | — | — | 3.7 | — | — | 37.5 | 8.3 | — | — | — | 2.8 | 2.6 | — | 1.0 | — |
| *C. substellata* | 6.4 | — | — | 2.9 | — | 15.3 | — | — | 4.0 | — | — | 23.2 | — | — | — | — | 0.4 | 3.2 | — | — | — |
| *Cladia aggregata* | 5.2 | — | — | — | — | 28.4 | — | — | 12.2 | — | — | 15.5 | 10.3 | 33.5 | — | — | — | — | — | — | — |
| *Cladina rangiferina* | 6.3 | — | — | 0.3 | — | 33.3 | — | — | 3.1 | — | — | 19.0 | 21.2 | 3.0 | — | — | 5.8 | 11.5 | — | 2.7 | — |
| *Dictyonema glabratum* | 4.9 | 1.6 | 3.0 | 3.2 | — | 49.2 | — | 2.1 | 0.8 | — | — | 8.2 | 7.9 | 18.8 | 0.9 | — | 4.7 | — | — | — | 0.3 |
| *Leptogium phyllocarpum* | 10.6 | — | — | 13.3 | — | 15.2 | — | — | 7.7 | — | — | 21.0 | — | — | — | — | — | 19.0 | — | — | 23.7 |
| *Parmotrema delicatum* | 13.2 | — | — | — | — | 35.3 | — | — | — | — | — | 30.2 | 34.5 | — | — | — | — | — | — | — | — |
| *P. mantiqueirense* | 8.1 | — | — | 35.9 | — | 19.3 | — | — | — | — | — | 44.7 | — | — | — | — | — | — | — | — | — |
| *P. shindler* | 12.2 | — | — | 7.6 | — | 18.1 | — | — | — | — | — | 16.5 | 12.8 | — | 44.9 | — | — | — | — | — | — |
| *Ramalina celastri* | 3.2 | — | — | 0.8 | — | 18.9 | 2.3 | — | 0.8 | — | — | 7.2 | 14.7 | 36.2 | 19.0 | — | — | — | — | — | — |
| *R. usnea* | 3.2 | — | — | — | 1.3 | 65.2 | | — | 4.3 | — | — | 29.1 | — | — | — | — | — | — | — | — | — |

**TABLE 3**
**Fatty Acid Composition of the Lipids Present in the *n*-Heptane-Isopropanol Phase from 21 Lichens[a]**

| | Triglycerides | Fatty acid | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lichen | Yield (%) | 12:0 | 13:0 | 14:0 | 15:0 | 16:0 | 16:1 | 17:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 21:0 | 22:0 | 22:2 | 23:0 | 24:0 |
| *C. islandica* | 14.0 | 2.0 | 1.0 | 7.9 | 4.0 | 62.3 | — | 2.0 | 17.8 | — | — | — | 3.0 | — | — | — | — | — |
| *C. clathrata* | 23.7 | — | — | 3.4 | 5.3 | 49.5 | — | 2.7 | 35.5 | — | — | — | 3.4 | — | 2.8 | — | — | — |
| *C. connexa* | 22.5 | — | — | 10.7 | — | 61.5 | — | — | 27.7 | — | — | — | — | — | — | — | — | — |
| *C. crispatula* | 21.5 | — | — | 2.3 | 5.0 | 60.5 | — | 3.6 | 24.2 | — | — | — | 2.1 | 0.8 | 1.5 | — | — | — |
| *C. furcata* | 37.3 | — | — | — | — | 66.2 | — | — | 33.8 | — | — | — | — | — | — | — | — | — |
| *C. ibitipocae* | 26.6 | — | — | 3.6 | 6.0 | 60.0 | — | 3.6 | 23.4 | — | — | — | 1.8 | — | 1.5 | — | — | — |
| *C. imperialis* | 35.0 | — | — | 8.2 | 4.9 | 41.1 | — | 5.2 | 38.6 | — | — | — | — | — | 1.9 | — | — | — |
| *C. miniata* | 29.8 | — | — | 5.0 | 8.7 | 53.4 | — | 4.2 | 25.3 | — | — | — | 1.7 | | 1.7 | — | — | — |
| *C. penicillata* | 37.0 | — | — | 3.5 | 2.7 | 57.9 | — | 2.9 | 26.1 | — | — | — | 2.9 | — | 3.9 | — | — | — |
| *C. salmonea* | 26.4 | — | — | 2.9 | 3.0 | 60.5 | — | 2.8 | 27.9 | — | — | — | 1.6 | | 1.2 | — | — | — |
| *C. signata* | 32.0 | — | — | 1.8 | 1.7 | 49.7 | — | 4.6 | 35.2 | — | — | — | 3.0 | — | 1.7 | — | 1.3 | 0.8 |
| *C. substellata* | 26.6 | — | — | 4.5 | 4.5 | 39.2 | — | 1.8 | 51.8 | — | — | — | — | — | — | — | — | — |
| *C. aggregata* | 22.1 | — | — | — | — | 33.9 | — | 3.2 | 19.0 | 11.6 | 29.7 | — | — | — | — | 2.8 | — | — |
| *C. rangiferina* | 29.3 | — | — | 2.2 | 1.6 | 46.6 | — | 2.4 | 22.4 | — | — | — | 6.0 | — | 14.2 | — | 2.2 | 2.4 |
| *D. glabratum* | 21.6 | — | — | 2.1 | 3.3 | 75.0 | — | 3.6 | 16.1 | — | — | — | — | — | — | — | — | — |
| *L. phyllocarpum* | 20.8 | — | — | — | — | 100 | — | — | — | — | — | — | — | — | — | — | — | — |
| *P. delicatum* | 11.9 | — | — | — | — | 69.4 | — | — | 30.5 | — | — | — | — | — | — | — | — | — |
| *P. mantiqueirense* | 27.3 | — | — | — | — | 74.6 | — | — | 25.3 | — | — | — | — | — | — | — | — | — |
| *P. shindler* | 16.0 | — | — | 2.1 | — | 67.0 | — | — | 30.8 | — | — | — | — | — | — | — | — | — |
| *R. celastri* | 18.3 | — | — | — | — | 17.3 | 3.0 | 1.0 | 8.1 | 18.6 | 38.1 | 13.7 | — | — | — | — | — | — |
| *R. usnea* | 11.4 | — | — | 3.1 | 5.0 | 51.8 | — | 5.2 | 30.1 | — | — | — | 1.3 | — | 2.0 | — | 1.3 | — |

[a]See Table 2 for lichen abbreviations.

**TABLE 4**
**Unusual Lipids Present in the *n*-Heptane-*iso*propanol Phase from 19 Lichens**

| Lipid[a] | | Lichen[c] | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *m/z* in parentheses | $T_M$[b] | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| Hexanedioic acid (143, 114, 111, 101, 74, 55, 54) | 0.82 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 7.0 | — | — | — |
| Octananedioic acid (171, 138, 129, 97, 74, 69, 55) | 0.92 | 0.6 | — | — | — | — | — | 1.1 | — | — | — | — | — | — | — | 1.5 | — | — | — | — |
| Nonanedioic acid (185, 152, 143, 111, 83, 74, 69, 55) | 0.98 | 2.4 | 3.6 | 1.4 | 3.1 | — | 1.5 | — | 4.7 | 2.5 | 2.6 | 9.7 | 3.2 | 1.3 | — | 3.6 | 6.7 | 1.3 | — | 1.4 |
| Decanedioic acid (199, 166, 138, 125, 98, 74, 69, 55) | 1.05 | — | — | — | 1.1 | — | — | — | — | 0.8 | 0.6 | — | — | — | — | — | — | — | — | — |
| Undecanedioic acid (213, 171, 152, 139, 98, 74, 69, 55) | 1.11 | — | 1.1 | — | 1.2 | 1.1 | — | — | 1.5 | — | — | 1.3 | — | — | — | — | — | — | — | — |
| 10-Nonadecanol (265, 264, 215, 201, 171, 157, 109, 83) | 1.36 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 3-Hydroxy-decanoic acid (155, 129, 103, 71, 69, 57, 43) | 0.85 | — | — | — | — | 2.6 | — | — | — | — | — | — | — | — | — | — | — | — | 1.5 | — |
| 9-Oxo-nonanoic acid (159, 158, 143, 109, 87, 69, 43) | 0.98 | — | — | — | — | — | — | — | — | — | — | 4.4 | — | — | — | — | — | — | — | — |
| 9-Oxo-decanoic Acid (185, 169, 143, 111, 87, 83, 69, 43) | 1.04 | — | — | — | — | 1.1 | — | — | — | — | — | 1.3 | — | — | — | — | — | — | — | — |
| 6,10,14-$Me_3$-2-pentadecanone (250, 210, 109, 85, 71, 58, 43) | 0.99 | — | — | — | — | — | 0.6 | — | — | — | — | — | — | — | 1.0 | — | — | — | — | — |
| 6-Me-heptanoate (171, 137, 130, 115 , 103, 87, 74, 55) | 0.86 | — | — | — | — | — | — | — | — | — | — | 0.6 | — | — | 1.4 | — | — | — | — | — |
| 9-Me-tetradecanoate (256, 227, 199, 129, 101, 87, 74) | 0.92 | — | — | — | — | — | — | 1.3 | — | — | — | — | — | — | — | — | — | — | — | — |
| 11,14-Eicosadienoic acid (294, 263, 220, 215, 95, 81, 67) | 1.19 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 1.2 | — | — |

[a]Lipids determined as total. 1, *C. islandica*; 2, *C. clathrata*; 3, *C. connexa*; 4, *C. crispatula*; 5, *C. furcata*; 6, *C. ibitipocae*; 7, *C. imperialis*; 8, *C. penicillata*; 9, *C. salmonea*; 10, *C. signata*; 11, *C. substellata*; 12, *C. rangiferina*; 13, *C. aggregata*; 14, *D. glabratum*; 15, *P. delicatum*; 16, *P. mantiqueirense*; 17, *P. shindler*; 18, *R. celastri*; 19, *R. usnea*.
[b]Retention time ($T_M$) relative to palmitic acid methyl ester.
[c]All the lichens were studied (21), but only 19 species contained these types of lipid. See Table 2 for lichen abbreviations.

acetates, which were analyzed by GC–MS. The monosaccharides obtained after total hydrolysis (Table 5) show the presence of glucose, mannose, galactose, and xylose in all cases. Fucose was observed only in extracts of *C. islandica*, *P. mantiqueirense*, and *P. shindler*. Rhamnose was found in most lichens, but not in those of *C. aggregata*, *P. shindler, P. mantiqueirense*, and *R. celastri*.

## DISCUSSION

Extractions with various solvents gave rise to different yields and fatty acids; the best yields were obtained with extracts D, C, and E, although all of them contained different fatty acids. The procedure of Extract A was the most efficient for extracting short-chain fatty acids. The advantages of using EtOH/$H_2O$ (9:1, vol/vol) under reflux were apparent since it gave a high yield, was cheaper and less toxic. It is capable of extracting polar glycolipids, according to Leeden and Yu (22). However, for a more reliable examination of total fatty acids in tissues, a battery of different solvents is necessary. *Dictyonema glabratum* was chosen as the best candidate, as its fresh tissues were more readily obtainable in greater amounts.

Total lipids were examined, and palmitic and stearic acids were present in all studied lichens. This observation was supported by the natural biosynthetic route of lipids in fungi and by the fact that most organisms produce fatty acids with various chain lengths ($C_{12}$–$C_{20}$), as described by Wassef (12). Since lichens contain ~90% mycobiont (16), similar results would be expected. The presence of odd-number fatty acids was shown by Lynen (cited in Ref. 12), and these arose using propionate as primer in the substitution of acetate and the use of isobutyrate, leucine, valine or isoleucine, which gave rise to iso- and anteiso-branched fatty acids (Baraud, cited in Ref. 12). In our upper organic phases, other unusual lipids were detected, namely they were aldehyde, keto, hydroxy, and dicarboxylic fatty acids. These have not been previously described in lichens. We also showed the presence of dicarboxylic acids, and the main one found was nonanedioic (azelaic) acid (Table 4); these are probably formed by ω-oxidation (23). Nonanedioic and hexanedioic acids have a cytotoxic effect against squamous carcinoma affecting cell proliferation (24). However, why are these compounds present in lichens? Other types of unusual lipids were the aldehyde and keto-derivatives, 9-oxo-nonanoic and 9-oxo-decanoic acids, which can be associated with cold-acclimation (25). Another reason to justify this hypothesis is the presence of large amounts of unsaturated fatty acids in lichens when they are submitted to cold conditions (4).

The family Cladoniaceae includes 11 genera with polyphyletic assemblage (26). We have now investigated lichens of the *Cladonia* and *Cladina* genera for their carbohydrate and FAME compositions, both of total fatty acids and isolated TG. Although the relationship of these genera is not totally clear (27), the dendogram derived from FAME data shows that they have similar profiles on fatty acid analysis. The cluster analysis evaluated for *Cladonia*, *Cladina*, and other

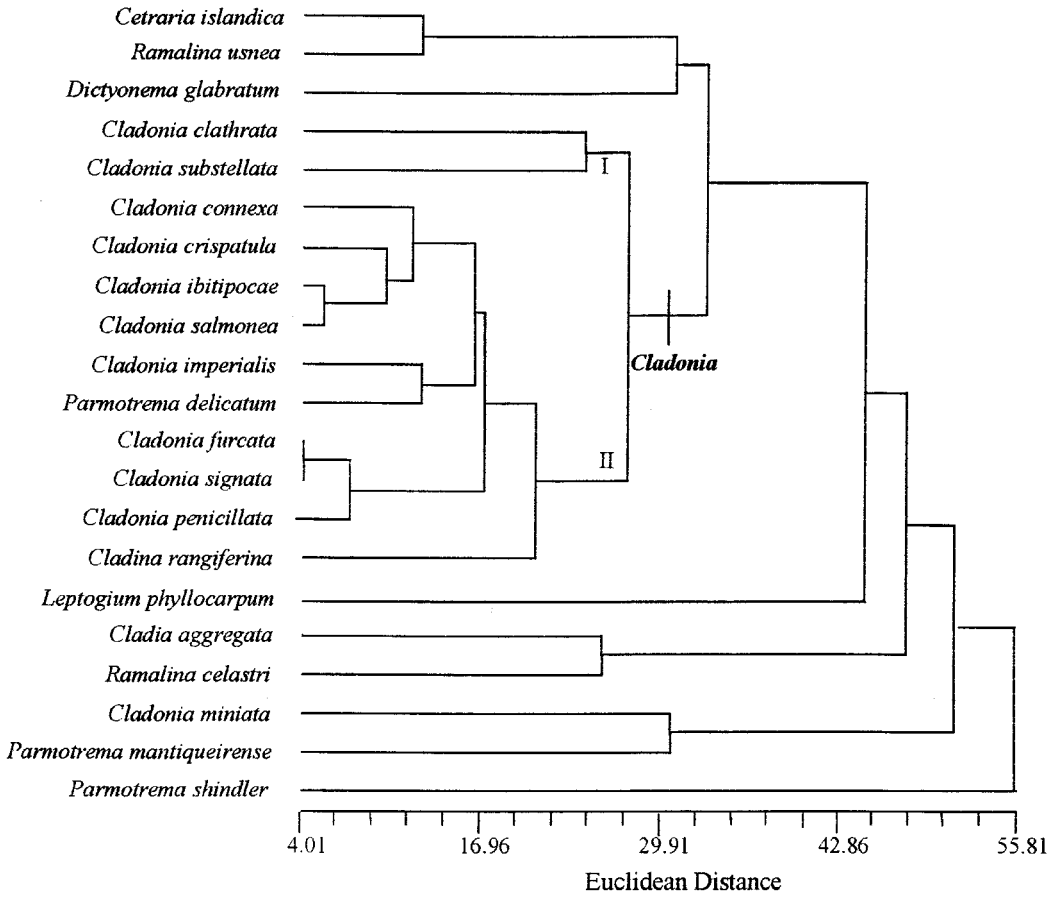


**FIG. 1.** Unweighted pair group with mean average from pairwise calculated distances obtained comparing fatty acid methyl ester profiles from 21 lichen species performed using the NTSys program. (I and II) *Cladonia* subclusters.

lichens from these data enabled the consistent conjunction of *Cladonia* species. Although these lichens were subjected to different growth conditions, this criterion is important: the option of laboratory culture in synthetic media of so many lichens is very laborious. The data suggest a similar profile of fatty acids for the *Cladonia* genus. *Cladia miniata* showed a different fatty acid composition and was positioned outside the group. This agrees with morphological, chemical, and ecological characteristics evaluated by Stenroos *et al.* (27), who carried out cluster analysis of *Cladonia* and *Cladina* genera. On the other hand, *C. rangiferina* clustered with *Cladonia* species, emphasizing a pronounced relationship between these genera. It should be observed that the genus *Cladina* is not well established. In the Americas, Asia, Australasia, and Russia, it is now usually recognized, while in Europe most lichenologists consider it a subgenus of *Cladonia* (27). *Cladia aggregata* did not show the same pattern of fatty acids as obtained for the lichens of the genera *Cladonia* and *Cladina*. This observation could have taxonomic value (12), since *C. rangiferina* belongs to the same family as those of the genus *Cladonia*. The basidiolichen *D. glabratum* appeared to be interesting for comparison of its fatty acid profile, which was quite different from that of lichenized ascomycetes (28), with the presence of the short-chain fatty acids, 10:0, 12:0, and 16:2. The lichen *L. phyllocarpum* is ascomycetous and with a cyanobacterium symbiosis, and its fatty acids were similar to those of other ascolichens. These observations (ascomycetous and basidiomycetous) showed that the fatty acids might be derived from the mycobionts, since they are different and phycobionts are cyanobacteria.

It is possible that some lichen fatty acids could be formed directly by the phytobiont and modified by the mycobiont. An

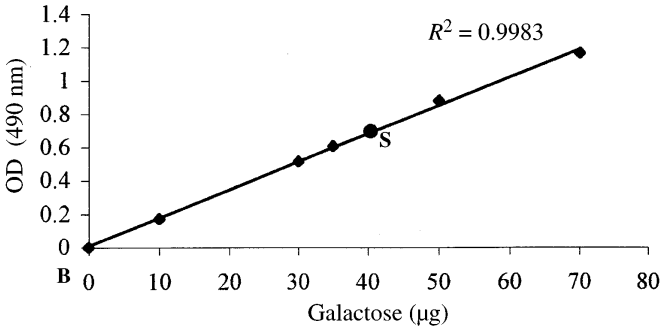


**FIG. 2.** Blank tube (B) containing 0.5 mL of M trifluoroacetic acid and the phenol-sulfuric acid mixture. Standard (S) containing 24 µg of galactose and 2 mg of lipid extract *(Cetraria islandica)*. Calibration was carried out with standards of 10, 30, 50, and 70 µg of galactose. OD, optical density.

**TABLE 5**
**Monosaccharide Composition and Carbohydrate in Total Lipid Extracts, Obtained from 21 Lichen Species**

| Lichen | Yield (%)[a] | (%) Monosaccharide content | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Glycerol | Rhamnose | Fucose | Arabinose | Xylose | Mannose | Galactose | Glucose |
| *C. islandica* | 0.9 | 1.2 | 2.5 | 3.1 | 32.4 | 2.1 | 5.4 | 9.1 | 40.6 |
| *C. clathrata* | 0.9 | 1.8 | 3.9 | — | 43.0 | 0.8 | 11.5 | 12.3 | 20.8 |
| *C. connexa* | 0.5 | 0.9 | 2.5 | — | 18.0 | 5.9 | 15.6 | 23.3 | 34.4 |
| *C. crispatula* | 0.2 | 1.0 | 6.1 | — | 33.9 | 7.1 | 17.0 | 12.3 | 20.3 |
| *C. furcata* | 0.8 | 0.6 | 2.5 | — | 22.2 | 3.3 | 23.0 | 29.6 | 18.8 |
| *C. ibitipocae* | 1.0 | 0.8 | 0.9 | — | 10.9 | 5.3 | 27.6 | 25.0 | 29.8 |
| *C. imperialis* | 0.9 | 0.5 | 19.8 | — | 47.0 | 14.2 | 4.3 | 2.8 | 10.1 |
| *C. miniata* | 2.5 | 0.3 | 0.4 | — | 3.2 | 0.2 | 38.4 | 22.9 | 34.0 |
| *C. penicillata* | 0.8 | 1.0 | 1.8 | — | 28.2 | 4.2 | 22.0 | 13.6 | 29.2 |
| *C. salmonea* | 0.9 | 0.3 | 1.7 | — | 15.4 | 9.8 | 16.9 | 19.1 | 36.8 |
| *C. signata* | 0.8 | 3.6 | 13.6 | — | 37.6 | 11.8 | 4.3 | 7.2 | 20.8 |
| *C. substellata* | 0.6 | 1.1 | 0.8 | — | 7.7 | 3.8 | 38.2 | 18.8 | 29.4 |
| *C. aggregata* | 0.8 | 0.8 | — | — | 91.3 | 3.3 | 2.6 | 1.0 | 1.0 |
| *C. rangiferina* | 2.3 | 2.1 | 14.6 | — | 34.4 | 12.0 | 13.9 | 4.6 | 15.1 |
| *D. glabratum* | 3.3 | 3.8 | 8.0 | — | 25.3 | 3.1 | 7.1 | 28.5 | 17.1 |
| *L. phyllocarpum* | 1.9 | 0.9 | 2.7 | — | 3.2 | 2.4 | 49.7 | 18.2 | 22.6 |
| *P. delicatum* | 0.9 | 0.5 | 9.2 | — | 36.5 | 7.7 | 15.9 | 10.1 | 20.0 |
| *P. mantiqueirense* | 1.1 | 0.8 | — | 1.8 | 1.7 | 1.6 | 64.2 | 15.3 | 10.1 |
| *P. shindler* | 1.9 | 2.6 | — | 5.8 | — | 19.3 | 15.8 | 25.1 | 26.2 |
| *R. celastri* | 0.8 | 0.5 | — | — | 93.2 | 0.5 | 3.6 | 1.2 | 0.8 |
| *R. usnea* | 1.3 | 1 | 2.8 | — | 24.5 | 5.5 | 6.5 | 47.6 | 12 |

[a]SD (standard deviation), triplicates of total sugar concentration varied from ± 0.009 to ± 0.021. See Table 2 for lichen abbreviations.

example of this process occurs in the transformation of free aldoses to polyalcahols as suggested by Ahmadjian (29), although evidence has not yet been observed in lichens that fatty acids, or any other metabolite, can be synthesized in the mycobiont and later modified or transferred to the phycobiont (16,29). It appears likely that lichen fatty acids should arise from the mycobiont, which comprises ~90% of the mycelial biomass and which is formed in large amounts in fungi (16). Many of the fatty acids are similar to those in lichenized fungi (16,29); these observations may show the lichen fatty acids as markers for taxonomical study just like those observed in fungi (13). For better results, more cladistic analyses should be carried out on FAME profiles in other well-classified genera, although the value of the FAME profiles appears to be an important additional parameter that can be used in lichen taxonomy.

The colorimetric estimation of the total sugar in pigmented material is a common problem. The use of direct TFA-hydrolyzed material instead of water in the conventional method did not interfere with the optical densities, enabling accurate determinations with the phenol-sulfuric acid method.

Monosaccharide analysis of the 21 lichens showed high levels of total galactose, expected since the major phycobiont glycolipids are galactolipids, but the presence of high levels of arabinose, mannose, and glucose was not. It may be significant that the presence of their corresponding polyalcohols in lichens has been observed (29). The presence of arabinitol and mannitol could be explained by the carbohydrate movement (29), the glucose produced by the phycobiont being rapidly converted to mannitol and arabinitol by the mycobiont. Although *C. aggregata* showed high levels of arabinose (91.3%), when compared to total monosaccharide, this suggests a difference in taxonomical position, in relationship to the genus *Cladonia* (Table 5). The presence of high levels of polyalcohols and saccharide derivatives would be expected, since they are more soluble in hot organic solvents. Another possible explanation is that aldoses were liberated by hydrolysis from lipopolysaccharides, since this polymer is produced by some algae and fungi (30,31), and is soluble in organic solvents.

## ACKNOWLEDGMENTS

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# A New Ceramide from the Basidiomycete *Russula cyanoxantha*

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**ABSTRACT:** A new phytosphingosine-type ceramide (**1**) was isolated along with nine other compounds—5α,8α-epidioxy-(22*E*,24*R*)-ergosta-6,22-dien-3β-ol, 5α,8α-epidioxy-(24*S*)-ergosta-6-en-3β-ol, (24*S*)-ergosta-7-ene-3β,5α,6β-triol, (22*E*,24*R*)-ergosta-7,22-dien-3β,5α,6β-triol, inosine, adenine, L-pyroglutamic acid, fumaric acid, and D-allitol from the ethanol and chloroform/methanol extract of the fruiting bodies of the basidiomycete *Russula cyanoxanotha* (Schaeff.) Fr. The structure of (**1**) was established as (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino) octadecane-1,3,4-triol by means of spectroscopic and chemical methods.

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The family Russulaceae is one of the largest in the subdivision Basidiomycotina in Whitthaker's Kingdom of Fungi and comprises hundreds of species (1). Although secondary metabolites occurring in the fruiting bodies of European *Lactarius* species have well been investigated, the *Russula* mushrooms have received less attention, notwithstanding the larger number of existing species (2). Recently some new terpenoids from *Russula* species have been reported (2,3).

The fruiting bodies of *Russula cyanoxantha* have long been used as foods and medicinal agents in China; an extract of its fruiting bodies has been shown to be active against tumors (4). In a preceding paper, we reported on two sphingadienine-type glucocerebrosides isolated from *R. ochroleuca* (5). In a continuation of our study on the bioactive metabolites of the higher fungi in Yunnan Province, the chemical constituents of *R. cyanoxantha* collected at Ailao Mountains in Yunnan Province of the People's Republic of China were investigated. Ten compounds were isolated from the fruiting bodies of *R. cyanoxantha*, and the structure of **1** was established as (2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino) octadecane-1,3,4-triol. The present report deals with the isolation and structure elucidation of the new ceramide (**1**) from the $CHCl_3$-soluble fraction of the EtOH and $CHCl_3$/MeOH extract of the fruiting bodies of this fungus by repeated column chromatography (CC).

## EXPERIMENTAL PROCEDURES

*Chromatographic and instrumental methods.* Melting points were obtained on an XRC-1 apparatus (Sichuan, People's Republic of China) and uncorrected. Optical rotations were measured on a Horiba SEPA-300 polarimeter (Horiba, Tokyo, Japan). The nuclear magnetic resonance (NMR) spectra ($^1H$, $^{13}C$ NMR and two-dimensional NMR) were recorded on Bruker AM-400 and DRX-500 NMR instrument (Karlsruhe, Germany) at 500 MHz for $^1H$ and 100 MHz for $^{13}C$ NMR; tetramethylsilane was used as an internal standard and coupling constants were represented in Hertz. Mass spectra were carried out with a VG Autospec3000 mass spectrometer (VG, Manchester, England). Infrared (IR) spectra were obtained in KBr pellets on a Bio-Rad FTS-135 infrared spectrophotometer (Bio-Rad, Richmond, CA). Gas chromatography–mass spectrometry (GC–MS) was performed on a Finnigan 4510 GC–MS spectrometer (San Jose, CA) employing the electron impact (EI) mode (ionizing potential 70eV) and a capillary column (30 m × 0.25 mm) packed with 5% phenyl-dimethylsilicone on HP-5 (Hewlett-Packard, Palo Alto, CA). Helium was used as carrier gas; column temperature 160–240°C (rate of temperature increase: 5°C/min).

*Materials.* CC was conducted over silica gel (200–300 mesh; Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China). Thin-layer chromatography (TLC) analysis was carried out on plates precoated with silical gel $F_{254}$ (Qingdao Marine Chemical Ltd.). Reversed-phase chromatography was carried out on LiChroprep® RP-8 (40–63 μm) (Merck, Darmstad, Germany).

Fresh fruiting bodies of *R. cyanoxantha* were collected from Ailao Mountains of Yunnan Province in August 1998 and identified by Prof. P.G. Liu and X.H. Wang (Kunming Institute of Botany, the Chinese Academy of Sciences, Kunming, Yunnan, People's Republic of China). A voucher specimen is deposited at the Herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences, People's Republic of China.

*Extraction and isolation.* Dried fruiting bodies (300 g) of *R. cyanoxantha* were extracted with 95% EtOH (1.2 L × 3), followed by extraction with $CHCl_3$/MeOH (1:1, vol/vol) at

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Abbreviations: CC, column chromatography; DMSO, dimethyl sulfoxide; EI–MS, electron impact–mass spectrometry; FAB–MS, fast atom bombardment–mass spectrometry; GC–MS, gas chromatography–mass spectrometry; HMBC, heteronuclear multiple bond connectivity; HR–EI–MS, high resolution–electron impact–mass spectrometry; IR, infrared spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

room temperature. The combined extracts were concentrated *in vacuo* to give a crude extract, which was partitioned between $H_2O$ and $CHCl_3$ to provide a $CHCl_3$ extract (17 g) and a water-soluble fraction. The $CHCl_3$-soluble fraction was subjected to CC elution with a solvent mixture of petroleum ether/acetone (50:1–1:1, vol/vol) to give 18 fractions. Fraction 4 after crystallization from *n*-hexane furnished 5α,8α-epidioxy-(22*E*,24*R*)-ergosta-6,22-dien-3β-ol (73 mg). Fraction 6 subjected to CC (petroleum ether/EtOAc 7:3, vol/vol) afforded 5α,8α-epidioxy-(24*S*)-ergosta-6-en-3β-ol (38 mg) and (24*S*)-ergosta-7-ene-3β,5α,6β-triol (15 mg), respectively. Recrystallization of Fraction 9 from petroleum ether/acetone produced (22*E*,24*R*)-ergosta-7,22-dien-3β,5α,6β-triol (5 mg). Fraction 16 was submitted to repeated CC ($CHCl_3$/MeOH 8:2, vol/vol), and then recrystallized from petroleum ether/acetone (6:4) to give **1** (50 mg). The concentrated $H_2O$ fraction was dissolved in warm MeOH, and the resulting MeOH-soluble fraction (24.2 g) was subjected to CC ($CHCl_3$/MeOH 9:1–4:6, vol/vol) to give 13 fractions. Preparative TLC ($CHCl_3$/MeOH/$H_2O$ 8.5:1.5:0.02, by vol) purification of Fraction 5 afforded inosine (11.2 mg). Fractions 3–4 were further purified by LiChroprep RP-8 CC (using a gradient of 5%–10% MeOH/$H_2O$, vol/vol, 20 min, ultraviolet detector) afforded adenine (7.6 mg). Fraction 8 was chromatographed over silica gel ($CHCl_3$/MeOH/ $H_2O$ 9:1:0.05, by vol) to yield L-pyroglutamic acid (20 mg). Further CC purification of Fraction 10 gave fumaric acid (21 mg). Fraction 14 afforded D-allitol (6.2 g) after crystallization from MeOH/$H_2O$.

*(2*S*,3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino) octadecane-1,3,4-triol (**1**).* White amorphous powder. mp 140–142°C (petroleum ether/acetone); $[\alpha]_D$ = +9.4° (*c* = 0.21, pyridine). IR (KBr) ν 3340, 3220 (OH), 2919, 2850, 2487, 2395,
1619 (N–C=O), 1544 (NH), 1468, 1353, 1068, 1027, 723 $cm^{-1}$; $^1H$ and $^{13}C$ NMR (500 and 100 MHz, pyridine-$d_5$) see Table 1; high resolution–electron impact–mass spectrometry (HR–EI–MS) *m/z* 683.6407 $[M]^+$ ($C_{42}H_{85}NO_5$ calcd. 683.6427); EI–MS (70 eV) *m/z* (relative intensity %) 683 $[M]^+$ (2), 665 $[M - H_2O]^+$ (11), 651 $[M - CH_2OH - 1]^+$ (5), 456 $[M - CH_3(CH_2)_{13}CHOH]^+$ (13), 439 $[456 - OH]^+$ (18), 409 $[439 - CHOH]^+$ (22), 384 $[CH_3(CH)_{21}CH(OH)CONH_2 + H]^+$ (24), 357 $[M - CH_3(CH_2)_{21} - OH]^+$ (27), 339 (4), 320 (7), 227 (13).

*(2*S*,3*S*,4*R*,2′*R*)-2-(2′-acetoxytetracosanoylamino) octadecane-1,3,4-triacetoxyl (**1a**).* Compound **1** (6.9 mg) was dissolved in pyridine (1.1 mL); the mixture was treated with $Ac_2O$ (1.1 mL) and was left standing overnight at room temperature. The reaction solution was then diluted with 3 mL of water and extracted with EtOAc (3 × 10 mL). The EtOAc extract was washed with brine and dried over $Na_2SO_4$, then evaporated to dryness under reduced pressure. The residue obtained was subjected to silica gel CC (petroleum ether/ethyl acetate 8:2, vol/vol) to give 5.5 mg of the peracetate (**1a**) as white powder solids. EI–MS (70 eV) *m/z* (relative intensity, %) 851 $[M]^+$ (9), 611 $[M - 4 \times CH_3COOH]^+$ (2), 543 $[M - CH_3(CH_2)_{21} - H]^+$ (85). Positive fast atom bombardment–mass spectroscopy (FAB–MS) *m/z* 853 $[M + 1]^+$ (29); $^1H$ NMR (400 MHz, $CDCl_3$, in ppm) δ 4.01 (1H, *dd*, *J* = 11.6, 3.1 Hz, 1-Ha), 4.34 (1H, *dd*, *J* = 11.6, 5.4, 1-Hb), 4.44 (*m*, 2-H), 5.10 (*m*, 3-H), 4.95 (*m*, 4-H), 1.82 (*m*, 2H, 5-$H_2$), 1.63 (*m*, 2H, 6-$H_2$), 1.25 (28 × $CH_2$ *brs*), 0.88 (6H, *t*, *J* = 6.5 Hz, 2 × $CH_3$), 5.10 (*m*, 2′-H), 1.63 (*m*, 3′-$H_2$), 2.18 (*s*, OAc), 2.09 (*s*, OAc), 2.06 (*s*, OAc), 2.03 (*s*, OAc), 6.61 (1H, *d*, *J* = 9.2 Hz, NH).

*Methyl 2-(*R*)-hydroxytetracosanoate (**1b**).* Compound **1** (28 mg) was refluxed with 2.2 mL of 0.9 mol/L HCl in 82% aqueous methanol at 80°C for 16 h. The reaction mixture was extracted with petroleum ether, and the petroleum ether layer was concentrated and chromatographed using silica gel (petroleum ether/ethyl acetate 9:1–7:3, vol/vol; ratios changed as 9:1, 8:2, 7:3) to give a methyl ester of fatty acid (**1b**) as

**TABLE 1**
**$^1H$ and $^{13}C$ Nuclear Magnetic Resonance (NMR) Spectral Data[a] for Compound 1 in Pyridine-$d_5$**

| Atom no. | $^{13}C$ in ppm (*J* in Hz) | $^1H$ in ppm (*J* in Hz) | $^1H$-$^1H$ COSY selected | HMQC selected | HMBC selected |
|---|---|---|---|---|---|
| 1 | 62.14 (*t*) | 4.52 (*dd*, 10.6, 4.5)<br>4.43 (*dd*, 10.6, 5.2) | H-2 | H-1 | H-2, 3 |
| 2 | 53.10 (*d*) | 5.12 (*m*) | NH/$H_2$-1/H-3 | H-2 | H-1′, 1, 3 |
| 3 | 76.89 (*d*) | 4.35 (*dd*, 6.5, 4.0) | H-2/H-4 | H-3 | H-1, 2, 4, 5 |
| 4 | 73.12 (*d*) | 4.28 (*m*) | H-3/H-5 | H-4 | H-2, 3, 5, 6 |
| 5 | 34.23 (*t*) | 1.93 (*m*) | | H-5 | H-3, 4, 6 |
| 6 | 26.66 (*t*) | 1.70 (*m*) | | | |
| 7–17 | 29.63–32.16 (*t*) | 1.25–1.41 | | | |
| 18 | 14.28 (*q*) | 0.86 (*t*, 6.7) | | H-18 | |
| 1′ | 175.37 (*s*) | | | | |
| 2′ | 72.56 (*d*) | 4.62 (*dd*, 7.6, 4.0) | H-3′ | H-2′ | H-1′, 3′, 4′ |
| 3′ | 35.75 (*t*) | 2.24, 2.04 (*m*) | H-2′/H-4′ | H-3′ | H-2′, 4′ |
| 4′ | 25.86 (*t*) | 1.76 (*m*) | H-3′ | H-4′ | H-2′, 3′ |
| 5′–23′ | 29.63–32.16 (*t*) | 1.25–1.41 | | | |
| 24′ | 14.28 (*q*) | 0.86 (*t*, 6.7) | | H-24′ | |
| NH | | 8.57 (*d*, 8.8) | H-2 | | H-1′ |

[a]COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond connectivity.

white solid, which was subjected to GC–MS. The result showed that **1b** was a methyl 2-hydroxytetracosanoate which displayed major ion peaks at *m/z* 398 $[M]^+$, 339 $[M - 59]^+$; the GC retention time was 35 min. $[\alpha]_D = 4.5°$ (*c* = 0.83, $CHCl_3$); EI–MS (70 eV) *m/z* 398 $[M]^+$; $^1H$ NMR (400 MHz, $CDCl_3$, in ppm) δ 4.19 (1H, *dd, J* = 4.2, 7.4 Hz, H-2), 3.79 (3H, *s*, $OCH_3$), 2.74 (1H, *bs*, OH), 1.76 (1H, *m*), 1.63 (1H, *m*), 1.10–1.25 (40 H, *m*), and 0.88 (3H, *t, J* = 7.0 Hz, $CH_3$).

*2-Acetoamino-1,3,4-triacetoxyoctadecane (**1c**).* The aqueous methanol layer was neutralized with saturated $Na_2CO_3$ and concentrated to dryness, and then heated with $Ac_2O$/pyridine (1:1) for 1.5 h at 70°C. The reaction mixture was diluted with $H_2O$ and extracted with EtOAc. The residue of the EtOAc fraction was chromatographed using silical gel (*n*-hexane/EtOAc 8:2, vol/vol) as eluent to furnish an acetate (**1c**) of the long-chain base as a white solid. $[\alpha]_D = +10.9°$ (*c* = 0.67, $CHCl_3$); EI–MS (70 eV) *m/z* (relative intensity %): 486 $[M + 1]^+$ (1), 426 $[M + 1 - HOAc]^+$ (2), 366 $[M + 1 - 2 \times HOAc]^+$ (9), 305 $[M - 3 \times HOAc]^+$ (24.5), 245 $[M + 1 - 4 \times HOAc]^+$ (0.5); $^1H$ NMR (400 MHz, $CDCl_3$) δ 5.97 (1H, *d, J* = 9.2 Hz, NH), 5.10 (1H, *dd, J* = 8.5 Hz, 3.1 Hz, 3-H), 4.93 (1H, *dt, J* = 9.8, 3.1 Hz, 4-H), 4.47 (1H, *m*, 2-H), 4.29 (1H, *dd, J* = 11.6, 4.3 Hz, 1-Ha ), 4.00 (1H, *dd, J* = 11.6, 3.1 Hz, 1-Hb), 2.08 (3H, *s*, 3-OAc), 2.05 (6H, *s*, 1-OAc, 4-OAc), 2.03 (3H, *s*, HNAc), 1.12–1.70 (26H, *m*), 0.88 (3H, *t, J* = 6.1 Hz, $CH_3$).

*5α,8α-Epidioxy-(22*E*,24*R*)-ergosta-6,22-dien-3β-ol.* Colorless crystals, mp 182–184°C, $[\alpha]_D = -34°$ (*c* = 0.6, $CHCl_3$); IR (KBr) ν: 3525, 3309, 2957, 2873, 1653, 1459, 1377, 1046, 1029, 985, 970, 969, 935, 858 $cm^{-1}$; EI–MS (70 eV) *m/z* (relative intensity %): 428 $[M]^+$ (5), 410 (4), 396 (100), 363 (35), 271 (7), 251 (14), 152 (30), 107 (22), 81 (43), 69 (63); $^{13}C$ NMR (100 MHz, $CDCl_3$, in ppm) δ 34.7, 30.2, 66.5, 37.0, 82.1, 135.4, 130.8, 79.4, 51.2, 37.0, 23.4, 39.4, 44.6, 51.7, 20.6, 28.6, 56.3, 12.9, 18.2, 39.7, 20.9, 135.2, 132.4, 42.8, 33.1, 19.9, 19.6, 17.6; $^1H$ NMR (400 MHz, $CDCl_3$, in ppm) δ 3.94 (1H, *m*, H-3), 6.22 (1H, *d, J* = 8.5 Hz, H-6), 6.48 (1H, *d, J* = 8.5 Hz, H-7), 0.86 (3H, *s*, $H_3$-18), 1.06 (3H, *s*, $H_3$-19), 0.97 (3H, *d, J* = 6.6 Hz, $H_3$-21), 5.11 (1H, *dd, J* = 15.3, 8.0 Hz, H-22), 5.19 (1H, *dd, J* = 15.1, 7.5 Hz, H-23), 0.83 (3H, *d, J* = 5.0 Hz, $H_3$-26), 0.82 (3H, *d, J* = 5.0 Hz, $H_3$-27), 0.89 (3H, *d, J* = 5.3 Hz, $H_3$-28). The above spectral data were in accord with those reported.

*5α,8α-Epidioxy-(24*S*)-ergosta-6-en-3β-ol.* Colorless crystals, mp 143–145°C, IR (KBr) ν: 3372, 2957, 2874, 1650, 1465, 1379, 1047, 1029, 956, 935, 859 $cm^{-1}$; EI–MS (70 eV) *m/z* (relative intensity %): 430 $[M]^+$ (24), 412 (41), 398 (100), 379 (17), 365 (49), 339 (25), 271 (7), 251 (9), 152 (57), 107 (30), 95 (40), 81 (51), 69 (46); $^{13}C$ NMR (100 MHz, $CDCl_3$, in ppm) δ 35.8, 30.2, 66.5, 39.1, 82.2, 135.4, 130.8, 79.5, 51.2, 37.0, 23.5, 39.1, 44.8, 51.6, 20.7, 28.2, 56.4, 12.6, 18.2, 39.5, 18.8, 33.6, 30.6, 39.5, 31.5, 17.7, 20.5, 15.5; $^1H$ NMR (400 MHz, $CDCl_3$, in ppm) δ 3.95 (1H, *m*, H-3), 6.22 (1H, *d, J* = 8.5Hz, H-6), 6.49 (1H, *d, J* = 8.5 Hz, H-7), 0.77 (3H, *s*, $H_3$-18), 0.89 (3H, *s*, $H_3$-19), 0.86 (3H, *d, J* = 5.4 Hz, $H_3$-21), 0.75 (3H, *d, J* = 6.8 Hz, $H_3$-26), 0.75 (3H, *d, J* = 6.7 Hz, $H_3$-27), 0.88 (3H, *d, J* = 6.5 Hz, $H_3$-28). The above spectral and physical data were in agreement with those reported.

*(24*S*)-Ergosta-7-ene-3β,5α,6β-triol.* Colorless crystals, mp 235–237°C, $[\alpha]_D = 69.4°$ (*c* = 0.16, pyridine). IR (KBr) ν: 3441 (OH), 2958, 2871, 1657, 1465, 1382, 1050, 1031, 969, 940 $cm^{-1}$; negative FAB–MS *m/z* 585 $[M + 153]^-$; EI–MS (70 eV) *m/z* (relative intensity %): 414 (100), 399 (53), 396 (72), 381 (71), 287 (12), 269 (18), 251 (27), 105 (31), 95, 81, 69; $^{13}C$ NMR (100 MHz, pyridine-$d_5$, in ppm) δ 32.6, 33.8, 67.6, 42.0, 76.1, 74.3, 120.4, 141.6, 43.8, 38.1, 22.4, 40.1, 43.9, 55.2, 23.5, 28.2, 56.5, 12.3, 18.8, 37.0, 19.3, 34.0, 31.2, 39.4, 31.8, 17.8, 20.7, 15.7; $^1H$ NMR (400 MHz, pyridine-$d_5$, in ppm) δ 4.83 (1H, *m*, H-3), 3.03 (2H, *dd, J* = 12.2, 12.2 Hz, $H_2$-4), 4.33 (1H, *bd, J* = 5.1 Hz, H-6), 5.74 (1H, *bd, J* = 5.1 Hz, H-7), 0.63 (3H, *s*, $H_3$-18), 1.53 (3H, *s*, $H_3$-19), 0.97 (3H, *d, J* = 6.8 Hz, $H_3$-21), 0.85 (3H, *d, J* = 6.8 Hz, $H_3$-26), 0.79 (3H, *d, J* = 6.8 Hz, $H_3$-27), 0.78 (3H, *d, J* = 6.8 Hz, $H_3$-28). The above data were identical with those reported.

*(22*E*,24*R*)-Ergosta-7,22-dien-3β,5α,6β-triol (= cerevisterol).* Colorless crystals, mp 224–227°C; EI–MS (70 eV) *m/z* (relative intensity %): 430 $[M]^+$ (7), 412 $[M - H_2O]^+$ (18), 394 $[M - 2H_2O]^+$ (26), 379 $[M - 2H_2O - CH_3]^+$ (12), 376 $[M - 3H_2O]^+$ (4), 305 $[M - C_9H_{17}]^+$ (3), 269 $[M - 2H_2O - C_9H_{17}]^+$ (6), 251 $[M - 3H_2O - C_9H_{17}]^+$ (13), 107 (25), 95 (36), 81 (45), 69 (53); $^1H$ NMR (400 MHz, $CDCl_3$, in ppm) δ 5.30 (1H, *bd, J* = 4.9 Hz, H-7), 5.21 (1H, *dd, J* = 15.2, 7.0 Hz, H-23), 5.16 (1H, *dd, J* =15.2, 7.8 Hz, H-22), 4.06 (1H, *m*, H-3), 3.60 (1H, *bd, J* = 4.9 Hz, H-6), 0.57 (3H, *s*, $H_3$-18), 1.06 (3H, *s*, $H_3$-19), 1.00 (3H, *d, J* = 6.6 Hz, $H_3$-21), 0.89 (3H, *d, J* = 6.9 Hz, $H_3$-28), 0.82 (3H, *d, J* = 6.6 Hz, $H_3$-26), 0.80 (3H, *d, J* = 6.5 Hz, $H_3$-27). The above spectral data agree with the literature values.

*Inosine (= 1,9-dihydro-9-β-*D*-ribofuranosyl-6H-purin-6-one).* White amorphous powder, mp 213°C (dec.). $[\alpha]_D = -45°$ (*c* = 0.6, $H_2O$); EI–MS (70 eV) *m/z* (relative intensity %): 268 $[M]^+$ (28), 250 $[M - H_2O]^+$ (8), 237 $[M - CH_2OH]^+$ (41), 178 (49), 164 (99), 135 $[M - \text{ribosyl}]^+$ (100), 108 (38), 73 (16), 55 (19); negative FAB–MS *m/z*: 420 $[M - H + 153]^-$ (100), 266, 188, 134; $^{13}C$ NMR dimethylsulfoxide [(DMSO)-$d_6$, 100 MHz, in ppm)] δ 152.3 (C-2, *d*), 149.1 (C-4, *s*), 119.3 (C-5, *s*), 156.1 (C-6, *s*), 139.8 (C-8, *d*), 87.9 (C-1′, *d*), 70.6 (C-2′, *d*), 73.4 (C-3′, *d*), 85.8 (C-4′, *d*), 61.6 (C-5′, *t*); $^1H$ NMR (DMSO-$d_6$, 400 MHz, in ppm) δ 5.86 (1H, *d, J* = 6.2 Hz, H-1′), 5.43 (1H, *dd, J* = 4.5, 4.6 Hz, H-2′), 5.19 (1H, *br.d, J* = 4.3 Hz, H-3′), 4.59 (1H, *br.d, J* = 5.3 Hz, H-4′), 3.95–4.13 (2H, *br.dd, J* = 3.2, 3.5 Hz, $H_2$-5′), 8.33 (1H, *s*, H-8), 8.12 (1H, *s*, H-2), 7.33 (1H, *br.s*, OH). The above data are in agreement with those reported.

*Adenine (= 2-aminopurine).* White amorphous powder, mp > 338°C (dec.); EI–MS (70 eV) *m/z* (relative intensity %): 135 $[M]^+$ (100), 108 (35), 81 (14), 54 (12); $^{13}C$ NMR (DMSO-$d_6$, 100MHz) δ 151.5 (*d*, C-2), 151.9 (*s*, C-4), 116.6 (*s*, C-5), 154.4 (*s*, C-6), 139.8 (*d*, C-8); $^1H$ NMR (DMSO-$d_6$, 400 MHz, in ppm) δ 7.62 (*s*, 1H, H-8), 7.59 (*s*, 1H, H-2), 6.59 (*brs*, 1H, $NH_2$). These data are in agreement with those

reported.

*L-Pyroglutamic acid [= (S)-2-pyrrolidone-5-carboxylic acid].* Colorless crystals, mp 156–158°(C (MeOH), $[\alpha]_D$ = –11° ($H_2O$); EI–MS (70 eV) *m/z* (relative intensity %): 129 $[M]^+$ (34), 101 (18), 84 (82), 56 (100); negative FAB–MS *m/z* 128 $[M - H]^-$; $^{13}C$ NMR ($CD_3OD$ 100 MHz, in ppm ) δ 181.5 (*s*, COOH), 176.1 (*s*, CO), 57.0 (*d*, CH), 30.4 (*t*, C-3), 26.0 (*t*, C-4); $^1H$ NMR ($CD_3OD$, 400 MHz, in ppm) δ 6.75 (1H, *s*), 4.24 (1H, *dd, J* = 4.6, 8.6 Hz, 2-H), 2.49 (1H, *m*, 3-$H_a$), 2.14 (1H, *m*, 3-$H_b$), 2.31 (2H, *m*, 4-$H_2$). The above data are in accord with those reported.

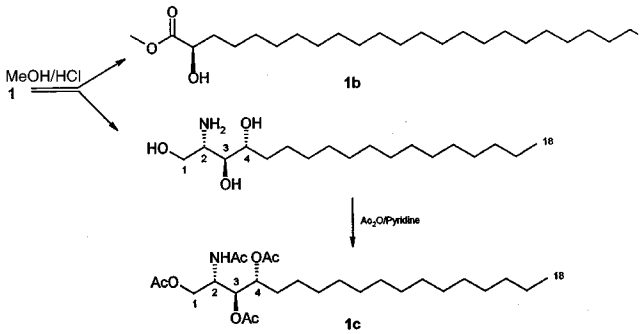
*Fumaric acid (= E-butenedioic acid).* Leaf-like crystals, mp 210°C (MeOH/$CHCl_3$, sublimation). EI–MS (70 eV) *m/z* (relative intensity %): 116 $[M]^+$ (43), 98 $[M - H_2O]^+$ (100), 88 $[M - CO]^+$ (27), 81 $[M - HO - H_2O]^+$ (20), 72 $[M - CO_2]^+$ (37), 71 $[M - COOH]^+$ (34), 55 (29), 53 (87); $^{13}C$ NMR (100 MHz, acetone-$d_6$, in ppm) δ 166.06 (*s*), 134.65 (*d*); $^1H$ NMR (400 MHz acetone-$d_6$, in ppm) δ 8.03. The above data are consistent with those reported.

*D-Allitol.* Colorless needles, $[\alpha]_D$ = 0° (c = 0.36, $H_2O$); mp 154.5–156°C (MeOH/$H_2O$). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3271, 2959, 2936, 1461, 1377, 1351, 1332, 1304, 1092, 1025; $^1H$ NMR (400 MHz, DMSO-$d_6$, in ppm) δ 4.42 (2H, *d, J* = 5.4 Hz, H-1, 6), 4.36 (2H, *t, J* = 5.4 Hz, H-3, 4), 3.46 (2H, *d, J* = 1.8 Hz, H-1, 6), 3.36 (2H, *q, J* = 5.4 Hz, H-2, 5); $^{13}C$ NMR (100 MHz, pyridine-$d_5$, in ppm) δ 73.45 (*d*, C-3, 4), 72.33 (*d*, C-2, 5), 65.54 (*t*, C-1, 6); EI–MS (70 eV) *m/z* (relative intensity %): 183 $[M + H]^+$ (36), 146 (15), 133 (70), 115 (26), 103 (73), 93 (53), 85 (46), 74 (84), 73 (100), 61 (89). The above data are consistent with literature values.

## RESULTS AND DISCUSSION

The $CHCl_3$-soluble fraction of the EtOH and $CHCl_3$/MeOH extract from the fruiting bodies of *R. cyanoxantha* was subjected to repeated column chromatography to yield (2*S*,3*S*, 4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino) octadecane-1,3,4-triol (**1**).

Compound **1** was obtained as a white amorphous powder, $[\alpha]_D$ +9.4° (*c* = 0.21, pyridine). The HR–EI–MS spectrum of **1** indicated a molecular formula of $C_{42}H_{85}NO_5$ ($M^+$ 683.6407, calcd. 683.6427). The IR spectrum of **1** revealed the absorption bands of hydroxyls at 3340 and 3220 $cm^{-1}$, a secondary amide at 1544 and 1619 $cm^{-1}$, and the long aliphatic chains at 723 $cm^{-1}$. The $^1H$ NMR spectrum of **1** showed the presence of two terminal methyls at δ 0.86 ppm (6H, *brt, J* = 6.7 Hz) and methylenes at δ 1.25–1.41 ppm (*ca.* 56H, *brs*), an amide proton signal at δ 8.57 ppm (1H, *d, J* = 8.8 Hz). In the $^{13}C$ NMR (distortion enhancement by polarization transfer) spectrum of **1** the signals for carbons (1 × C, 4 × CH, 35 × $CH_2$, 2 × $CH_3$) were recognized in which the presence of one quaternary carbon at δ 175.37 ppm (CONH, C-1′), four methines at δ 53.10 (CHNH, C-2), 72.56 (CHOH, C-2′), 73.12 (CHOH, C-4), and 76.89 ppm (CHOH, C-3) and a methylene at 62.14 ppm ($CH_2OH$, C-1) were followed from NMR data. Compound **1** possesses five characteristic signals of protons geminal to hydroxyls at δ 4.28 (1H, *m*), 4.35 (1H, *dd, J* = 6.5, 4.0 Hz), 4.62 (1H, *dd, J* = 7.6, 4.0 Hz), 4.43 (1H, *dd, J* = 10.6, 5.2 Hz), and 4.52 ppm (1H, *dd, J* = 10.6, 4.5 Hz). A sixth signal at low field appeared as a multiplet at δ 5.12 ppm and was assigned as a methine proton vicinal to the nitrogen atom. Therefore, all of the above spectral data revealed that **1** should be a phytosphingosine-type ceramide containing a 2-hydroxy fatty acid (6,7). Furthermore, compound **1** was considered to possess normal- type side chains since the carbon atom signals due to terminal methyl groups were observed at δ = 14.28 (*normal* form) (8) in the $^{13}C$ NMR spectrum of **1** (Table 1).



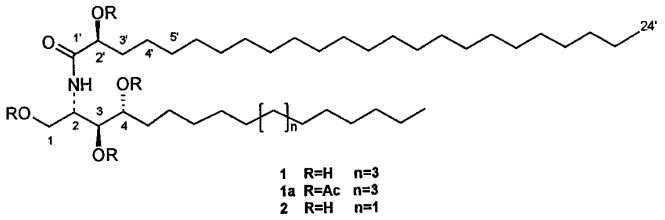

To determine the numbers of hydroxyl groups, compound **1** was acetylated with $Ac_2O$-pyridine at room temperature to yield the corresponding tetra-acetylated product **1a** which gave prominent peaks at *m/z* 851 $[M]^+$, 611 $[M - 4 \times CH_3COOH]^+$ in the EI–MS. The peracetate **1a** showed four ester methyl proton signals at δ 2.18, 2.09, 2.06, and 2.03 ppm in the $^1H$ NMR spectrum; thereby the presence of four hydroxyl groups in the original structure of **1** was confirmed.

Acidic methanolysis (6) of **1** with 0.9 N HCl solution in 82% aqueous MeOH yielded a fatty acid methyl ester and a long-chain base (Scheme 1). The fatty acid methyl ester was identified as methyl 2′-hydroxytetracosanoate (**1b**) by the help of GC–MS analysis. The existence of this fatty acyl moiety in **1** was also confirmed by the significant fragment ion peaks at *m/z* 384 $[CH_3(CH_2)_{21}CH(OH)CONH_2 + H]^+$ and 357 $[M - CH_3(CH_2)_{21}(OH]^+$ in the EI–MS. In addition, the $^1H$ NMR spectrum and optical rotation ($[\alpha]_D$ = –4.5°) of **1b** are in good accord with the data reported in the literature (6),

**TABLE 2**
**$^1H$ NMR Spectral Data[a] and Optical Rotations of Compound 1, Natural Ceramide 2, and Synthetic Ceramide 2 in Pyridine-$d_5$**

| | **1** | **2** natural[b] | **2** synthetic[c] |
|---|---|---|---|
| 1-Ha | 4.52 (*dd*, 10.6, 4.5) | 4.53 (*dd*, 10.7, 4.5) | 4.52 (*dd*, 10.7, 4.5) |
| 1-Hb | 4.43 (*dd*, 10.6, 5.2) | 4.43 (*dd*, 10.7, 4.5) | 4.43 (*dd*, 10.6, 5.0) |
| 2-H | 5.12 (*m*) | 5.12 (*m*) | 5.12 (*m*) |
| 3-H | 4.35 (*dd*, 6.5, 4.0) | 4.35 (*dd*, 6.5, 4.6) | 4.36 (*dd*, 6.6, 4.6) |
| 4-H | 4.28 (*m*) | 4.29 (*m*) | 4.29 (*m*) |
| 2′H | 4.62 (*dd*, 7.6, 4.0) | 4.63 (*dd*, 7.6, 3.7) | 4.63 (*dd*, 7.6, 4.0) |
| $[\alpha]_D$ | +9.4° | +11.5° | +9.1° |

[a]*J* in parentheses, δ in ppm. For abbreviations see Table 1.
[b]Data from Reference 8.
[c]Data from Reference 9.




therefore the absolute configuration at C-2′ in **1b** is also supposed to be *R*. The long-chain base, namely phytosphingosine, is a $C_{18}$ aliphatic amino alcohol unit containing three hydroxyls and an amino group. It was confirmed by treatment of methanolysis product of **1** with $Ac_2O$/pyridine at 70°C to afford a tetraacetylphytosphingosine, i.e., 2-acetoamino-1,3,4-triacetoxyoctadecane (**1c**). The $^1H$ NMR spectrum and optical rotation ($[\alpha]_D = +10.9^\circ$) for **1c** was found to be identical to that of the known counterpart (7).

The relative stereochemistry at C-2, C-3, C-4, and C-2′ was proposed as 2*S*,3*S*,4*R*,2′*R*, since the chemical shifts and coupling constants of 1-H, 2-H, 3-H, 4-H, and 2′-H in **1** were in good agreement with those of the natural ceramide, (2*S*, 3*S*,4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino) hexadecane-1,3,4-triol (**2**) isolated from the starfish *Acanthaster planci* (Table 2) (8) and which was confirmed by synthesis (9). The above fact and the comparison of the optical rotations of **1** with compound **2** (natural, $[\alpha]_D = +11.5°$; synthetic, $[\alpha]_D = +9.1°$) (8,9) suggested that **1** has the same absolute configuration as that of the natural one for the core structure like positions 2, 3, 4, 2′ chiral centers. Accordingly, the above evidence led to the establishment of the structure of **1** as (2*S*,3*S*, 4*R*,2′*R*)-2-(2′-hydroxytetracosanoylamino) octadecane-1,3,4-triol, whose structure as shown in Scheme 2 was verified by further two-dimensional NMR experiments: $^1H$-$^1H$ correlation spectroscopy heteronuclear multiple bond connectivity, and heteronuclear multiple quantum coherence.

Based upon comparison of spectroscopic (MS, IR, $^1H$ and $^{13}C$ NMR) and physical data with the literature, the structures of the other nine known compounds were characterized as 5α, 8α-epidioxy-(22*E*,24*R*)-ergosta-6,22-dien-3β-ol (10,11), 5α, 8α-epidioxy-(24*S*)-ergosta-6-en-3β-ol (10), (24*S*)-ergosta-7-ene-3β,5α,6β-triol (12,13), (22*E*,24*R*)-ergosta-7,22-dien-3β,5α,6β-triol (12,14), inosine (15), adenine (5), L-pyroglutamic acid (16), fumaric acid (17), and D-allitol (5), respectively.

The ceramides, cleavage products of various sphingolipids including gangliosides and cerebrosides, are involved in various signal transduction pathways (18). Many extracellular stresses, such as tumor necrosis factors-α and human immunodeficiency virus, have been shown to activate sphingomyelinases that release ceramides which inhibit cell growth and induce apoptosis (19,20). Because of the importance of ceramides, the chemistry and biology of ceramides have been a vital subject of research in recent years (9,21,22).

The occurrence of the ceramide-containing $C_{18}$-phytosphingosine and an α-hydroxy fatty acid is rather common in the bonding form in mushrooms. More recently, Jennemann *et al.* (23) reported on a series of glycoinositolphosphoceramides possessing this type of ceramide from higher mushrooms (*Agaricus*). However, except for the fact that this type of ceramide (phytosphingosine/α-fatty acid) itself is a normal constituent of the glycosylphosphoinositolcermides of fungi in general, it has been reported previously to occur in the free state only in the fungus *Phellinus pini* (7). This probably represents a precursor of these glycolipids. One functional aspect of the hydroxyl group cluster of this ceramide, especially in the neighborhood of a phosphoinositol, may indeed be to strengthen the structures where it occurs.

These ergostane-type compounds were previously obtained from marine organisms (11–13) and mushroom (10,14). Biogenetically, $\Delta^6$-ergosterol peroxides and $\Delta^7$-polyhydroxysterols seem quite obviously to originate from ergosterol distributed widely in both fungi and marine organisms (12,13). The occurrence of closely related ergostane derivatives and ceramides in taxonomically remote species is interesting and may indicate the connection with a common producer, probably symbiotic microorganisms. The above fact suggests that a close correlation between terrestrial fungi and marine organisms appears to exist, which is of evolutionary value.

## ACKNOWLEDGMENTS

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# *Ab initio* and Density Functional Theory Studies for the Explanation of the Antioxidant Activity of Certain Phenolic Acids[1]

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**ABSTRACT:** *Ab initio* and density functional theory molecular orbital calculations were carried out at both the HF/6–31+G(d) and B3LYP/6–31+G(d) levels for the four antioxidants, *p*-hydroxycinnamic acid derivatives, namely, the *p*-coumaric, caffeic, ferulic, and sinapinic acid and the corresponding radicals, in an attempt to explain the structural dependency of the antioxidant activity of these compounds. Optimized resulting geometries, vibrational frequencies, absolute infrared intensities, and electron-donating ability are discussed. Both the high degree of conjugation and the extended spin delocalization in the phenoxyl radicals offer explanation for the scavenging activity of the four acids. In structurally related compounds, the calculated heat of formation value in radical formation appears as a meaningful molecular descriptor of antioxidant activity in accordance with experimental data. This becomes more clear at the B3LYP level.

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Structure-activity relationship studies using theoretical methods are gaining interest among scientists for prediction or elucidation of differences in the activity of series of molecules (1–6). Phenolic antioxidants such as tocopherols, flavonoids, and phenolic acids are the most intensively examined categories because of their broadly accepted biological function (7–10). Difficulties in theoretical approach are related to the various factors that determine the performance of a molecule as an antioxidant; scavenging of free radicals, metal chelation, and lipophilicity are the most important ones (11,12). Although a considerable number of molecular descriptors have been examined, only a few of them prioritize the molecules in accordance to conclusions derived by experimental data. Various factors have been considered so far for the characterization of free radical scavenging activity of antioxidants, for instance, (i) the difference in the heat of formation (ΔHOF) between the antioxidant and the free radical (1–4), (ii) the spin distribution in the radical (4), (iii) the dissociation energy of the phenolic O–H bond (5,6,13) and (iv) the energy-eigenvalue of the highest-occupied molecular orbital (HOMO) (2,13–16). Owing to the large size of the antioxidant molecules, semiempirical quantum-chemical methods have been employed in most cases and *ab initio* calculations are quite rare (4–6,13,17–19).

Korzekwa *et al.* (20) proposed that a linear relationship exists between stability of radicals and activation energies of hydrogen abstraction in the reaction of a flavonoid with a radical in a series of analogous substrates. Consequently, the calculated enthalpy difference (ΔHOF) between the parent antioxidant molecule and its potential radical indicates the relative order of hydrogen atom abstraction, and the difficult task of determining transition states could be avoided for the moment. Based on these, the above hydrogen abstraction scheme is generally accepted (1,21) for the phenolic acid antioxidants, the parent molecule phenoxyl radical couple exhibiting the lowest theoretical ΔHOF value will be considered the one that most easily allows hydrogen atom abstraction by any free radical. The derived radical could then scavenge another one. Hydrogen atom abstraction depends mostly on enthalpy differences (as reflected by calculated ΔHOF values), whereas radical scavenging depends on the spin distribution. The calculated ΔHOF values of the parent molecule phenoxyl radical couples correlate well with the experimental antioxidant activity trend of the same molecules. This was also found by both Zhang (1,2) for certain phenolic antioxidants and van Acker *et al.* (4) for certain flavonoids. Zhang derived ΔHOF values from semiempirical calculations, whereas van Acker *et al.,* from *ab initio* ones; still the antioxidants studied by both exhibited profound structural differences. Moreover, van Acker *et al.* effectively used the radical spin distribution to

[1]Supplementary material, Tables S1–S9, are available from the author E.G.B. upon request.

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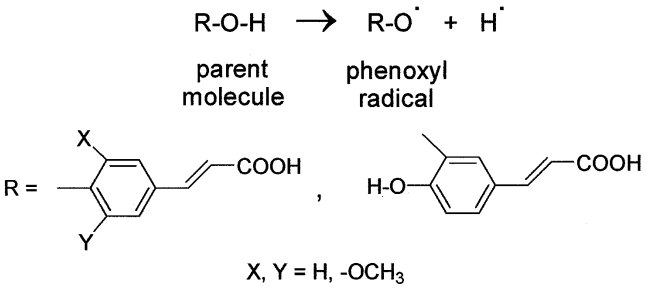
Abbreviations: ARP, antiradical power; B3LYP, Becke's three parameter hybrid functional using the Lee-Yang-Parr correlation functional; DFT, density functional theory; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; $EC_{50}$, efficient concentration; HF, Hartree-Fock; HOMO, highest occupied molecular orbital; IP, induction period; PF, protection factor.




**SCHEME 1**

account for the ease of a second radical scavenging. Hence, the molecular descriptors considered to be worth examining are the ΔHOF values and the radical spin distribution.

In the present study, high level *ab initio* and density functional theory (DFT) molecular orbital calculations, at both the HF/6-31+G(d) and B3LYP/6-31+G(d) levels, are carried out to optimize the geometries of four structurally related antioxidants, *p*-hydroxycinnamic acid derivatives, namely caffeic, sinapinic, ferulic and *p*-coumaric acids (parent molecules) and the corresponding phenoxyl radicals (Scheme 1). The geometries of the parent molecules and their phenoxyl radicals, the vibrational frequencies and the electron-donating ability of the parent molecules are examined and discussed.

The ΔHOF values, giving information about the ease with which the radical is formed, are compared, too. The radical spin distributions, which are measures of the radical stability since they provide information about the degree of delocalization as well as the electron-donating ability, are also calculated and considered. Calculations for the molecular descriptors considered in this study are CPU-intensive and physical memory-demanding. To our knowledge, no *ab initio* and/or DFT results are available regarding energetics and structural parameters for the molecules under examination. The only relative paper is that of Hueso-Urea *et al.* (22), in which, some molecular descriptors have been calculated, using semiempirical approaches, to establish structure-liquid chromatographic retention time relationships for certain phenolic compounds. Neither structural nor detailed frequency data were given in that paper. The only experimental data available for the four acids under study are solid-state X-ray crystallographic studies for caffeic and ferulic acids (23,24) and solid-state infrared spectroscopic data for all of them (25).

The *p*-hydroxycinnamic acids under study are widely examined experimentally, and the order of their activity is well established in practice. Additional experiments are also carried out to support theoretical results.

## MATERIALS AND METHODS

*Quantum-chemical calculations*. The geometries of all minimum energy structures for all four hydroxycinnamic acid derivatives and the corresponding radicals were fully optimized, employing the Hartree-Fock (HF) (26) theory (namely, the restricted HF for the parent molecules and unrestricted HF for the radicals) and the Becke's Three Parameter Hybrid Functional using the Lee-Yang-Parr correlation functional theory (B3LYP) (27) of available DFT ones, both with the 6–31+G(d) basis set (28–33). To form the radical, an H· was removed from the phenolic hydroxyl group in each acid. Diffuse functions (32) were added to heavy atoms, e.g., C and O, and inclusion of *d* polarization functions (33) on heavy atoms was considered necessary for the calculation of molecular properties. Pure *d* functions were used in the *d* shells, i.e., the *d* functions have five components. $C_1$ point group symmetry for each species was assumed as the initial geometry of the optimization procedure, and all redundant internal coordinates (34) were fully optimized (35). All calculations investigating the structural parameters of the molecules addressed here are based on final frequency calculations that provide energy minima with certainty. For each molecule full optimization calculations were performed in an attempt to determine the closest structure to the global minimum. The nonappearance of negative frequencies was assumed to be evidence for a global potential energy minimum of each calculated structure. The GAUSSIAN-94 software package (36), installed on a Hewlett-Packard 9000 series workstation (model J210 with 128 MB of memory; Hewlett-Packard, Palo Alto, CA), was used throughout this study.

The 298 K theoretical difference of heat of formation, ΔHOF, between the parent phenolic antioxidant, HOF*m*, and its free radical, HOF*f*, produced after the H-abstraction equals

$$\Delta\text{HOF} = \text{HOF}f - \text{HOF}m + \text{E}h + RT \quad [1]$$

where HOF*f*, HOF*m*, and E*h* are the sum of electronic, zero-point and thermal energies of the radical, the parent molecule, and the H atom, respectively; *R* is the gas constant, and *T* is the absolute temperature.

*Samples and standards.* Commercial refined olive oil was from Elais S.A. (Piraeus, Greece). Sinapinic acid and *p*-coumaric acid were from Sigma Chemical Co. (St. Louis, MO), whereas ferulic and caffeic acids were from Fluka (Buchs, Switzerland). Silicic acid (100–200 mesh size, Sigma Chemical Co.), celite (Riedel de Häen, Seelze, Germany), commercial sucrose, activated carbon (100 mesh size, Aldrich, Dorset, England), and *n*-hexane (Riedel de Häen) were used for column chromatography. 2,2-Diphenyl-l-picrylhydrazyl radical (DPPH) (approximately 90%) was from Sigma Chemical Co., and 2-propanol for spectroscopy was from Merck (Darmstadt, Germany). All solvents used were of appropriate grade.

*Estimation of antiradical activity of the four* p*-hydroxycinnamic acids by DPPH and antioxidant activity on olive oil triacylglycerol fraction*. The antiradical activity of the molecules under study was determined using the free radical DPPH ($6 \times 10^{-5}$ M) in ethanol (37). The reduction of DPPH was followed by monitoring the decrease in its absorbance at 515 nm, automatically recorded every 60 s. The exact initial DPPH concentration ($C_{\text{DPPH}}$) in the reaction medium was calculated from a calibration curve using the equation

$$\text{Abs}_{515\,\text{nm}} = 11{,}589 \times (C_{\text{DPPH}}) + 0.0027$$

(correlation coefficient, $r = 0.999$) [2]

where Abs = absorbance.

Different concentrations (expressed as the number of antioxidant moles per mole of DPPH) were used, and for each antioxidant, the reaction kinetics were plotted. From these graphs, the percentage of DPPH remaining at the steady state was determined. These values were transferred onto another graph showing the percentage of residual stable radical at the steady state as a function of the molar ratio of antioxidant to DPPH. The latter was used to determine the efficient concentration ($EC_{50}$), which is the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%. The lower the $EC_{50}$, the higher the antioxidant activity is. Then, the antiradical power (ARP) was calculated as $ARP = 1/EC_{50}$.

Commercial refined olive oil was purified in our laboratory using column chromatography (38) and stored at −18°C till use. The quality characteristics of the substrate were evaluated as reported elsewhere (39). Samples (2.5 g) of purified olive oil containing phenolic antioxidants dissolved in ethanol at a concentration of $2.8 \times 10^{-3}$ M and control samples were prepared, and induction periods (IP) at 120°C were estimated using a Rancimat apparatus (679 Rancimat; Metrohm Ltd., Herisau, Switzerland.). IP is considered to be the time period over which the oil is resistant to oxidation with or without the presence of antioxidants. Protection factors (PF) were calculated as the ratio of IP in the presence of the antioxidant/IP of control, i.e., PF = IP (time units)/$IP_{control}$ (time units).

## RESULTS AND DISCUSSION

*Equilibrium geometries.* All tables with the structural data for all acids and phenoxyl radicals under examination are given as Supplementary Material (Tables S1–S5), available upon request from the author. Owing to the lack of structural experimental studies in the gas phase for the same acids, the X-ray solid-state ones for caffeic (23) and ferulic (24) acids are also given in Table S1. Although comparison between results in the gas and the solid state is not allowed, it is simply mentioned that the corresponding values do not deviate more than 1%. The resulting structures for the parent molecules (**I**, *p*-coumaric acid; **II**, caffeic acid; **III**, ferulic acid; **IV**, sinapinic acid), together with the adopted numbering scheme, are given in Figure 1; those of their radical species (**V**, *p*-coumaric acid radical; **VI**, caffeic-H acid radical; **VII**, caffeic-open radical; **VIII**, caffeic acid radical; **IX**, ferulic acid radical; **X**, sinapinic acid radical) in Figure 2. This was easily accomplished, since in all structures, with only a few exceptions, the dihedral angle values were either ±180° or 0°; planar structures were derived for the substituents OH and OMe, in excellent agreement with assumptions made by Wu and Lai (40). Moreover, owing to the very low deviations derived between the corresponding structural values of the parent molecules and the phenoxyl radicals, calculated at a particular level, mean structural values are given in both Figures 1 and 2. In the case of caffeic acid, possessing two neighboring hydroxyl groups in the phenyl ring, three different radical structures were considered. The first two correspond to the radical structures deriving from the OH group at C(3), in which the intramolecular hydrogen bond is retained (**VI**), or eliminated (**VII**), the third to the structure in which an $H^{\cdot}$ was eliminated from the OH group at C(4) (**VIII**), leading also to a nonhydrogen bonding structure. The reason for this separate consideration stems from the fact that an intramolecular hydrogen bond further stabilizes any molecule.

An unexpected discrepancy, found between the HF and B3LYP levels of theory geometrical parameters, lies in the dihedral angle between the phenyl ring and the carboxyl group of the caffeic acid, being 8.5° in the former and zero in the latter. This discrepancy leads to a symmetry point group of $C_1$ for the caffeic acid at the HF level and a corresponding one of $C_s$ at the B3LYP level. The planar calculated structures for all acids and radicals at the latter imply that the molecules are completely conjugated, hence a $C_s$ symmetry point group is also assumed for them.

As shown in both Figures 1 and 2, the phenyl ring and the carboxyl groups are *trans* to each other around the connecting carbon-carbon double bond. This is the case at both levels of theory. A *trans* configuration around the connecting carbon-carbon double bond accounts well for the correctness of our results concerning both series of compounds studied, since a *trans* configuration leads, in general, to a more energetically stable structure than a *cis* one.

An inspection of the numbers appearing in Figures 1 and 2 clearly shows that the computed bond distances at the B3LYP level are constantly slightly higher than those calculated at the HF level. With only a few exceptions, the computed bond lengths obtained from both levels of theory are in agreement within 1.5 %.

It is also observed that, when a substituent hydroxyl or methoxy group is added to the *p*-coumaric acid at position *ortho* to the aroxyl group (leading to any of the three other acids under study), particular distortions at bond lengths and angles appear. It is noteworthy that the existing C(4)–O bond distance becomes longer when a hydroxyl group is added, e.g., caffeic acid by *ca*. 0.01 Å at both levels of theory, and shorter when a methoxy is added, e.g., ferulic and sinapinic acids. Furthermore, the existing phenolic O–H bond distance is slightly shorter in caffeic acid and slightly longer in ferulic and sinapinic acids, as compared to the *p*-coumaric one, at both levels of theory. This molecular descriptor may be useful in the explanation of the antioxidant activity in a series of homologs, considering that for larger bond length the hydrogen can be removed more easily, thus forming the phenolic hydroxyl. Phenolic OH bond lengths along with the inhibition of lipid oxidation values of all parent molecules studied are given in Table 1.

An inspection of the figures presented in the table clearly shows that the B3LYP O-H bond-length values are consistently higher than the HF ones. It is clear that caffeic acid, the experimentally most active molecule, is the one having the shortest OH bond length, whereas *p*-coumaric acid, the weakest antioxidant experimentally among the four studied, ex-

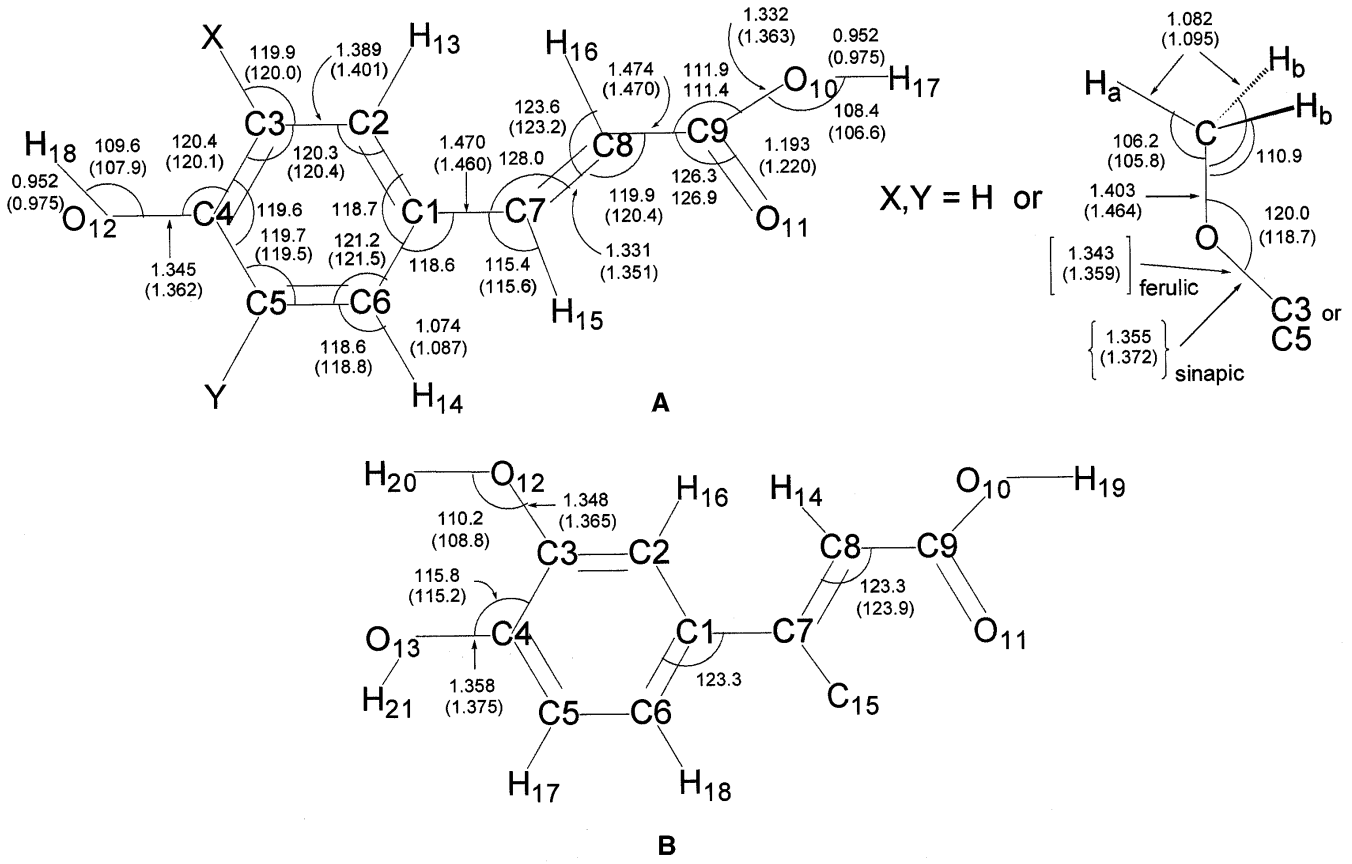



**FIG. 1.** Equilibrium structural parameters for the **I**, **III** and **IV** (A), and **II** acids (B). Density functional theory (DFT) values are given in parentheses. Where a particular structural parameter had identical values at both levels of theory, this is given once. Absence of a particular structural parameter value denotes identity to the corresponding one of the other acids. The C2-C3-X angle shown is the one for X = H; the one for X = O is 125.8° (126.1°).

hibits the second-shortest OH bond length. This could mean that phenolic OH bond length is not a fruitful molecular descriptor by itself, at this level of theory, to compare antioxidant activity of phenolic antioxidants. Zhang also reached an analogous conclusion by performing Austin Model 1 (AM1) semiempirical calculations on flavonoid antioxidants (1).

**TABLE 1**
**O—H Bond Length and HOMO Values, and Inhibition of Lipid Oxidation of the Four *p*-Hydroxycinnamic Acid Antioxidants**

| | II | IV | III | I |
|---|---|---|---|---|
| O–H[a,b] | 0.9694 | 0.9744 | 0.9742 | 0.9704 |
| O–H[c] | 0.9475 | 0.9502 | 0.9503 | 0.9478 |
| HOMO[d] | –0.3090 | –0.3029 | –0.3021 | –0.3100 |
| HOMO[e] | –0.2294 | –0.2236 | –0.2245 | –0.2333 |
| Inhibition (PF)[f] | 6.2 | 2.4 | 1.1 | 1.05 |
| Inhibition (ARP)[g] | 4.5 | 2.5 | 1.8 | 0.008 |

[a]All O–H bond lengths in Å.
[b]Parent molecule phenolic O–H bond lengths (DFT level).
[c]Parent molecule phenolic O–H bond lengths (HF level).
[d]Parent molecule HOMO energy (eV) values (HF level).
[e]Parent molecule HOMO energy (eV) values (DFT level).
[f,g]This work. HOMO, highest occupied molecular orbital; PF, protection factor; ARP, antiradical factor; DFT, density functional theory; HF, Hartree-Fock.

Formation of a hydrogen bond in both caffeic and ferulic acids is substantiated by the increase of the C(2)-C(3)-O angle by approximately 6.0° when a methoxy group is added to *p*-coumaric acid, compared to a 6.6° decrease of the C(3)-C(4)-O angle when a hydroxyl group is added instead. Sinapinic acid exhibits the same hydrogen bond structural effects with ferulic acid, as expected.

There is considerable C–C bond length alteration in the benzene ring on going from the parent molecules to the phenoxyl radicals. As a matter of fact, contrary to the equality of all C–C bonds in the acids [*ca.* 1.390 (1.400) Å], the two pairs of C–C bonds, C(1)-C(2), C(1)-C(6) and C(4)-C(3), C(4)-C(5) in the benzene ring in the radicals are longer than the C(2)-C(3) and C(5)-C(6) ones by *ca.* 0.04–0.06 Å, indicating quinoid structure. These deviations however, were larger for the former pair of C–C bonds corresponding to the C(4)-O˙ edge, than the latter corresponding to the C(1)-chain edge. Wu and Lai (40) also found very similar geometries for the B3LYP/6-31G(d) level radical structures of both phenol and anisole. Moreover, the C–O bond of the phenoxyl radical has considerable double-bond character, as indicated by the short bond length of *ca.* 1.238 (1.250) Å. The increase of the C(3)-C(4)-O(13) angle, on going from **VI** to **VII**, is probably

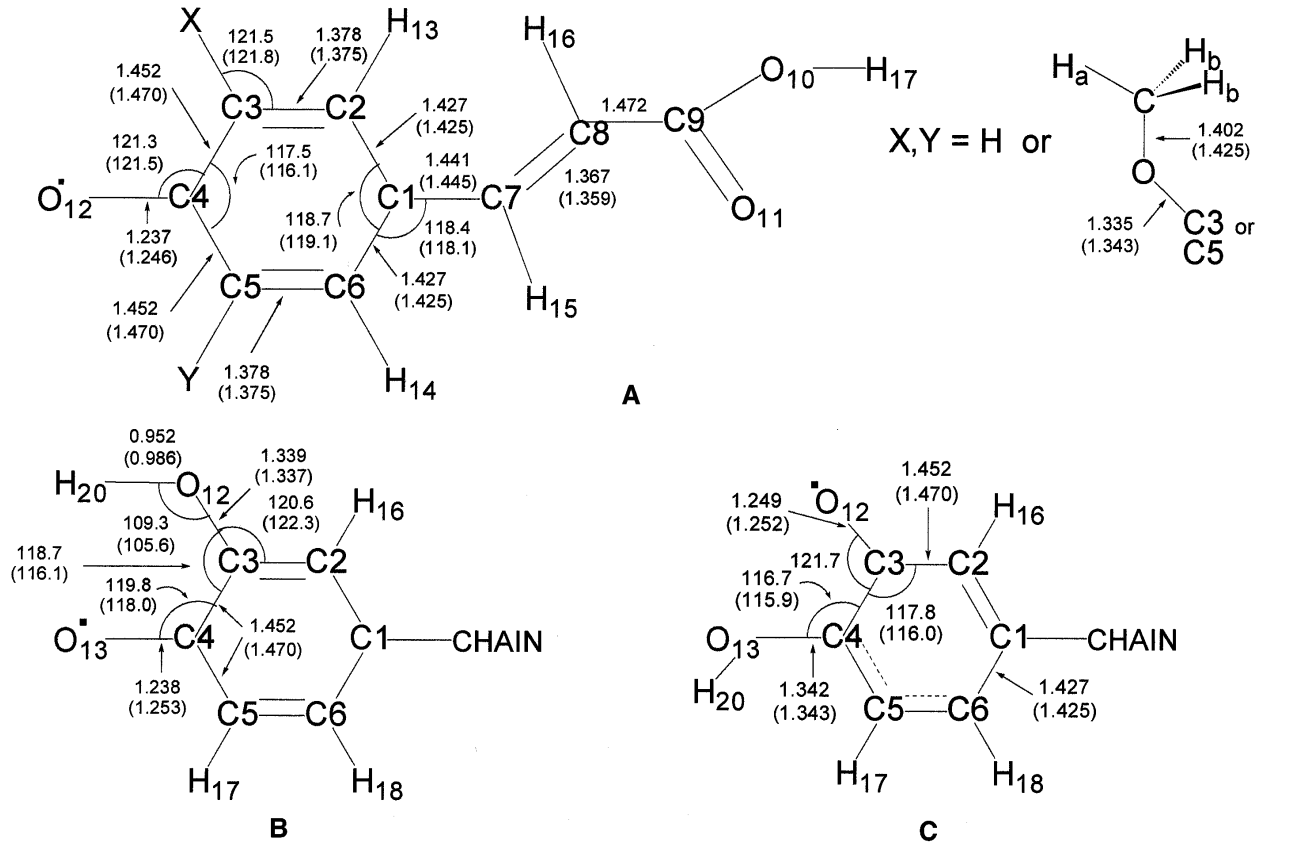



**FIG. 2.** Equilibrium structural parameters for the **V**, **IX** and **X** (A), **VI** (B) and **VII** (C) radicals. DFT values are given in parentheses. Where a particular structural parameter had identical values at both levels of theory, this is given once. Absence of a particular structural parameter value denotes identity to the corresponding one of the other acids. The C2-C3-X angle shown is the one for X = H; the one for X = O is 125.3° (125.5°). The C2-C3-O12, O13-C4-C3, C3-O12-H20, and C4-C3-O12 angle values for the **VIII** radical are 123.6° (123.9°), 121.7°, 111.3° (110.4°), and 116.2° (115.2°), respectively. For abbreviation see Figure 1.

due to the elimination of the O(13)…(H(20) hydrogen bonding. Moreover, the reduction of the C(4)-C(3)-O(12) angle and the concomitant increase of the C(2)-C(3)-O(12) one could be attributed to the H(20)…(H(16) repulsion.

Agreement between the computed values for the bond angles, obtained at both levels of theory, is within 1% or better. Dihedral angles calculated at both levels of theory are identical. However, this is mainly observed between *p*-coumaric and ferulic molecules and less for the sinapinic and caffeic ones (within 1%). The C–C and C–C–C phenyl-ring bonds and angles, and the phenolic C–O and O–H bonds are in excellent agreement with others derived theoretically (41) for the 2,6-dimethylphenol at the UHF/6–31G(d) level of theory; still, this is also the case with the carboxylic C–O, C=O and O–H bonds (42).

*Vibrational frequencies.* Frequency values calculated at the HF level contain known systematic errors (electron correlation is not taken into account, whereas B3LYP calculations include it) that produce an overestimate of about 10% compared to the experimental values. Therefore, it is usual to scale frequencies predicted at the HF level by an empirical factor of 0.8929 (43–45) along with one of 0.9613 (43–45) for the B3LYP model of DFT theory. The harmonic oscillator approach, which is used for calculated frequencies, usually produces higher values than the fundamental ones. Owing to the large size of the frequency tables for all acids under study, their unscaled calculated harmonic frequencies and infrared intensities are given as Supplementary Material (Tables S6–S9), available upon request from the author. An inspection of the figures presented in those tables clearly shows that the B3LYP frequency values are, contrary to those of the bond lengths, consistently lower than the HF ones with a minor exception for those between 200 and 450 $cm^{-1}$.

From all frequency values in Tables S6–S9 (available from the author upon request), only a few have been properly scaled and their values are given in Table 2. Those were chosen based upon the importance of the bond and the intensity of the corresponding absorption. Carboxylic acids, for instance, are mostly characterized by the O–H and C=O stretching bands (46). Table 2 includes the scaled calculated frequency values for these latter bands, as well as those for the phenolic O–H ones, along with their intensities, vibrating atoms, corresponding experimental frequencies in the vapor phase (47,48), and percentage differences between scaled calculated and experimental ones. The above frequencies are well characterized as stretch bands, since both their atom's

**TABLE 2**
**The Most Important Scaled (sc.) Calculated Harmonic Frequencies of the Four Acids, at Both Levels of Calculations, Along with Their Corresponding Experimental (exp.) Ones and the Unscaled Infrared Intensities[a]**

| | | HF | | | | DFT | |
|---|---|---|---|---|---|---|---|
| Acid | Bond | $\nu_{exp.}$[b] ($cm^{-1}$) | A[c] | $\nu_{sc.}$ ($cm^{-1}$) | DP | $\nu_{sc.}$ ($cm^{-1}$) | DP |
| **I** | O–H(20) | 3651 | 119 | 3656 | 0.14 | 3596 | 1.53 |
| | O–H(19) | 3582 | 166 | 3606 | 0.66 | 3545 | 1.04 |
| | C=O | 1762 | 478 | 1762 | 0.00 | 1715 | 2.74 |
| **II** | O–H(21) | 3651 | 143 | 3665 | 0.38 | 3616 | 0.97 |
| | O–H(20) | 3651 | 151 | 3638 | 0.36 | 3574 | 2.15 |
| | O–H(19) | 3582 | 169 | 3614 | 0.88 | 3556 | 0.73 |
| | C=O | 1762 | 923 | 1772 | 0.56 | 1710 | 3.04 |
| **III** | O–H(24) | 3582 | 172 | 3630 | 1.32 | 3552 | 0.84 |
| | O–H(23) | 3582 | 169 | 3606 | 0.67 | 3544 | 1.07 |
| | C=O | 1762 | 482 | 1763 | 0.06 | 1714 | 2.80 |
| **IV** | O–H(28) | 3582 | 178 | 3633 | 1.40 | 3547 | 0.99 |
| | O–H(27) | 3582 | 171 | 3606 | 0.67 | 3546 | 1.01 |
| | C=O | 1762 | 477 | 1764 | 0.11 | 1715 | 2.74 |

[a]The % $\nu_{sc.}/\nu_{exp.}$ discrepancy percentage (DP) is also shown.
[b]The corresponding experimental values are from References 46–48.
[c]Intensity of absorption at the HF level (arbitrary units).

orientation and eigenvector orientation lie on a single plane.

The first band examined corresponds to the phenyl O–H stretching frequencies. Those appear in the region of 3665–3630 $cm^{-1}$, where caffeic and ferulic acids define its two far ends, respectively. The corresponding absorption of *p*-coumaric acid is found at 3656 $cm^{-1}$, since there are no neighboring groups that would lead to a variation of the expected value. The formation of an intramolecular hydrogen bond between the two properly oriented neighboring hydroxy groups in caffeic acid leads to both a strengthening of the free O–H bond and a concomitant weakening of the one participating in the hydrogen bond, as compared to that of *p*-coumaric acid. In effect, this leads to both a shift toward higher frequency values (3665 $cm^{-1}$) for the former bond and a shift toward lower frequency values (3638 $cm^{-1}$) for the latter, followed by a simultaneous narrowing of both bands. This narrowing is further substantiated by its frequency intensity, since a high frequency intensity results in a narrow absorption bandwidth. This band narrowing will be discussed in the following section with respect to the corresponding absolute infrared intensities. Owing to the intramolecular hydrogen bond formation, the existing O–H covalent bond in the newly formed five-membered ring becomes weaker in all acids except *p*-coumaric. The corresponding absorption appears at a lower value (3633 $cm^{-1}$) in sinapinic acid rather than in *p*-coumaric one (3656 $cm^{-1}$). The O–H phenyl group frequency value for ferulic acid (3630 $cm^{-1}$) is very close to that of sinapinic acid because of the formation of an analogous five-membered intramolecular hydrogen-bonded ring. The lower frequency values derived for these latter two acids, as compared to that of caffeic (3665 $cm^{-1}$), is in excellent agreement with the known fact that the hydroxy group is a better electron donor than the methoxy one (49).

In a similar manner, the scaled calculated carboxylic O–H stretching frequency values range between 3606–3614 $cm^{-1}$ and are always lower than the scaled phenolic ones (see also Table 2). Moreover, it is easily seen that there are no large discrepancies between the carboxylic carbonyl C=O group stretching bands for all acids.

So far, each particular theoretical frequency value shown in Table 2 has been both assigned and explained regarding its relative shift in the infrared spectrum region. Frequency information from infrared and Raman spectra in the gas phase was not available for any of the molecules under study. Vapor phase frequency values (46–48) of the most characteristic groups closely match the calculated ones of the four acids under study. In particular, the OH stretching frequency of *m*- and *p*-substituted alkylated phenols, in vapor phase, appears as a single band in the region of 3360–3642 $cm^{-1}$ (48), and its mean value, 3651 $cm^{-1}$, is representative for comparison with *p*-coumaric acid. A frequency value lower than 3651 $cm^{-1}$ should be considered for the vapor phase of caffeic acid owing to formation of an intramolecular hydrogen bond (46). The OH stretching frequency is found at 3595–3569 $cm^{-1}$, when phenol exhibits an –OR group as an *o*-substituent (48). Hence, the mean value, 3582 $cm^{-1}$, of this latter frequency range was considered as the corresponding one for the vapor phase phenolic OH frequency values for both ferulic and sinapinic acids. The OH stretching frequency is found at 3585–3580 $cm^{-1}$, in the α,β-unsaturated monomeric carboxylic acids (47); its mean value, 3582 $cm^{-1}$, was considered as the corresponding one for the vapor-phase carboxylic OH frequency values for all acids. Finally, the vapor phase C=O band is near 1764–1760 $cm^{-1}$ in α,β-unsaturated carboxylic and its mean value, 1762 $cm^{-1}$, was considered as the vapor phase carboxylic C=O frequency for all acids.

An inspection of the values given in Table 2 clearly indicates that: (i) the calculated results coming from either level of theory are in excellent agreement with those in the vapor phase to within 1.5 %; (ii) most of the calculated frequency values are larger than the available vapor phase ones; (iii) the scaled calculated frequencies at the HF level better approximate vapor phase ones; (iv) scaled calculated HF frequencies are always larger than the DFT ones.

*Absolute infrared intensities.* The *ab initio* unscaled absolute infrared intensities for the four acids under study are also given in Table 2. An inspection of those values clearly shows that the intense infrared modes occur within the 1500–1800 and 3500–3700 $cm^{-1}$ regions. Unfortunately, there are no available experimental intensities in the gas phase for any of the four acids under study to compare with. Usually, individual band intensities cannot be accurately measured experimentally due to overlap with neighboring frequencies. However, calculated intensities of nonoverlapping bands occasionally deviate more than 50% from experimental ones (50).

As it was discussed before, formation of a five-membered intramolecular hydrogen-bond ring results in both a shift and a narrowing of the band corresponding to the covalent participating OH bond. The narrowing of the band is also substan-

tiated by its frequency intensity, since a high frequency intensity leads to a narrow absorption bandwidth. Based upon the calculated frequency intensities, it was shown that sinapinic acid exhibits the largest intensity value for the O–H stretching frequency, whereas *p*-coumaric acid the smallest. As a matter of fact, the effect of hydrogen bond formation is to increase the IR intensity and decrease the O–H stretching frequency (46). Sinapinic and ferulic acids, and to a minor extend caffeic acid, do exhibit stronger intensities of absorption as compared to those of *p*-coumaric acid due to intramolecular hydrogen-bond ring formation. It is well known that the stronger the hydrogen bond, the larger the OH stretching frequency shift toward a lower value (46), which predicts that hydrogen bonds formed in ferulic and sinapinic acids will be stronger than those in caffeic acid.

*Heat of formation, ΔHOF values.* Both Figures 1 and 2 clearly show that planar structures were derived. Structure planarity strongly supports complete conjugation within parent and radical molecular species. Intramolecular H-bond interactions found between the two neighboring phenolic hydroxyl groups in caffeic acid and between the phenolic hydroxyl group and the methoxy one in ferulic and sinapinic acids, as well as in caffeic-H acid radical, further strengthen stabilization of these molecules. The calculated ΔHOF values of the parent molecule-radical couples are shown in Table 3, which clearly states that the latter exhibits the lowest ΔHOF value among all radicals studied at both levels of theory. Caffeic acid has been reported to be more active than its counterparts (51–57). Sinapinic and ferulic acids present some antioxidant activity, whereas *p*-coumaric is almost inactive. These findings also are in agreement with experimental data given in Table 3.

ΔHOF numbers in Table 3 also indicate that there are significant energy differences between most phenoxyl radicals studied; the corresponding energy differences are in the range of 2–19 kJ/mol (DFT) and 3–23 kJ/mol (HF). The significant energy differences between the ΔHOF values at both levels of theory provide a secure and ready way to put the structurally related compounds under study in an order according to their antioxidant activity. Such differences have not been found by using semiempirical levels of theory (1). The inconsistency observed at the HF level, between the calculated ΔHOF value-trend and the experimental scavenging activity trend for some of the radicals studied, could be due to the fact that at this theoretical level electron correlation is not taken into account, whereas DFT calculations include it. The energy differences between ferulic and *p*-coumaric (DFT) and sinapinic and ferulic (HF) acids are small (2–3.3 kJ/mol). This is also the case with the experimental PF and ARP values of the former pair of compounds. The hydrogen-bonding interaction has a large stabilizing effect of *ca.* 23 kJ/mol (HF) or 34 kJ/mol (DFT) on the **VI** as compared to that of **VII**. Analogous stabilization effects have been observed in the case of the catechol radical where the corresponding energy gain was in the range of 15–25 kJ/mol (4). The higher value derived for the stabilization effect of the H-bond at the DFT level could be attributed to the functional model used. Actually, the precise choice of nonlocal functionals remains a matter of some uncertainty for the H-bonding interaction calculation. According to Gresh *et al.* (58) the Local Density Approximation seriously overestimates at 37 kJ/mol, whereas various nonlocal functionals provide lower energy results.

The spin density values of the atoms constituting the radicals studied are shown in Figure 3. An inspection of the numbers presenting in Figure 3 shows that the computed spin density values at the HF level are consistently higher than those calculated at the B3LYP level. If oxidation takes place in the phenolic OH, the spin delocalization spreads over all atoms participating in the extended conjugation system of the molecule. At each particular theoretical level, the spin density values on similar atoms are almost identical, so that the delocalization is large in all radicals studied. The planarity of the rad-

**TABLE 3**
**ΔHOF Values of the Phenoxyl Radicals, and Inhibition of Lipid Oxidation of the Four *p*-Hydroxycinnamic Acid Antioxidants**

| | VI | X | IX | V | VII | VIII |
|---|---|---|---|---|---|---|
| HOF*m*[a,b] | –1,702,667.85 | –2,106,364.34 | –1,805,790.91 | –1,505,191.55 | –1,702,667.85 | –1,702,667.85 |
| HOF*f*[c] | –1,701,069.03 | –2,104,748.41 | –1,804,154.88 | –1,503,557.31 | –1,701,032.67 | –1,701,021.89 |
| HOF*m*[d] | –1,692,808.52 | –2,094,074.43 | –1,795,190.96 | –1,496,289.63 | –1,692,808.52 | –1,692,808.52 |
| HOF*f*[e] | –1,691,350.62 | –2,092,592.33 | –1,793,705.10 | –1,494,819.00 | –1,691,325.58 | –1,691,318.76 |
| ΔHOF[f,g] | 291.56 | 308.66 | 328.77 | 326.97 | 327.91 | 338.70 |
| ΔHOF[h] | 156.00 | 180.19 | 183.94 | 168.72 | 181.03 | 187.86 |
| Inhibition (PF)[i] | 6.2 | 2.4 | 1.1 | 1.05 | — | — |
| Inhibition (ARP)[j] | 4.5 | 2.5 | 1.8 | 0.008 | — | — |

[a] All energies in kJ/mol.
[b] Sum of electronic and thermal energies of parent molecule (DFT level).
[c] Sum of electronic and thermal energies of free radical produced after H-abstraction (DFT level).
[d] Sum of electronic and thermal energies of parent molecule (HF level).
[e] Sum of electronic and thermal energies of free radical produced after H-abstraction (HF level).
[f] Calculated at the DFT level.
[g] The sum of electronic and thermal energies of the H atom are –1309.75 and –1304.39 kJ/mol in the DFT and the HF levels of theory, respectively.
[h] Calculated at the HF level.
[i,j] This work. For abbreviations see Table 1.

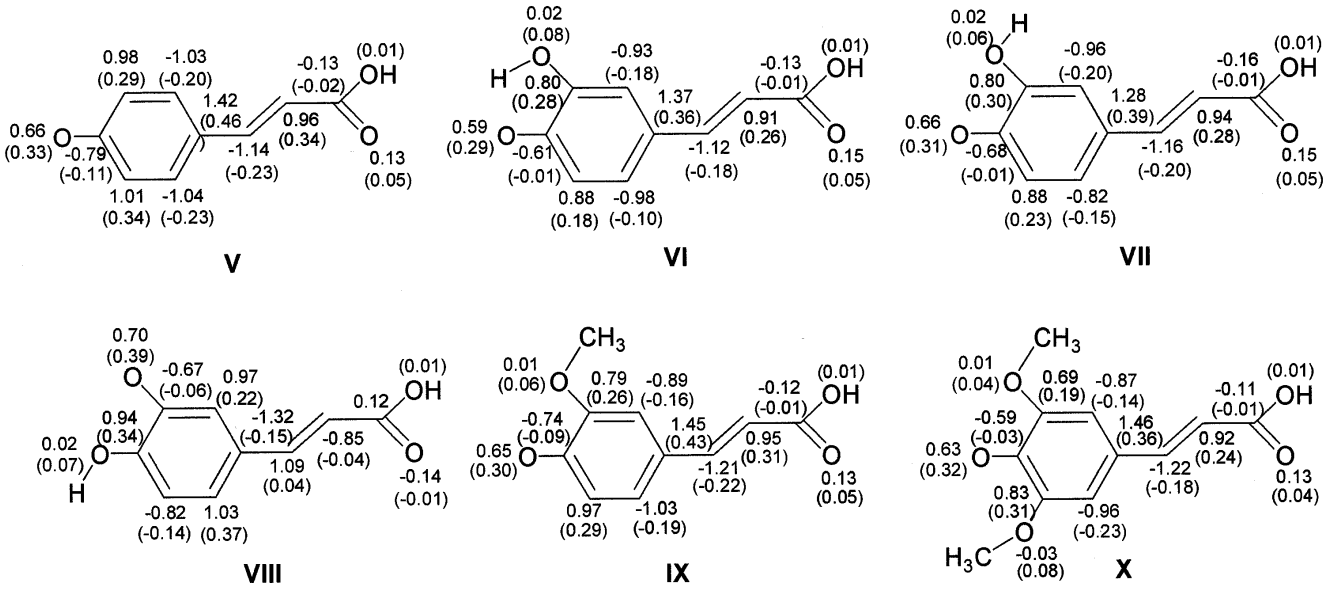



**FIG. 3.** Calculated atomic spin densities (DFT values are given in parentheses). Values of ≥0.01 are only shown; the benzene ring and the olefinic hydrogens, possessing 0.01 spin values, are omitted for clarity. For abbreviation see Figure 1.

icals leads to their full conjugation, and this, in turn, to an extended spin delocalization. Van Acker *et al.* (4) reached an analogous conclusion. The excellent delocalization possibilities of the radicals under study could account for their potential radical scavenging activity. Absence of a high amount of localized spin in these compounds may diminish the possibility to initiate a radical chain reaction (4). It is worth mentioning that, contrary to the HF spin density values for the **VIII**, the DFT ones clearly show that almost all spin remains in the benzene ring and its substituents, and the delocalization possibilities are limited. This fact could account for a lower antioxidant activity. The inferior antioxidant activity of **VIII**, as compared to that of **VII**, could be due to its higher calculated ΔHOF value, hence to its relatively harder tendency for hydrogen atom abstraction.

*Molecular electron-donating ability.* In an attempt to investigate whether the antioxidant activity correlates with the redox potentials, the HOMO energy-eigenvalues were calculated at both levels of theory. HOMO is a parameter representing molecular electron-donating ability. Calculated HOMO values are also listed in Table 1. Numbers in the table clearly show that *p*-coumaric acid, exhibiting the lowest experimental antioxidant activity, is the one with the lowest HOMO energy-eigenvalue at both levels of theory. This could account for its low experimental antiradical activity. However, caffeic acid, despite having the highest experimental antioxidant activity, exhibits the second-lowest HOMO energy-eigenvalue. Considering that an electron-donating group, for instance, the second hydroxyl of caffeic acid, should increase the HOMO energy-eigenvalue and reduce the O–H bond strength at the same time (16), the relative HOMO-energy order of the two acids appears correct. Nevertheless, the ferulic acid HOMO energy-eigenvalue, which involves a less electron donating group (methoxy) than the hydroxyl one of caffeic acid, should lie in between those of *p*-coumaric and caffeic acids. Consequently, HOMO energy-eigenvalue differences among those three acids are not suitable to predict antioxidant activity, although redox potential is expected to be directly related to their antioxidant activity. Zhang reached an analogous conclusion (16) by performing semiempirical calculations on phenolic antioxidants.

With the exception of the ΔHOF value, none of the remaining molecular descriptors by themselves could be safely used for the explanation of the antioxidant activity of the four molecules under examination. All parent-molecule phenoxyl-radical couples under study exhibit a high degree of conjugation, owing to their planarity. All radicals have almost equal, although large, spin delocalization. Both findings could account for their potential radical-scavenging activity. The higher antioxidant activity of caffeic acid, among the rest, should be attributed to its lower ΔHOF value, which arises from the less-energy-demanding hydrogen atom abstraction. It is also observed that relatively large ΔHOF value differences could lead to important changes in reactivity of a molecule *in vitro* and possibly *in vivo*. However, **VII** exhibits less antioxidant activity than **VI**, due to both its higher ΔHOF value and its limited spin delocalization. Considering that the structures of the four antioxidants studied exhibit slight differences and only one parameter is considered, the data obtained from experimental procedures and theoretical calculations are in good agreement. DFT calculations afford a good descriptor, ΔHOF, to correlate well with the antioxidant activity in molecules exhibiting similar structural parameters.

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# Formation of Cleavage Products by Autoxidation of Lycopene

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**ABSTRACT:** The cleavage products formed by autoxidation of lycopene were evaluated in order to elucidate possible oxidation products of lycopene in biological tissues. Lycopene solubilized at 50 μM in toluene, aqueous Tween 40, or liposomal suspension was oxidized by incubating at 37°C for 72 h. Among a number of oxidation products formed, eight products in the carbonyl compound fraction were identified as 3,7,11-trimethyl-2,4,6,10-dodecatetraen-1-al, 6,10,14-trimethyl-3,5,7,9,13-pentadecapentaen-2-one, acycloretinal, apo-14′-lycopenal, apo-12′-lycopenal, apo-10′-lycopenal, apo-8′-lycopenal, and apo-6′-lycopenal. These correspond to a series of products formed by cleavage in the respective 11 conjugated double bonds of lycopene. The maximal formation of acycloretinal was 135 nM in toluene, 49 nM in aqueous Tween 40, and 64 nM in liposomal suspension. Acycloretinoic acid was also formed by autoxidation of lycopene, although its formation was lower in the aqueous media than in toluene. The pig liver homogenate had the ability to convert acycloretinal to acycloretinoic acid, comparable to the conversion of all-*trans*-retinal to all-*trans*-retinoic acid. These results suggest that lycopene might be cleaved to a series of apolycopenals and short-chain carbonyl compounds under the oxidative conditions in biological tissues and that acycloretinal is further enzymatically converted to acycloretinoic acid.

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Provitamin A carotenoids are metabolized to vitamin A through enzymatic cleavage at central double bond to retinal in intestinal cells of vertebrates (1,2). Eccentric cleavage, by which double bonds of provitamin A were cleaved at random position, was also proposed as an additional pathway for retinoid synthesis (3). Thus, the enzymatic oxidations of provitamin A carotenoids have an essential role to provide vertebrates with vitamin A. However, the cleavage products such as retinal and β-apocarotenals with different carbon chain length also have been known to be produced from β-carotene by nonenzymatic oxidation under various conditions: autoxidation in solvents; oxidation with peroxy radical initiators, singlet oxygen, and cigarette smoke; and cooxidation by lipoxygenase (4–10). Canthaxanthin was also reported to give a series of cleavage products by oxidation with nickel peroxide (11). Moreover, 4-oxo-retinoic acid was identified as an oxidation product of canthaxanthin that had been incubated in a cell-culture medium and was found to activate retinoic acid receptor (RAR-β) gene promoter and to enhance gap junctional communication (12,13). The oxidation product of β-carotene, 5,8-endoperoxy-2,3-dihydro-β-apocarotene-13-one, has been shown to inhibit growth and cholesterol synthesis of breast cancer cells (14). A urinary metabolite of canthaxanthin in rats was identified as 3-hydro-4-oxo-7,8-dihydro-β-ionone (15), and one of the astaxanthin metabolites in primary culture of rat liver was found to be a glucuronide of 3-hydroxy-4-oxo-β-ionone (16). These two products were cleavage products at the 9-10 double bond of the respective carotenoid. Therefore, these reports suggest that oxidation of carotenoids including non-provitamin A carotenoids gives eccentric cleavage products in biological tissues and that some of the cleavage products are biologically active compounds as retinoids. In other words, some of the biological effects of carotenoids, whether provitamin A or not, are potentially dependent on their oxidation products formed in tissues. Thus, it is worth evaluating oxidation products of carotenoids formed under oxidative conditions in tissues and their biological effects.

Lycopene, one of non-provitamin A carotenoids, has attracted much attention for its beneficial effect on human health. The epidemiological studies showed that the high consumption of tomato and tomato-based foods rich in lycopene and the high level of plasma lycopene have been associated with a reduction of the risk of prostate cancer (17,18). Lycopene was also suggested to have a protective role in the development of arteriosclerosis (19,20). In animal experiments, dietary lycopene was demonstrated to repress the formation, induced by carcinogens, of aberrant crypto foci in the intestine (21–23). Induction of differentiation, inhibition of proliferation, and enhancement of gap junctional communication in several cancer cell lines were also observed as biological effects of lycopene (24–28). However, mechanisms underlying these biological effects still remain to be elucidated, although the chemical nature of lycopene such as radical scavenging and singlet oxygen quenching activities would be responsible for some of biological effects (29–31). Oxidation products of lycopene might be involved in biological effects

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Abbreviations: GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; RAR, retinoic acid receptor; RXR, retinoid X response; UV–VIS, ultraviolet–visible.

as mentioned above. Several oxidation products of lycopene have been reported, but little is known about their biological effects. Photosensitized oxidation of lycopene produced 2-methyl-2-hepten-6-one and apo-6′-lycopenal (32), and oxidation with hydrogen peroxide and *m*-chloroperbenzoic acid produced lycopene-1,2-epoxide and lycopene-5,6-epoxide (33,34). Khachik *et al.* (35) found 5,6-dihydroxy-5,6-dihydrolycopene in human serum as an oxidation product of lycopene. However, the formation of cleavage products of lycopene has not been documented and has received little attention. In the present study, the formation of cleavage products from lycopene under several oxidative conditions was evaluated *in vitro,* in order to investigate possible oxidation products formed in biological tissues and their relationship to the biological effects of lycopene.

## MATERIALS AND METHODS

*Materials.* Tomato oleoresin (Lyc-O-Mato™ 6%) was obtained from Ajinomoto Takara Co. (Tokyo, Japan). All-*trans*-retinal, all-*trans*-retinoic acid, Tween 40, and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine were purchased from Sigma Chemical Co. (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). *d*-α-Tocopherol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals and solvents were of reagent grade.

*Preparation of acycloretinal, apolycopenals, and acycloretinoic acid.* Lycopene, which was purified from tomato oleoresin by crystallization, was subjected to ozonolysis in ice-cold dichloromethane. Ozone gas, which was generated with a Matsui MO-5A ozone generator (Matsui Co., Tokyo, Japan) equipped with an ultraviolet (UV) lamp (8W), was bubbled into 2 mM lycopene dissolved in dichloromethane at a flow rate of 260 mL/min. The bubbling was stopped when lycopene was decreased to 10% of the initial concentration. The reaction mixture was evaporated *in vacuo* to dryness. The residue was dissolved in hexane/ethyl acetate (99:1, vol/vol), applied to a silica gel column (kiesel gel, 30–70 mesh; Merck, Darmstadt, Germany) which was preconditioned with the same solvent as above. The column was washed with an adequate amount of hexane/ethyl acetate (99:1, vol/vol), and carbonyl compounds were eluted with hexane/ethyl acetate (95:5, vol/vol).

The eluate (carbonyl compound fraction) was analyzed by HPLC with an MCPD-3600 photodiode array detector (Otsuka Electronics Co. Ltd., Osaka, Japan) and liquid chromatography–mass spectrometry (LC–MS) analyses on a TSK-Gel ODS 120T column (Tosoh Co., Tokyo, Japan) 4.6 × 250 mm. The solvent system consisted of acetonitrile/water (90:10, vol/vol) containing 0.1% ammonium acetate (solvent A) and methanol/ethyl acetate (70:30, vol/vol) containing 0.1% ammonium acetate (solvent B). A linear gradient from solvent A (100%) to solvent B (100%) was applied for 10 min at a flow rate of 1 mL/min, followed by isocratic elution with solvent B (100%) for an additional 10 min. Eight major peaks were detected at 6–12 min. They were assigned as 3,7,11-trimethyl-2,4,6,10-dodecatetraen-1-al, 6,10,14-trimethyl-3,5,7,9,13-pentadecapentaen-2-one, acycloretinal (3,7,11,15-tetramethyl-2,4,6,8,10,14-hexadecahexaen-1-al), apo-14′-lycopenal, apo-12′-lycopenal, apo-10′-lycopenal, apo-8′-lycopenal, and apo-6′-lycopenal, based upon its UV–visible (VIS) spectra and $[M + H]^+$ ion. The $\lambda_{max}$ values in their UV–VIS absorption spectra were 340, 365, 400, 420, 445, 460, 470, and 490 nm, respectively.

Acycloretinal was separated from the carbonyl compound fraction on a LiChroprep RP-18 column (40–63 μm, 11 × 200 mm; Merck) with a linear gradient from acetonitrile/water (90:10, vol/vol) to methanol/ethyl acetate (70:30, vol/vol) for 20 min at flow rate of 2 mL/min. The acycloretinal fraction was further purified on a TSK-Gel Silica 60 column (Tosoh Co.), 4.6 × 250 mm with hexane/ethyl acetate (92:8, vol/vol) as a mobile phase. $^1$H nuclear magnetic resonance (NMR) data of the purified acycloretinal were consistent with the values reported (36,37). Acycloretinoic acid (3,7,11,15-tetramethyl-2,4,6,8,10,14-hexadecahexaenoic acid) was prepared from the purified acycloretinal with Tollens reagent by the method of Barua and Barua (38). It was further purified on a TSK-Gel Silica 60 column, 4.6 × 250 mm with hexane/ethyl acetate (92:8, vol/vol) containing 0.1% acetic acid as a mobile phase. The purified acycloretinoic acid had the following spectral data: UV–VIS ($\lambda_{max}$, ethanol) 365 nm, E (1%, 1 cm) 1960; $^1$H NMR (600 MHz, $CDCl_3$) δ ppm, 1.62 (3H, *s*, C15-C$H_3$), 1.69 (3H, *s*, C16-*H*), 1.84 (3H, *s*, C11-C$H_3$), 2.01 (3H, *bs*, C7-C$H_3$), 2.13 (4H, *bs*, C12-*H,* and C13-*H*), 2.36 (3H, *s*, C3-C$H_3$), 5.10 (1H, *m*, C14-*H*), 5.80 (1H, *s*, C2-*H*), 5.98 (1H, *d*, *J* = 10.3 Hz, C10-*H*), 6.19 (1H, *d*, *J* = 12.3 Hz, C6-*H*), 6.24 (1H, *d*, *J* = 15.3 Hz, C8-*H*), 6.32 (1H, *d*, *J* = 15.2 Hz, C4-*H*), 6.60 (1H, *dd*, *J* = 15.3, 10.3 Hz, C9-*H*), 7.04 (1H, *dd*, *J* = 15.2, 12.3 Hz, C5-*H*); $^{13}$C NMR (150 MHz, $CDCl_3$) δ ppm, 13.0 (C7-*C*$H_3$), 14.2 (C3-*C*$H_3$), 17.3 (C11-*C*$H_3$), 17.8 (C15-*C*$H_3$), 25.4 (C16), 26.2 (C13), 41.0 (C12), 117.8 (C2), 124.1 (C14), 125.6 (C10), 127.4 (C9), 130.2 (C6), 131.6 (C5), 134.3 (C15), 134.6 (C8), 135.4 (C4), 138.0 (C11), 140.5 (C7), 155.0 (C3), 172.0 (C1). The carbonyl compound fraction, the purified acycloretinal and acycloretinoic acid were used as references to analyze cleavage products of lycopene by HPLC.

*HPLC analyses.* Lycopene was analyzed by HPLC on a TSK-Gel ODS 80Ts column (Tosoh Co.) 4.6 × 250 mm, attached to a precolumn (2 × 20 mm) of Pelliguard LC-18 (Supelco, Inc., Bellefonte, PA). Methanol/ethyl acetate (70:30, vol/vol) containing 0.1% ammonium acetate was used as a mobile phase at a flow rate of 1 mL/min with the photodiode array detector. The retention time of lycopene was 14.6 min. Lycopene was monitored at 470 nm and was quantified from the peak area by use of standard curve with lycopene. Acycloretinal and apolycopenals were analyzed by HPLC on the same column as above. The solvent system consisted of acetonitrile/methanol/water (75:15:10, by vol) containing 0.1% ammonium acetate (solvent A) and methanol/ethyl acetate (70:30, vol/vol) containing 0.1% ammonium acetate (solvent B). A linear gradient from solvent A (100%) to sol-

vent B (100%) was applied for 10 min at a flow rate of 1 mL/min, followed by isocratic elution with solvent B (100%) for an additional 10 min. They were monitored at each $\lambda_{max}$ with the photodiode array detector as described in section *"Preparation of acycloretinal, apolycopenals and acycloretinoic acid."* Acycloretinal was quantified from the peak area by use of standard curves of purified acycloretinal. Apolycopenals were quantified from the peak area by use of a standard curve of acycloretinal and the ratio of their extinction coefficients to that of acycloretinal. Extinction coefficient (ε) values used were as follows: acycloretinal, 56,888 (39); apo-12′-lycopenal, 84,700; apo-10′-lycopenal, 101,000; apo-8′-lycopenal, 118,300 (40); apo-6′-lycopenal, 108,290 (41). Apo-14′-lycopenal was not determined because of lack of published data on its extinction coefficient.

Acycloretinoic acid was analyzed by HPLC on the same column as above with acetonitrile/methanol/water (70:20:10, by vol) containing 0.1% acetic acid used as a mobile at a flow rate of 1 mL/min. It was monitored at 365 nm with a photodiode array detector and was quantified from its peak area of HPLC by use of a standard curve of purified acycloretinoic acid.

*Autoxidation of lycopene.* Autoxidation of lycopene, solubilized at 50 μM in toluene, aqueous Tween 40 or liposomal suspension, was carried out by incubating under atmospheric oxygen at 37°C for 72 h. Lycopene dissolved in 1 mL of toluene was placed in a long test tube (1.3 × 16 cm) and incubated at 37°C. After incubation, 0.2 mL of 0.1% α-tocopherol/ethanol was added, and the mixture was stored at −80°C until extractions. Lycopene solubilized in an aqueous 5% Tween 40 was prepared as follows. One milliliter of 5% Tween 40/acetone and 50 nmol of lycopene dissolved in dichloromethane were mixed in a test tube (1.3 × 10 cm), and solvents were removed with a stream of argon gas. The residue was dissolved in 1 mL of deionized water. Lycopene solubilized in a liposomal suspension was prepared as follows. Five micromoles of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 50 nmol of lycopene dissolved in dichloromethane were mixed in a test tube (1.3 × 10 cm), and the solvent was removed under a stream of argon and then *in vacuo* for 30 min. The residue was dispersed in 1 mL of 0.01 M Tris-HCl buffer, pH 7.4, containing 0.5 mM of diethylenetriamine pentaacetic acid by mixing with a vortex mixer for 1 min and sonicating with a Branson 1210J ultrasonicate (Branson, Danbury, CT) for 30 s at ambient temperature. Lycopene solubilized in an aqueous Tween 40 and liposome was incubated at 37°C with continuous shaking at 120 rpm. After incubation, 1 mL of 0.02% α-tocopherol/ethanol was added, and the mixture was stored at −80°C until extractions. For evaluation of acycloretinoic acid formation in the aqueous media, the incubation mixture was scaled up from 1 to 20 mL because of the limited formation.

*Extraction of oxidation products*. Residual lycopene and its oxidative products formed in toluene were extracted as follows. The stored mixture was evaporated to dryness under argon gas, and the residue was redissolved in 2 mL of acetone. A 20-μL aliquot was subjected to an HPLC analysis under conditions used for the analysis of lycopene. For extraction of acycloretinal and apolycopenals, the stored mixture was evaporated to dryness under argon gas and the residue was redissolved in 300 μL of hexane/ethyl acetate (99:1, vol/vol). The residue was applied to a Bond Elut solid-phase cartridge (SI 100 mg; Varian, Harbor, CA), preconditioned with the same solvent as above. The cartridge was washed with 1 mL of hexane/ethyl acetate (99:1, vol/vol). The eluate with 3 mL of hexane/ethyl acetate (95:5, vol/vol) was dried and the residue was dissolved in 200 μL of acetonitrile. A 100-μL aliquot of the eluate was subjected to an HPLC analysis under conditions used for the analysis of acycloretinal and apolycopenals. For extraction of acycloretinoic acid, the stored mixture was evaporated to dryness under argon gas. The residue was dissolved in 1 mL of ethanol, and then 1 mL of 0.1 N NaOH was added. After washing three times with 2 mL of hexane, the aqueous phase was acidified with 30 μL of 6 N HCl, and acycloretinoic acid was extracted three times with 2 mL of hexane. The combined extract was dried and the residue was dissolved in 200 μL of acetonitrile/methanol/water (70:20:10, by vol) containing 0.1% acetic acid. A 100-μL aliquot of the extract was subjected to HPLC analysis.

Residual lycopene and its oxidation products formed in an aqueous Tween 40 and liposomal suspension were extracted as follows. Lycopene was extracted three times with 2 mL of hexane from the stored mixture. The combined extract was evaporated to dryness under argon gas, and the residue was dissolved in 2 mL of acetone. A 20-μL aliquot of the final extract was subjected to an HPLC analysis under conditions used for analysis of lycopene. Acycloretinal and apolycopenals were extracted as in the case of lycopene, and the extract was dissolved in 300 μL of hexane/ethyl acetate (99:1, vol/vol). The extract was fractionated with a Bond Elut solid-phase cartridge and subjected to HPLC analysis as described above. For the extraction of acycloretinoic acid, 2 mL of 1 N NaOH was added to the stored mixture (40 mL). The mixtures were washed three times with 40 mL of hexane. The aqueous phase was acidified with 600 μL of 6 N HCl, and then acycloretinoic acid was extracted three times with 40 mL of hexane. The combined extracts were evaporated to dryness under argon gas and dissolved in 200 μL of hexane/ethyl acetate (92:8, vol/vol). A 50 μL aliquot of the extract was subjected to fractionation by HPLC on a TSK-Gel Silica 60 column, 4.6 × 250 mm, attached to a precolumn (2 × 20 mm) of Pelliguard LC-SI (Supelco, Inc.) with hexane/ethyl acetate (92:8, vol/vol) containing 0.1% acetic acid as a mobile phase at flow rate of 1 mL/min. The combined fraction of acycloretinoic acid from three-time fractionations was evaporated to dryness under argon gas, and the residue was redissolved in 200 μL of acetonitrile/methanol/water (70:20:10, by vol) containing 0.1% acetic acid. A 100-μL aliquot of the acycloretinoic acid fraction was subjected to HPLC analysis.

*Conversion of acycloretinal to acycloretinoic acid by liver homogenate.* The pig liver was homogenized with a Potter-Elvehjem homogenizer in 5 vol of 0.1 M $KH_2PO_4$–$K_2HPO_4$ buffer, pH 7.4, and 0.15 M KCl. A supernatant solution of homogenate, after centrifugation at 9,000 × *g* for 30 min, was

used to evaluate metabolic activity of liver homogenate for conversion of acycloretinal to acycloretinoic acid under the modified conditions reported previously (42). The final reaction mixture contained 10 μM acycloretinal or all-*trans*-retinal, 0.5 M Tricine-KOH buffer, pH 8.0, 150 mM KCl, 2 mM NAD, 2 mM dithiothreitol, and homogenate (9.1 mg protein) in a total volume of 1 mL. The mixture of all the components except for acycloretinal or retinal was first preincubated at 37°C for 10 min. The reaction was then initiated by adding 5 μL of 2 mM acycloretinal or all-*trans*-retinal in dimethyl sulfoxide, and the mixture was incubated at 37°C for 60 min. The reaction was stopped by adding 3 mL of 0.025 N KOH in ethanol. The mixture was washed two times with 6 mL hexane. The aqueous phase was then acidified with 0.12 mL of 6 N HCl, and acycloretinoic acid was extracted three times with 6 mL of hexane containing 0.001% butylated hydroxytoluene. The combined extract was dried under a stream of argon gas, dissolved in 200 μL of acetonitrile/methanol/water (70:20:10, by vol) containing 0.1% acetic acid and subjected to HPLC analysis. Protein concentration in the homogenate was determined by Bradford's method (43) with the bovine serum albumin as standard.

*Spectral analyses.* To identify the cleavage products obtained from ozonolysis of lycopene, the carbonyl compound fraction was subjected to an LC–MS analysis. Positive-ion mass spectra were obtained using a model M-1200AP mass spectrometer equipped with an atmospheric pressure chemical ionization–MS interface, and a model L-7100 gradient HPLC system (Hitachi Co., Tokyo, Japan).

For GC–MS analysis, the acycloretinoic acid formed by autoxidation of lycopene in toluene was purified by HPLC both on a silica column and on an ODS column under the condition described in section *"Preparation of acycloretinal, apolycopenals, and acycloretinoic acid."* The purified acycloretinoic acid and the standard acycloretinoic acid synthesized from acycloretinal were converted to methyl ester with diazomethane. The methyl ester of acycloretinoic acid was analyzed by GC–MS. The separation of acycloretinoic acid methyl ester was carried out on an MP65HT fused-silica column with 65% phenyldimethylsiloxane as a bonded phase, 10 m × 0.25 mm (Quadrex Co., Woodbridge, CT), using helium gas at a flow rate of 1.8 mL/min. The splitless injector and interface were both at 250°C. The column temperature was raised from 60 to 250°C at the rate of 40°C/min and then held at 250°C for 10 min. The GC column was coupled to a Shimadzu QP-5000 MS system (Shimadzu Co., Kyoto, Japan) with electron impact ionization at 70 eV.

The $^{1}H$ and $^{13}C$ NMR spectra of acycloretinal and acycloretinoic acid were obtained in a solution of $CDCl_3$ with a Bruker (Karlsruhe, Germany) DRX 600 spectrometer.

## RESULTS

Autoxidation of lycopene under the three different conditions, where lycopene was solubilized in toluene, aqueous Tween 40, or liposomal suspension, produced a number of compounds with absorption in the UV–VIS region. The crude extract from the autoxidation mixture was fractionated on a silica column (Bond Elut SI), and the eluate with hexane/ethyl acetate (95:5, vol/vol) showed clearly the presence of a series of carbonyl compounds that were formed by cleavage of conjugated double bonds of lycopene. Figure 1 shows an HPLC chromatogram of the carbonyl compound fraction obtained from the oxidized lycopene in liposomal suspension. The peaks from 1 to 8 were assigned by comparing retention time and UV–VIS spectra with those of the reference cleavage products prepared by ozonolysis of lycopene. They had a characteristic bell-shaped UV–VIS absorption spectrum similar to that of all-*trans*-retinal, but different with $\lambda_{max}$ (Fig. 2). Peak 3 (acycloretinal) was the cleavage product at the central double bond of lycopene. Peak 1 (3,7,11-trimethyl-2,4,6,10-dodecatetraen-1-al) and peak 5 (apo-12′-lycopenal) were the cleavage products at the C11–C12 double bond. Peak 2 (6,10,14-trimethyl-3,5,7,9,13-pentadecapentaen-2-one) and peak 4 (apo-14′-lycopenal) were the cleavage products at the C13–C14 double bond. Long-chain apolycopenals such as apo-10′-lycopenal (peak 6), apo-8′-lycopenal (peak 7) and apo-6′-lycopenal (peak 8) were also detected. Their structures are shown in Figure 3.

The extract under acidic conditions from the oxidized lycopene in toluene contained the oxidation product with the same retention time (9.8 min) and UV–VIS spectra as standard acycloretinoic acid in HPLC analysis (Fig. 4, A and D). The oxidation product was also detected when lycopene solubilized in an aqueous Tween 40, or a liposomal suspension

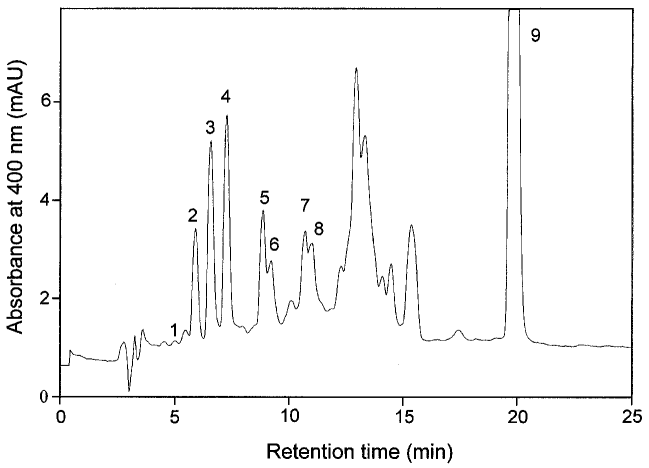


**FIG. 1.** High-performance liquid chromatography (HPLC) chromatogram of cleavage products by autoxidation of lycopene. Lycopene was solubilized at 50 μM in the liposomal suspension of 5 mM dimyristoylphosphatidylcholine and incubated at 37°C for 24 h. The cleavage products extracted from the incubation mixture were separated by HPLC on a TSK-Gel ODS 80Ts column, 4.6 × 250 mm, and were monitored at 400 nm with the photodiode array detector as described in the Materials and Methods section. Peak 1, 3,7,11-trimethyl-2,4,6,10-dodecatetraen-1-al (5.1 min); peak 2, 6,10,14-trimethyl-3,5,7,9,13-pentadecapentaen-2-one (5.9 min); peak 3, acycloretinal (6.6 min); peak 4, apo-14′-lycopenal (7.3 min); peak 5, apo-12′-lycopenal (8.9 min); peak 6, apo-10′-lycopenal (9.2 min); peak 7, apo-8′-lycopenal (10.7 min); peak 8, apo-6′-lycopenal (11.0 min); peak 9, lycopene (19.9 min).

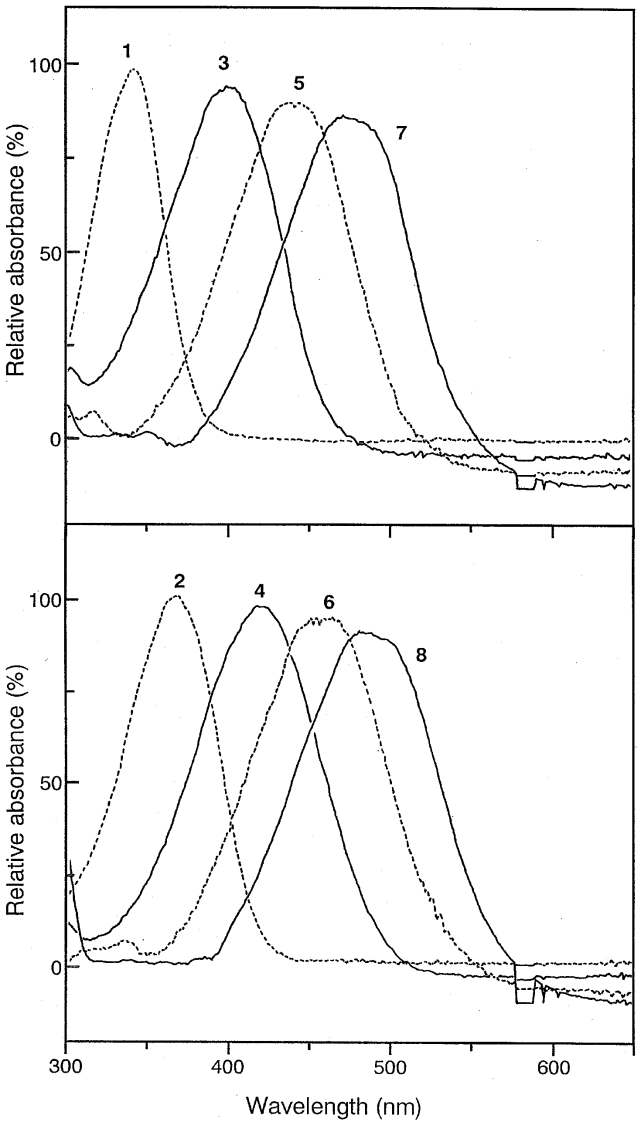


**FIG. 2.** Ultraviolet–visible (UV–VIS) spectra of the cleavage products formed from lycopene. The spectra of the cleavage products detected in Figure 1 were measured with the photodiode array detector. The number indicates the peak number in Figure 1.

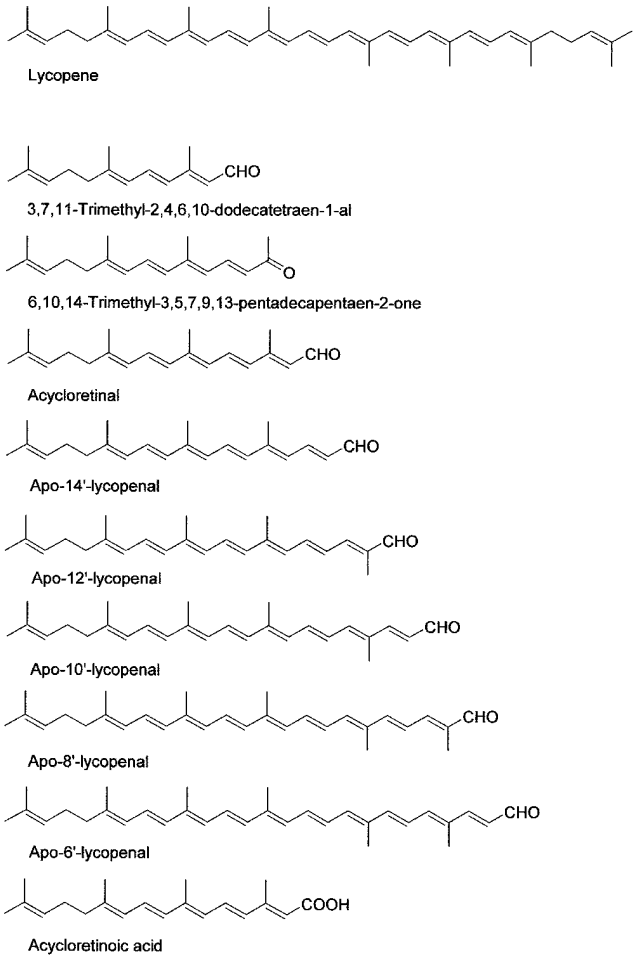


**FIG. 3.** Structure of cleavage products formed by autoxidation of lycopene.

was incubated (Figs. 4B and 4C). The acidic extract from the oxidized lycopene in the aqueous media had to be fractionated by HPLC on a silica column prior to detection of the oxidation product by HPLC on ODS column, because the amount of the oxidation product formed in these aqueous conditions was very low in comparison with those of the interfering substances. The oxidation product formed in toluene was isolated and purified by HPLC with both silica and ODS columns and then was methylated with diazomethane. The oxidation product at 9.8 min disappeared in the HPLC profile, while a new peak appeared with the same retention time (21.3 min) and UV–VIS spectrum as standard acycloretinoic acid methyl ester. The methylated oxidation product gave the same mass fragmentation as standard acycloretinoic acid methyl ester by GC–MS analyses (Fig. 5). A molecular ion at *m/z* 314 and $[M - 69]^+$, which lost a terminal dimethylallyl moiety, was observed. Thus, the oxidation product found in acidic extract from the oxidized lycopene under the three different conditions was assigned as acycloretinoic acid.

The decrease of lycopene and the formation of its oxidation products were quantitatively evaluated during the course of autoxidation at 37°C for 72 h under the three different conditions (Fig. 6). Among the eight cleavage products, 3,7,11-trimethyl-2,4,6,10-dodecatetraen-1-al, 6,10,14-trimethyl-3,5,7,9,13-pentadecapentaen-2-one, and apo-14′-lycopenal were not quantified because of the lack of their extinction coefficient in the literatures, although they showed remarkably large peaks in the HPLC profiles monitored at their wavelength of maximum UV–VIS absorption. Lycopene was more susceptible to autoxidation in toluene than in aqueous Tween 40 and in liposomal suspension. Lycopene in toluene was decreased from 50 to 0.9 μM after 48-h incubation. On the other hand, the lycopene solubilized in an aqueous Tween 40 or a liposomal suspension decreased to 9.3 and 26.9 μM, respectively. The amounts of total apolycopenals, including acycloretinal, apo-12′-lycopenal, apo-10′-lycopenal, apo-8′-lycopenal, and apo-6′-lycopenal, reached maxima of 819.3 and 387.0 nM after 24-h incubation in toluene and aqueous Tween 40, respectively, while in liposome, total apolycope-

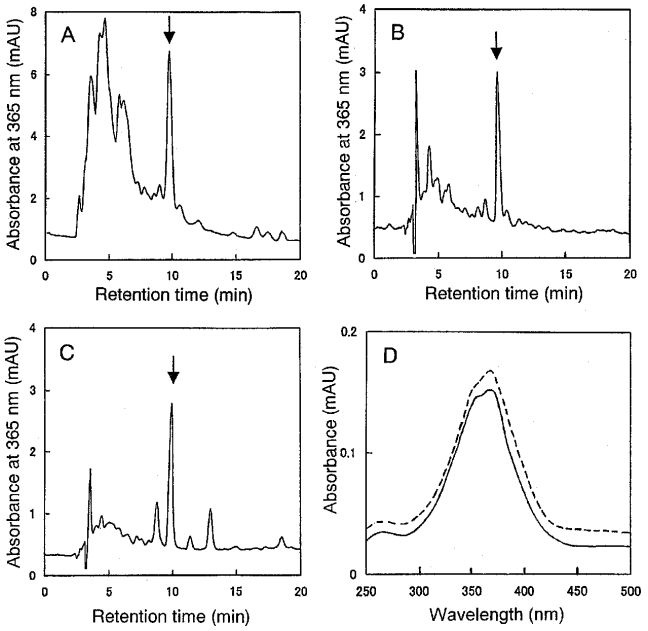


**FIG. 4.** HPLC analysis of acycloretinoic acid formed by autoxidation of lycopene. Lycopene was solubilized at 50 μM in toluene (A), in aqueous 5% Tween 40 (B), and in the liposomal suspension of 5 mM dimyristoylphosphatidylcholine (C). After incubation at 37°C for 24 h (A, B) or 72 h (C), the extracts of the incubation mixture were analyzed by HPLC as described in the Materials and Methods section. UV–VIS spectra (D) of the HPLC peaks of standard acycloretinoic acid (broken line) and of acycloretinoic acid formed (solid line) in toluene were measured with the photodiode array detector. The arrow indicated the retention time (9.8 min) of standard acycloretinoic acid. See Figures 1 and 2 for abbreviations.

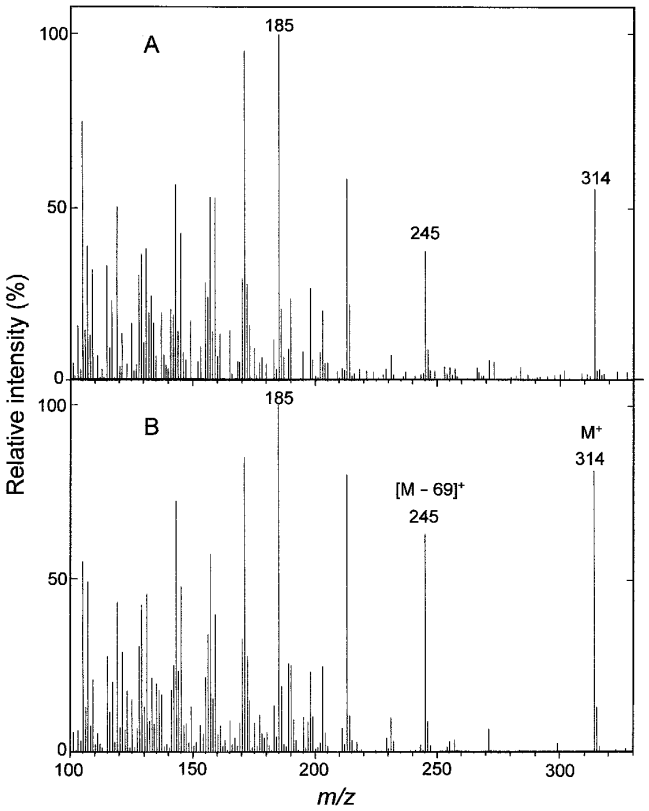


**FIG. 5.** Mass spectra of acycloretinoic acid methyl ester. (A) Methyl ester of the cleavage product formed by autoxidation of lycopene in toluene; (B) standard acycloretinoic acid methyl ester.

nals increased continuously to 238 nM at 72-h incubation. The ratio of maximal formation of acycloretinal to the initial level of lycopene (50 μM) was 0.3% (135 nM) in toluene, 0.1% (49 nM) in aqueous Tween 40, and 0.13% (64 nM) in liposomal suspension. Acycloretinoic acid reached 52.5 nM at 48 h in toluene with about 0.1% molar ratio to initial lycopene level. In an aqueous Tween 40 and liposome, acycloretinoic acid formation was also detected although its formation was at the lower level (1–2 nM) than in toluene. Formation of apo-6′-lycopenal was not observed in toluene and in an aqueous Tween 40, but a significant amount of apo-6′-lycopenal was detected in liposomal suspension (Table 1). The level of apo-10′-lycopenal in toluene was the highest among the aldehydes evaluated at any time point, while apo-8′-lycopenal was the highest in an aqueous Tween 40, and apo-6′-lyocpenal level was the second highest to acycloretinal in a liposomal suspension. Thus, acycloretinal and a series of apolycopenals were produced by autoxidation of lycopene in the three different conditions. However, acycloretinoic acid formation was significantly suppressed under the aqueous conditions.

To evaluate the metabolic conversion of acycloretinal into acycloretinoic acid in tissue homogenate, pig liver homogenate was incubated with 10 μM acycloretinal. Acycloretinal and all-*trans*-retinal were efficiently converted to the corresponding carboxylic acids by incubating with pig liver homogenate as shown in Table 2. No conversion in the control incubations of minus homogenate and heat-inactivated homogenate indicated the enzymatic catalysis. The conversion ratios of the incubated acycloretinal and retinal to the

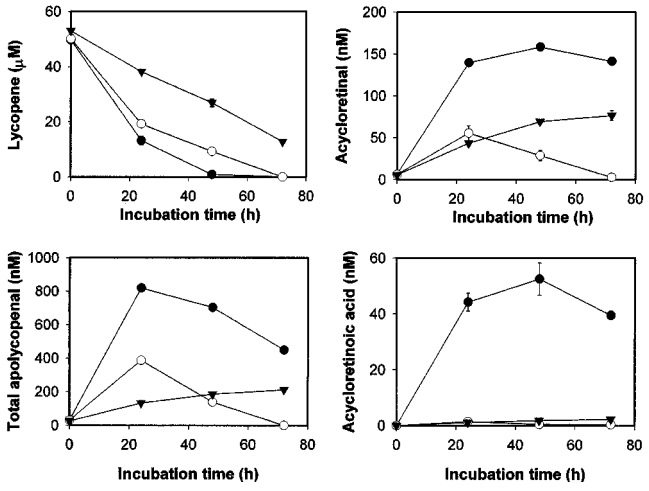


**FIG. 6.** Formation of cleavage products during autoxidation of lycopene under three different conditions. Lycopene was solubilized in toluene (●), 5% aqueous Tween 40 (○), and liposomal suspension of 5 mM dimyristoylphosphatidylcholine (▼) and incubated at 37°C for 72 h. Total apolycopenal was expressed as the combined amount of acycloretinal, apo-12′-lycopenal, apo-10′-lycopenal, apo-8′-lycopenal, and apo-6′-lycopenal. Values represent means ± SD ($n$ = 3).

**TABLE 1**
**Formation of Cleavage Products After 24-h Incubation of Lycopene at 37¡C[a]**

| | Solubilization of lycopene | | |
|---|---|---|---|
| | Toluene | Aqueous Tween 40 | Liposomal suspension |
| Residual lycopene (%) | 26.6 ± 3.0 | 38.3 ± 1.8 | 72.1 ± 0.4 |
| Acycloretinoic acid (nM) | 44.2 ± 3.2 | 1.4 ± 0.2 | 1.0 ± 0.1 |
| Acycloretinal (nM) | 129.0 ± 1.5 | 43.3 ± 9.0 | 32.2 ± 3.7 |
| Apo-12′-lycopenal (nM) | 135.4 ± 10.5 | 50.8 ± 5.3 | 13.8 ± 2.0 |
| Apo-10′-lycopenal (nM) | 362.2 ± 12.1 | 107.8 ± 8.8 | 12.3 ± 0.7 |
| Apo-8′-lycopenal (nM) | 168.6 ± 7.5 | 153.9 ±13.7 | 17.0 ± 1.7 |
| Apo-6′-lycopenal (nM) | ND[b] | ND | 30.8 ± 1.0 |

[a]Values represent means ± SD ($n$ = 3).
[b]ND, not detected (<1 nM).

acids were 96.3 and 79.1%, respectively, after incubation for 60 min.

## DISCUSSION

Carotenoids are highly susceptible to oxidation under certain oxidative conditions to produce a number of compounds. Recently several studies reported that oxidation products of carotenoids showed biological effects *in vitro*. Thus, some of the oxidation products might participate in biological effects of carotenoids reported in animal models and cell culture system. We have evaluated the *in vitro* oxidation products of lycopene, a typical non-provitamin A carotenoid, and have identified a homologous series of carbonyl compounds formed by cleavage at the conjugated double bonds and an analog of retinoic acid.

Numerous oxidation products appeared by autoxidation of lycopene under the three different conditions. In toluene, lycopene was homogeneously dissolved in nonaqueous media. Lycopene was solubilized as a micelle in an aqueous Tween 40 and was incorporated in a phospholipid membrane of liposome as a model of biological tissues. The carbonyl compounds were clearly separated in HPLC analysis after fractionation on a small silica column. The eight products identified were 3,7,11-trimethyl-2,4,6,10-dodecatetraen-1-al, 6,10,14-trimethyl-3,5,7,9,13-pentadecapentaen-2-one, acycloretinal, apo-14′-lycopenal, apo-12′-lycopenal, apo-10′-lycopenal, apo-8′-lycopenal, and apo-6′-lycopenal. Lycopene was oxidized rapidly in toluene, and considerable amounts of acycloretinal and apolycopenals were produced, compared with autoxidation in an aqueous Tween 40 and in liposomal suspension. The chain reaction in autoxidation of lycopene might be disturbed in the latter conditions, because of difficult interaction between lycopene molecules separated by Tween 40 and phosphatidylcholine bilayers. That formation of long-chain 6′-lycopenal was not detected in toluene, while it was a major apolycopenal in liposomal, suggests that 6′-lycopenal is further oxidized to a short-chain apolycopenal under the accelerated autoxidation in toluene.

These results were consistent with the autoxidation of β-carotene in organic solvent. Mordi *et al*. (5) proposed a cleavage reaction at the double bond through a dioxetane from peroxy radical of β-carotene. McClure and Liebler (6) identified apocarotenals as well as oxygenated apocarotenal as oxidation products of β-carotene initiated by azobis(2,4-dimethylvaleronitrile). They also identified β-apo-14′-carotenal, β-apo-10′-carotenal, and β-apo-8′-carotenal as products of β-carotene oxidized by singlet oxygen, suggesting 1,2-addition of singlet oxygen at each double bond to form dioxetane (7). Thus, taken together, the results in our study and in the previous reports suggest that the cleavage reaction to carbonyl compounds at the conjugated double bond by autoxidation, radical-mediated oxidation, and singlet oxygen occurs in any carotenoid with a long-chain of conjugated double bonds. These cleavage products may be produced *in vivo* if the tissues are exposed to an oxidative stress.

In terms of biological effects of oxidation products, it is important to know whether a compound analogous to retinoic acid is produced by oxidation of carotenoids, because retinoic acid is involved in regulation of gene expression through the nuclear receptors of RAR and RXR. Mordi *et al.* (5) suggested the formation of retinoic acid by autoxidation of β-carotene. In the present study, we clearly demonstrate the formation of acycloretinoic acid from lycopene by autoxidation in toluene at a significant amount comparable to each apolycopenal. Nikawa *et al.* (12) found 4-oxo-retinoic acid as oxidation product of canthaxanthin when incubated in a cell culture medium. We also found formation of acycloretinoic acid from lycopene in aqueous media, although the amount was much lower than that formed in toluene. The presence of water was suggested to suppress the formation of acycloretinoic acid from lycopene by autoxidation. Therefore, acycloretinoic acid formation would be very low if lycopene is oxidized in biological tissues. However, a conversion of acycloretinal to acycloretinoic acid might occur in biological tissues, because the biosynthesis of retinoic acid from retinol is mediated by microsomal retinol dehydrogenase and cytosolic retinal dehydrogenase (44). We found that the liver ho-

**TABLE 2**
**Conversion of Acycloretinal to Acycloretinoic Acid by Pig Liver Homogenate[a]**

| | Acycloretinoic acid | Retinoic acid |
|---|---|---|
| | (pmol/mg protein/h incubation) | |
| Complete incubation | 1,071 ± 24.2 | 902 ± 3.4 |
| Zero-time control | 6.6 ± 1.1 | 28.3 ± 1.3 |
| Minus homogenate control | 6.2 ± 1.3 | 22.0 ± 1.1 |
| Minus substrate control | ND[b] | 23.2 ± 1.1 |
| Heat-inactivated homogenate control[c] | 1.1 ± 0.0 | 18.2 ± 2.4 |

[a]Note: Acycloretinal and retinal were incubated at 10 μM with pig liver homogenate (9.1 mg protein/mL) at 37°C for 60 min, and the amounts of acycloretinoic and retinoic acids in the extracts were measured by high-performance liquid chromatography. Values are expressed as means ± standard deviations of triplicate incubations.
[b]ND, less than 0.1.
[c]Treatment of heat-inactivated homogenate was 100°C for 10 min.

mogenate had metabolic activity for conversion of acycloretinal to acycloretinoic acid, comparable to that for conversion of retinal to retinoic acid, although we did not evaluate whether apolycopenals were converted to the corresponding acids. Thus, acycloretinoic acid is potentially formed from acycloretinal, when lycopene is oxidized in biological tissues.

The results in the present study suggest that carotenoids including non-provitamin A carotenoids are nonenzymatically cleaved to a series of carbonyl compounds at conjugated C–C double bonds when the tissue is exposed to oxidative stress. Furthermore, central cleavage products among them may be enzymatically converted to the corresponding acids analogous to retinoic acid. This hypothesis is in part similar to the eccentric cleavage pathway (3) proposed for vitamin A formation in addition to the central cleavage (1). However, the eccentric cleavage was thought to occur by enzyme ubiquitously distributed in various tissues, while it was not evaluated whether non-provitamin A carotenoids were also cleaved by the same enzyme system.

In the present study, we demonstrated *in vitro* formation of acycloretinoic acid from lycopene by autoxidation. However, biological activities of acycloretinoic acid as an agonist of retinoic acid were not so marked as retinoic acid. Araki *et al.* (45) found that acycloretinoic acid could not activate a retinoic acid receptor element (RARE) of RAR-β gene and a retinoid X receptor element (RXRE) of human cellular retinol-binding protein Type II gene, but could bind to cellular retinoic acid binding protein. On the other hand, Stahl *et al.* (24) showed that acycloretinoic acid induced gap junctional communication and transactivated RAR-β2 promoter, although it was less active than retinoic acid. Several oxidation products of carotenoids other than lycopene are already known to have biological effects. 4-Oxo-retinoic acid, an oxidation product of canthaxanthin, was found to work as a ligand for RAR and to induce differentiation of cancer cells (12). 5,8-Endoperoxy-2,3-dihydro-β-apocarotene-13-one, an oxidation product of β-carotene, showed a growth inhibition of breast cancer cells (14). 5,6-Epoxy-β-carotene was reported to have significantly greater differentiation-inducing activity than β-carotene toward NB4 human leukemia cells (46). These results strongly suggest that oxidation products as well as intact carotenoids potentially have biological effects on human health. The formation of cleavage products of non-provitamin A carotenoids has never been demonstrated in the human body. However, 3-hydro-4-oxo-7,8-dihydro-β-ionone and 3-hydroxy-4-oxo-β-ionone were identified as the metabolites of canthaxanthin in rats and astaxanthin in rat hepatocytes, respectively (15,16). The results suggest the *in vivo* formation of the cleavage products of carotenoids. The detection and identification of oxidation products of carotenoids formed *in vivo* are needed to clarify their possible involvement in biological effects of carotenoids.

## ACKNOWLEDGMENTS

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# Cyclodehydration Reactions of Methyl 9,10-; 10,12-; and 9,12-Dioxostearates with 1,2-Diaminoethane Under Ultrasonic Irradiation

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**ABSTRACT:** Reaction of methyl 9,10-dioxostearate (**1**) and 9,12-dioxostearate (**2**) with 1,2-diaminoethane under concomitant ultrasonic irradiation (10–15 min, 60°C) in water furnished the corresponding 2,3-dihydropyrazine (**4**, 79%) and 1,2,3,4-tetrahydro-1,4-diazocine (**5**, 70%) derivatives, respectively. Reaction of methyl 10,12-dioxostearate (**3**) with 1,2-diaminoethane was successful only when glacial acetic acid was used instead of water under ultrasonic irradiation (4 × 10 min, 70°C) to give a 2,3-dihydro-1*H*-1,4-diazepine (**6**, 95%) derivative. The structures of these novel six-membered (**4**), seven-membered (**6**), and eight-membered (**5**) *N*-heterocyclic fatty ester derivatives were confirmed by a combination of infrared, nuclear magnetic resonance spectroscopic and mass spectral analyses.

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Many organic reactions under concomitant ultrasonic irradiation show significant increases in their rates of reaction (1). We have reported the successful preparation of numerous novel derivatives of fatty acid esters under such "extreme" conditions. Some of the products cannot be obtained when the same reactions are conducted under thermal conditions (2–4).

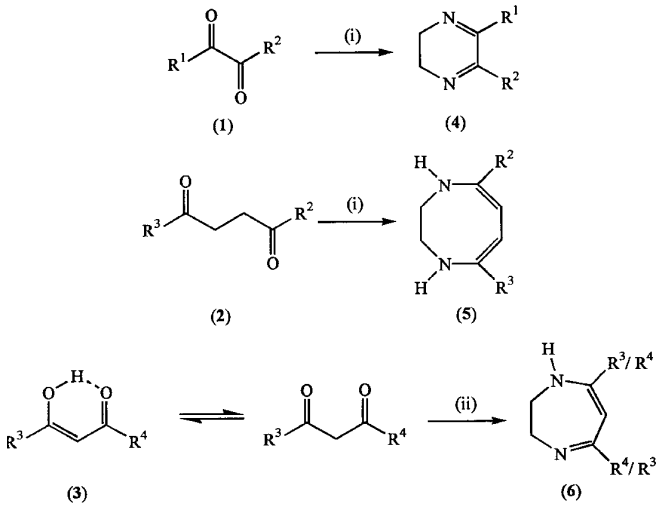
Fatty esters containing an *N*-heterocyclic nucleus (forming a part of the alkyl system of the fatty acid chain) were prepared by reaction of oxygenated $C_{18}$ fatty ester derivatives under concomitant ultrasonic irradiation. For example: methyl 12-azido-9-oxostearate reacted with triphenylphosphine in tetrahydrofuran to give a 1-pyrroline fatty ester derivative (2); ultrasound-assisted reaction of methyl 10,12-dioxostearate with hydrazines in water gave $C_{18}$ pyrazole derivatives (3); and methyl 9,12-dioxostearate reacted with hydrazines in water under ultrasound to yield a pyridazine derivative (4). It was interesting to realize that in the last two cases, the reactions were conducted entirely in water without any organic solvents despite the fact that the substrates were insoluble in water. Reactions carried out under ultrasonic irradiation appeared to overcome the problem of solubility of fatty ester substrates in the reaction medium, and at times such reactions furnished products that could not be achieved under thermal condition (5).

In this paper we report the successful cyclodehydration reactions of methyl 9,10-dioxostearate (**1**), methyl 9,12-dioxostearate (**2**), and methyl 10,12-dioxostearate (**3**) with 1,2-diaminoethane under concomitant ultrasonic irradiation (Scheme 1).

## MATERIALS AND METHODS

Methyl 9,10-dioxostearate (**1**) was prepared by oxidation of oleic acid with potassium permanganate in acetic anhydride (6) and followed by methylation. Methyl 9,12-dioxostearate (**2**) was obtained by hydration of methyl 12-oxo-9-octadecynoate (3), and methyl 10,12-dioxostearate (**3**) was derived from methyl ricinoleate by hydration of the double bond followed by chromic acid oxidation (4). 1,2-Diaminoethane was purchased from Merck KGaA (Darmstadt, Germany).

Ultrasonication was carried out using a 20 kHz (54 W/cm$^2$) ultrasound horn (Sonoreactor; Undatim Ultrasonic,




$R^1 = CH_3(CH_2)_7$; $R^2 = (CH_2)_7COOCH_3$; $R^3 = CH_3(CH_2)_5$; $R^4 = (CH_2)_8COOCH_3$

Reaction conditions: (i) $H_2NCH_2CH_2NH_2$, water, ))))), 60°C, 10–15 min

(ii) $H_2NCH_2CH_2NH_2$, AcOH, ))))), 70°C, 4 × 10 min

**SCHEME 1**

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Abbreviations: COSY, correlation spectroscopy; EIMS, electron impact mass spectrometry; HRMS, high-resolution mass spectrometry; IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy; $R_f$, retardation factor; TLC, thin-layer chromatography; TMS, trimethylsilane.

Louvain la Neuve, Belgium) with the reaction mixture contained in a water-jacketed cell (40 mm i.d., 80 mm length). Column chromatographic separation was performed on silica gel (Merck; Art. 7730, type 60, 70–230 mesh) as the adsorbent using gradient elution with a mixture of *n*-hexane/diethyl ether as the mobile phase. Infrared (IR) spectra were recorded on a Bio-Rad FTS-165 Fourier transform (FT)-IR spectrometer (Bio-Rad Inc., Hercules, CA). Samples were run as neat films on NaCl plates. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance $DPX_{300}$ (300 MHz) FT 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). High-resolution mass spectroscopy (HRMS) and electron-ionization mass spectroscopy (EIMS) spectra were recorded on a Finnigan Mat 95 mass spectrometer (Finnigan Mat Corp., San Jose, CA).

*General procedure for the synthesis of* N*-heterocyclic fatty esters (**4,5**) from methyl dioxostearates (**1,2**) as exemplified by the reaction of methyl 9,10-dioxostearate with 1,2-diaminoethane*. A mixture of methyl 9,10-dioxostearate (**1**, 0.3 g, 0.92 mmol) or methyl 9,12-dioxostearate (**2**, 0.3 g 0.92 mmol), 1,2-diaminoethane (0.110 g, 1.84 mmol), and water (25 mL) was irradiated under ultrasound for 10–15 min at 60°C. The reaction mixture was extracted with diethyl ether (3 × 30 mL), and the organic extract was successively washed with brine (10 mL), water (10 mL), and then dried ($Na_2SO_4$). The filtrate was evaporated, and the residue was chromatographed on a silica (15 g) column using a mixture of *n*-hexane/diethyl ether (5–20%, vol/vol, gradient elution) to give the corresponding 2,3-dihydropyrazine derivative (**4**, 0.26 g, 79%) as a pale yellow oil.

*Methyl 8-(5-octyl-2,3-dihydro-pyrazin-6-yl)-octanoate* (**4**, 79% yield). Thin-layer chromatography (TLC), $R_f$ = 0.3 (*n*-hexane/diethyl ether, 1:1 vol/vol, as developer); IR (neat) 2928, 2855, 1741, 1593, 1459, 1437, 1200, and 1160 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 0.88 (*t, J* = 6.7 Hz, 3H, C*H*$_3$), 1.2–1.7 (*m*, 18H, C*H*$_2$), 2.31 (*t, J* = 6.4 Hz, 2H, 2-*H*), 2.42 (*t, J* = 7.7 Hz, 4H, 8-*H* and 11-*H*), 3.32 (*s*, 4H, C*H*$_2$ of ring), and 3.66 (*s*, 3H, COOC*H*$_3$); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 14.03 (C-18), 22.59 (C-17), 24.79 (C-3), 25.89, 26.03, 28.92, 29.00, 29.06, 29.11, 29.29, 29.33, 29.55, 31.76 (C-16), 33.93 (C-2), 35.29 and 35.38 (C-8/C-11), 44.83 (*C*H$_2$ of ring), 51.32 (COO*C*H$_3$), 162.66 and 162.75 (C-9/C-10) and 174.05 (*C*OOCH$_3$); HRMS, found: $M^+$, 350.2936, $C_{21}H_{38}N_2O_2$ requires 350.2933; EIMS, *m/z* (peak intensity relative to base peak = 100%): 207 (32), 208 (100), 251 (26), 252 (89), 319 (21), 350 (33).

*Methyl 8-(9-hexyl-1,2,3,4-tetrahydro-[1,4]diazocin-5-yl)-octanoate* (**5**, 70% yield, pale yellow oil). TLC, $R_f$ = 0.6 (*n*-hexane/diethyl ether, 1:1 vol/vol, as developer); IR (neat): 3383, 3318, 2929, 2856, 1740, 1459, 1437, 1425, 1295, 1198, 1172, and 749 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 0.89 (*t, J* = 6.7 Hz, 3H, C*H*$_3$), 1.2–1.75 (*m*, 18H, C*H*$_2$), 2.31 (*t, J* = 7.5 Hz, 2H, 2-*H*), 2.52 (*t, J* = 7.8 Hz, 4H, 8-*H* and 13-*H*), 2.91 (*t, J* = 7.1 Hz, 2H, C*H*$_2$ of ring), 3.67 (*s*, 3H, COOC*H*$_3$), 3.82 (*t, J* = 7.1 Hz, 2H, C*H*$_2$ of ring), and 5.82 (*s*, 2H, 10-*H* and 11-*H*); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 14.25 (C-18), 22.77 (C-17), 25.06 (C-3), 26.85 and 26.91 (C-8/C-13), 28.78, 28.83, 29.21, 29.29, 29.51, 29.58, 31.90 (C-16), 34.21 (C-2), 42.88 and 46.28 (*C*H$_2$ of ring), 51.60 (COO*C*H$_3$), 104.11 (C-10 and C-11), 132.57 and 132.77 (C-9/C-12) and 174.44 (*C*OOCH$_3$); HRMS, found: $M^+$, 350.2930, $C_{21}H_{38}N_2O_2$ requires 350.2933; EIMS, *m/z* (peak intensity relative to base peak = 100%): 207 (100), 279 (89), 308 (67), 320 (98), 319 (22), 350 (73).

*Methyl 9-(7-hexyl-2,3-dihydro-1H-[1,4]diazepin-5-yl)-nonanoate* (**6**, 95% yield). A mixture of methyl 10,12-dioxostearate (**3**, 0.3 g, 0.92 mmol), 1,2-diaminoethane (0.110 g, 1.84 mmol), and glacial acetic acid (25 mL) was irradiated under ultrasound for four periods of 10 min at 70°C. A break of about 10 min between irradiation period was necessary to prevent the transducer of the ultrasonic horn from overheating. The reaction mixture was extracted with diethyl ether (3 × 30 mL), and the organic extract was successively washed with brine (10 mL), water (10 mL), and then dried ($Na_2SO_4$). Removal of the solvent under reduced pressure gave the 2,3-dihydro-1*H*-1,4-diazepine derivative as a viscous yellow oil (**6**, 0.31 g, 95%). TLC, $R_f$ = 0.1 (*n*-hexane/diethyl ether, 2:3 vol/vol, as developer); IR (neat): 3340, 2920, 2850, 1740, 1575, 1440, 1260, 920, and 730 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 0.86 (*t, J* = 6.4 Hz, 3H, C*H*$_3$), 1.2–1.7 (*m*, 20H, C*H*$_2$), 2.29 (*t, J* = 7.5 Hz, 2H, 2-*H*), 2.38 (*m*, 4H, 9-*H*, 13-*H*), 3.66 (*s*, 3H, COOC*H*$_3$), 3.70 (*brs*, 4H, C*H*$_2$ of ring), 4.59 (*s*, 1H, 11-*H*), and 10.6 (*brs*, 1H, N-*H*); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 14.12 (C-18), 22.60 (C-17), 24.96 (C-3), 28.87, 29.24, 29.35, 29.48, 31.67 (C-16), 34.08 (C-2), 37.49 (C-9/C-13), 48.62 (*C*H$_2$ of ring), 51.43 (COO*C*H$_3$), 88.70 (C-11), 170.28/170.39 (C-10/C-12), and 174.20 (*C*OOCH$_3$); HRMS, found: $M^+$, 350.2921, $C_{21}H_{38}N_2O_2$ requires 350.2933; EIMS, *m/z* (peak intensity relative to base peak = 100%): 124 (74), 137 (100), 194 (57), 207 (41), 280 (19), 293 (26), 319 (15), 350 (10).

## RESULTS AND DISCUSSION

Cyclodehydration was readily achieved when methyl 9,10-dioxostearate (**1**, a 1,2-diketo ester), and methyl 9,12-dioxostearate (**2**, a 1,4-diketo ester) were reacted with 1,2-diaminoethane in water under concomitant ultrasonic irradiation. However, no reaction occurred when methyl 10,12-dioxostearate (**3**, a 1,3-diketo ester) was treated under similar conditions unless the water medium was replaced by glacial acetic acid. The strong acidic condition required for the last reaction reflects the stability of methyl 10,12-dioxostearate (a 1,3-diketo), which prefers the enolic form when under neutral condition (7). Protonation of methyl 10,12-dioxostearate therefore permits the enol form to tautomerize to the less stable keto form. The amino groups of 1,2-diaminoethane are then able to attack the carbonyl groups of the 1,3-diketo tautomer to yield the requisite 2,3-dihydro-1*H*-1,4-diazepine derivative (**6**, 95% yield).

Reaction of methyl 9,10-dioxostearate (**1**) with 1,2-di-

aminoethane gave the 2,3-dihydropyrazine ester (**4**, 79% yield). The proton NMR spectrum showed the characteristic proton shifts of the two methylene groups of the dihydropyrazine ring system at $\delta_H$ 3.32 (*s*, 4H) (8). The 2,3-dihydropyrazine ring system was confirmed from the carbon shifts of the ring methylene groups [$\delta_C$ 44.83, and correlated with the signal at $\delta_H$ 3.32 in the $^{13}C$-$^{1}H$ correlation spectroscopy (COSY) spectrum] and from the shifts of the quaternary carbon atoms six-membered *N*-heterocyclic ring (at $\delta_C$ 162.66/162.75). The position of the 2,3-dihydropyrazine system in the alkyl moiety of the fatty ester was evident from the mass spectral analysis: *m/z* (peak intensity in parentheses) 207 (32), 208 (100, base peak), 251(26), 252 (89), 319 (M – 31, 21), 350 ($M^+$, 33).

Reaction of methyl 9,12-dioxostearate (**2**) with 1,2-diaminoethane gave the 1,2,3,4-tetrahydro-1,4-diazocine derivative (**5**, 70% yield). The IR spectrum showed two distinct vibrational bands at 3318 and 3383 $cm^{-1}$, which hinted to the presence of N-H bonds. The shift of the N-H protons appeared at an upfield region at $\delta_H$ 1.43 (very broad singlet), which was confirmed by deuterium NMR technique (by running the $^{1}H$-decoupled spectrum at 76.77 MHz of the sample after $D_2O$ exchange).The protons of the two methylene groups in the 1,2,3,4-tetrahydro-1,4-diazocine ring gave rise to two distinct triplets at $\delta_H$ 2.91 and 3.82, which were correlated to the carbon shifts at $\delta_C$ 42.88 and 46.28, respectively. Using a space-filled model of compound **5**, the geometric arrangement of the various atoms of the eight-membered *N*-heterocyclic ring system showed that one of the two methylene groups of the ring was located much closer to the conjugated diene system than the other methylene group. This stable configuration appeared to have affected the chemical environment of the nuclei of these methylene groups. And as a result, the two methylene groups in the ring were fully resolved. To ensure that the signals at $\delta_H$ 2.91 and 3.82 did arise from the shifts of the protons from two adjacent methylene groups, the $^{1}H$-$^{1}H$ COSY spectral analysis was performed. The result showed that the triplets at $\delta_H$ 2.91 and 3.82 were indeed correlated. From the $^{1}H$-$^{13}C$ COSY spectrum, the triplet at $\delta_H$ 2.91 was correlated to the carbon nucleus at $\delta_C$ 42.88, while the triplet at $\delta_H$ 3.82 was correlated to the signal at $\delta_C$ 46.82. The shift of the olefinic protons (10-*H* and 11-*H*) gave a singlet at $\delta_H$ 5.82, which was correlated to the carbon shift at $\delta_C$ 104.11. The quaternary olefinic carbon nuclei appeared $\delta_C$ 132.57/132.77 (C-9/C-12) and could not be further differentiated. The position of the 1,2,3,4-tetrahydro-1,4-diazocine system in the alkyl chain of the fatty ester was determined from the mass spectral analysis, *m/z*, intensity in brackets: 207 (100, base peak), 279 (89), 308 (67), 320 (98), 319 (M – 31), 350 ($M^+$).

A 2,3-dihydro-1*H*-1,4-diazepine derivative (**6**) was obtained from methyl 10,12-dioxostearate (**3**) with 1,2-diaminoethane in glacial acetic acid. The product showed a distinct band at 3340 $cm^{-1}$ for the N-H vibration in the IR spectrum. The proton shift of the N-H appeared at $\delta_H$ 10.6 (a very broad singlet and $D_2O$ exchangeable). Compound **6** exists as tautomers with the proton of N-H interchanging between the two nitrogen atoms of the ring. The rapid inversion of the half-chair-shaped seven-membered 2,3-dihydro-1*H*-1,4-diazepine ring was evident from the broad singlet obtained for the shifts of the ring methylene protons at $\delta_H$ 3.70. The corresponding signals of the ring methylene groups at $\delta_C$ 48.62 appeared also as broad signals in the $^{13}C$ NMR spectrum. Similar NMR observations were reported by Potter *et al.* (9) and Lloyd *et al.* (10–12) on 2,3-dihydro-1*H*-1,4-diazepinium salts. When the $^{1}H$-$^{13}C$ COSY experiment was performed on compound **6**, the correlation of the ring methylene protons and the corresponding ring methylene carbon nuclei did not show up in the spectrum. The nonobservance of such correlation was evidence of the high rate of inversion of the ring system at 27°C (operating temperature of the NMR instrument).

To slow down the inversion rate, compound **6** in $CDCl_3$ was shaken with $D_2O$. The proton–carbon correlation of the methylene groups in the ring system was then clearly shown when the hydrogen of the N-H group of compound **6** was deuterium-exchanged with $D_2O$ (Fig. 1, $^{1}H$-$^{13}C$ COSY). The deuterated form of compound **6** (containing N-D bonds) also allowed the broad signals arising from the shifts of the ring methylene protons and carbon nuclei to be "sharpened" up as compared to the corresponding signals observed for the nondeuterated form of compound **6**. With the hydrogen atom of N-H replaced by a deuterium, the isotopic effect caused the shifts of the ring methylene protons and carbon nuclei in the deuterated form of **6** to appear at $\delta_H$ 3.59 and $\delta_C$ 49.01, respectively. The replacement of the hydrogen of the N-H group by deuterium in compound **6** had apparently slowed down the rate of inversion of the half-chair-shaped seven-membered ring. The methylene groups of the ring system have become fixed in their orientation, hence the improved shape of the NMR signals. The position of the 2,3-dihydro-1*H*-1,4-diazepine system in the alkyl moiety of compound **6** was evident from the mass spectral analysis: *m/z* (intensity in brackets) 124 (74), 137 (100 = base peak), 194 (57), 207 (41), 280 (19), 293 (26), 319 (M – 31, 15), 350 (10).

Ultrasound-assisted experiments involving diketo fatty esters and diaminoethane allowed cyclodehydration reactions to be readily achieved in aqueous or acidic media. When these reactions were repeated under thermal (silent) condition, only compound **6** could be obtained in high yield from methyl 10,12-dioxostearate with 1,2-diaminoethane after refluxing the mixture for at least 1 h in glacial acetic acid. The role of ultrasound (at 60–70°C) in these reactions was therefore to enable the reacting groups to be brought sufficiently close for chemical reactions without the need for the substrates to be soluble in the reaction medium.

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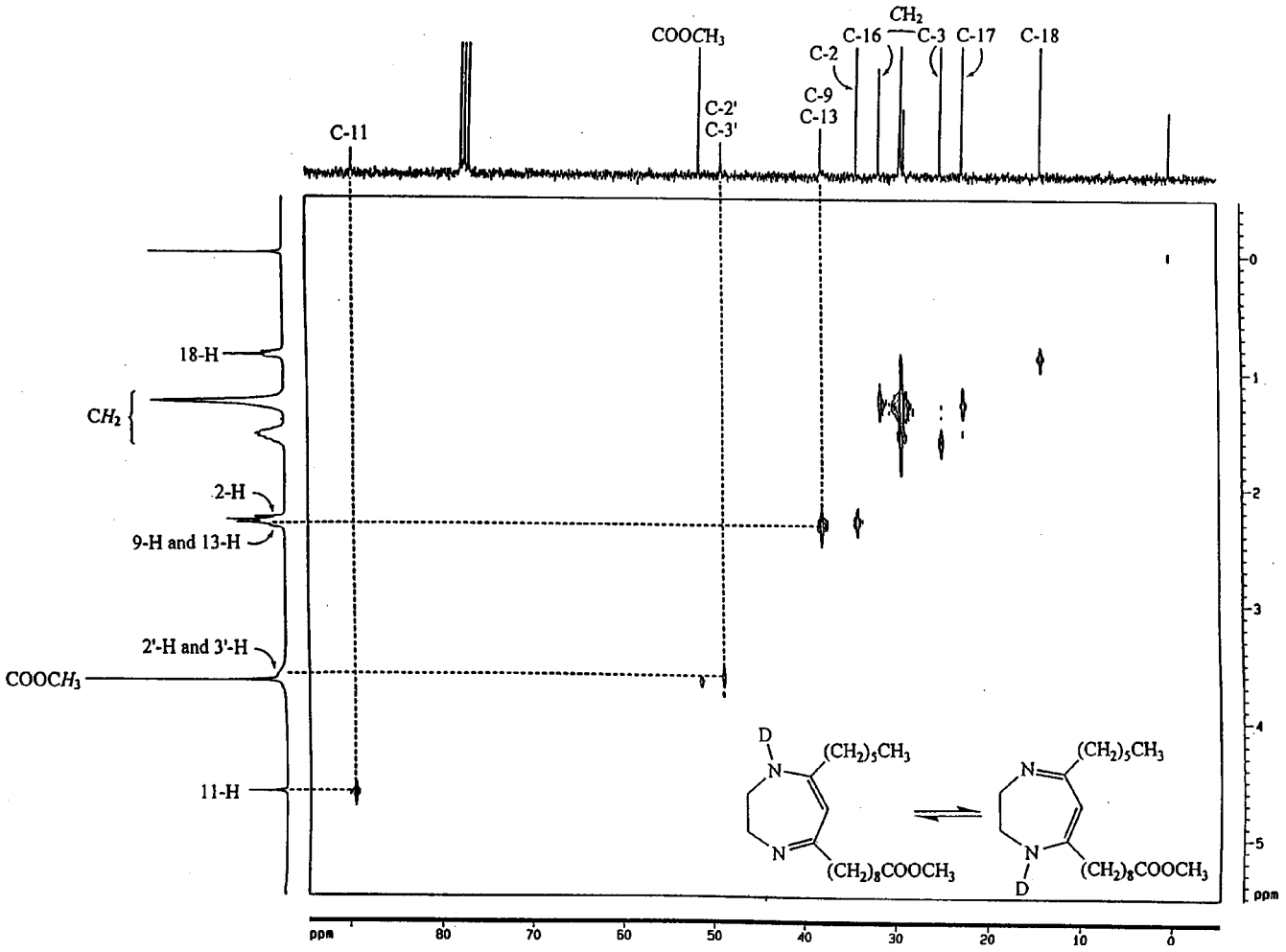


**FIG. 1.** $^{1}H$-$^{13}C$ nuclear magnetic resonance correlation spectrum of deuterated methyl 9-(7-hexyl-2,3-dihydro-1*H*-[1,4]diazepin-5-yl]-nonanoate (**6**).

able discussion on the NMR spectral results.

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METHOD

# Dialysis and Gel Filtration of Isolated Low Density Lipoproteins Do Not Cause a Significant Loss of Low Density Lipoprotein Tocopherol and Carotenoid Concentration

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**ABSTRACT:** The resistance of isolated low density lipoprotein (LDL) to copper-initiated oxidation is often used as a measure of effectiveness of an antioxidant intervention. Prior to oxidation, excess salt and EDTA are removed *via* dialysis or gel filtration of the LDL sample. However, there is concern over whether the antioxidant content of dialyzed or gel-filtered LDL is truly representative of native LDL extracted from a blood sample. Previously, the experiments done after the storage of native and dialyzed LDL at –80°C showed that the dialysis step can cause a loss of up to 60% in the tocopherol and carotenoid content of LDL. In the present study, a comparison of the micronutrient concentration in freshly prepared dialyzed and native LDL from 35 subjects showed that after the correction for cholesterol, only lycopene (13%, $P < 0.001$) and to a lesser extent α-carotene (8%, $P < 0.02$) were significantly decreased, and the absolute fall in concentration was far smaller than previously reported. Other experiments done with smaller numbers of samples suggested that there were minimal micronutrient losses following gel filtration and that it was important to include 10 μmol/L EDTA in the dialysis and elution buffer; otherwise micronutrient losses did occur. In summary, immediate dialysis of freshly isolated LDL in the presence of 10 μmol/L EDTA does not cause any major loss in the concentration of tocopherol and most carotenoids.

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Antioxidant components in low density lipoprotein (LDL) are believed to play a significant role in protecting LDL against oxidation both *in vivo* and *in vitro* (1,2). Several *in vivo* and *in vitro* supplementation studies have used antioxidant micronutrients such as vitamin E (3), carotenoids (4), flavonoids (5), and ubiquinols (6) in an attempt to increase the inherent antioxidant capacity of the LDL particle (7). The effectiveness of antioxidant intervention is mostly assessed by *ex vivo* measurement of the resistance of LDL to oxidation. Blood for such studies is mostly collected in preservatives such as EDTA. Likewise, during the isolation of LDL, salt solutions containing EDTA are used to prevent premature oxidation during isolation and storage. However, prior to the *in vitro* Cu-initiated oxidation of LDL (the most widely used method for measuring the oxidative resistance), excess EDTA has to be removed by gel filtration (8) or dialysis (9).

Scheek *et al.* (10) reported that the dialysis step leads to the loss of antioxidant micronutrients, and thus the postdialysis results may not be truly representative of freshly isolated or native LDL. Examination of literature showed that several groups have avoided dialysis and expressed their concerns in using the dialysis step (11–14) because of the results reported by Scheek *et al.* (10). In the present study we report the effect of dialysis and gel filtration on the freshly prepared LDL samples.

## METHODS

*Subjects.* The subjects were recruited from the University staff. Ethical approval was obtained from The University of Ulster Ethical Committee. Subjects were asked to fast for 8 h prior to giving blood.

*Blood collection and plasma preparation.* The blood was collected in plain tubes containing 1 mg/mL EDTA (sodium salt; Sigma Chemical Co., St. Louis, MO) and was centrifuged at $1000 \times g$ for 10 min to separate the plasma. Plasma was further centrifuged for 45 min at $1000 \times g$ to remove any remaining cell debris.

*LDL isolation.* The density of plasma was adjusted by the addition of 0.32 g of potassium bromide (KBr) per mL of plasma. The density-corrected plasma (4 mL) was layered underneath 7 mL of KBr solution (density 1.007 g/mL, pH 7.4, containing 1 mg/mL EDTA) in an Optiseal Polyallomer centrifuge tube (Beckman Instruments, London, United Kingdom). LDL was isolated at 7°C after ultracentrifugation for 2-1/2 h at 60 K in XL-70 Beckman ultracentrifuge using NVT65 rotor (Beckman Instruments). This procedure creates a density gradient such that LDL forms a discrete orange band *ca.* one-third of the way from the top of the tube. The LDL fraction was removed by puncturing the side wall of the tube with a hypodermic needle attached to a syringe. LDL samples

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Abbreviations: BHT, butylated hydroxytoluene; DLDL, dialyzed low density lipoprotein; GLDL; gel-filtered low density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; PBS, phosphate-buffered saline.

were immediately purged with nitrogen, and the lid additionally was sealed with Parafilm® to reduce the infiltration of oxygen; samples were then stored at 4°C. Electrophoresis of LDL using Paragon lipoprotein gels (Beckman, Fullerton, CA) showed that LDL was free of contamination from plasma proteins and high density lipoprotein.

*Dialysis of LDL.* LDL were dialyzed within 6 h of collection of blood. Samples (0.5 mL) were transferred to Visking dialysis tubing (Size 1, diameter 8/32″-6.3 mm; 30 m, Medicell International Ltd., Liverpool, United Kingdom) and dialyzed in buffer pH 7.4 containing 0.01 mol disodium hydrogen phosphate, 0.16 mol sodium chloride, 0.01 mmol EDTA (sodium salt), and 0.3 mmol of chloramphenicol per liter. Dissolved gases in the buffer were reduced by vacuum degassing for 20 min, followed by purging with nitrogen for 15 min. Samples were dialyzed for 24 h at 4°C, stored under nitrogen, and micronutrient analysis on native and dialyzed LDL was done on the day of preparation of the dialyzed sample.

*Gel filtration.* LDL was filtered using desalting columns (Econo-Pac 10 DG; BioRad, Hercules, CA) filled with Bio-Gel® P-6DG gel (bed volume of 10 mL and void volume of 3.3 mL). Desalting columns were stored in 0.01 mol/L phosphate buffer containing 0.01 mol/L sodium chloride and 0.02 % sodium azide; therefore, prior to the application of sample to the column, columns were washed with phosphate-buffered saline (PBS; Poole, Dorset, United Kingdom) containing 10 μmol/L EDTA.

For gel filtration, 1.0 mL of an LDL sample was run onto the column followed by 2.3 mL of 0.01 mmol $Na_2EDTA$ per liter PBS buffer pH 7.4 under gravity. Finally an additional 1 mL of PBS was added to the column, and the LDL was collected in 1 mL eluate. Nitrogen was purged through the samples for *ca.* 15 s, and they were stored at 4°C until ready for analysis. Micronutrient analysis was carried out on the same day as the preparation of the sample. Cu-initiated oxidation of gel-filtered and dialyzed LDL was carried out on the day of preparation of samples by the method described previously (15). Briefly, the final reaction mixture consisted of 0.25 mg LDL mass/mL (equivalent to 0.1 μmol LDL/L) and 11.7 μmol/L copper chloride. The diene conjugate formation was monitored at 234 nm, and the lag phase (resistance of LDL to oxidation) was calculated by drawing a tangent to the propagation phase to determine the intercept with the x-axis. The oxidation experiment was repeated on gel-filtered (GLDL) and dialyzed LDL (DLDL) prepared from plasma stored at –70°C for 4 wk (plasma samples were stored in 0.6% sucrose). The results are means of triplicate measurements.

*Cholesterol determination.* The cholesterol content of lipoproteins (native, dialyzed, and gel-filtered) was determined using CHOD-PAP kit from Boehringer (Mannheim, Germany). Intra-assay precision on freshly isolated LDL sample (10 measurements) was 2.5%.

*High-performance liquid chromatography (HPLC) analysis of micronutrients in lipoproteins.* The micronutrient content of the native, dialyzed, and gel-filtered LDL was determined using reversed-phase HPLC as described previously (16). Briefly, 0.1 mL of sample was mixed with 0.1 mL of sodium dodecyl sulfate and 0.2 mL of ethanolic tocopherol acetate (40 mg/mL). To the mixture, 1 mL of heptane was added and samples were vortexed vigorously for 3 min. Samples were centrifuged at $800 \times g$ for 10 min at 10°C. Heptane layer (0.7 mL) was removed, evaporated to dryness, and reconstituted in 0.1 mL of reconstitution mobile phase [i.e., mobile phase plus 90 mg butylated hydroxytoluene (BHT)/L]. Of the sample, 50 μL was injected onto a 3-μm Spherisorb ODS2 column (10 cm × 4 mm) and eluted with mobile phase pumped at 1 mL/min, and micronutrients were measured using a multichannel ultraviolet/visible detector set at the wavelengths 295 (for the detection of tocopherols) and 450 (for carotenoid detection) nm. Mobile phase consisted of acetonitrile/methanol/dichloromethane (500:500:128, by vol) and 0.01 g BHT/L. Data were collected and integrated using Maxima software (Waters, Millipore, Watford, United Kingdom). Within-assay precision of micronutrients analyzed in freshly prepared native and dialyzed LDL samples was less than 8%.

*Statistical analysis.* Data were examined for normality and found to be skewed both before and after transformation to logarithms. Therefore, nonparametric tests were used, particularly the Wilcoxon Paired Rank Sum to calculate the significance of changes observed following the dialysis.

## RESULTS

Table 1 shows results for cholesterol and micronutrient analyses of freshly prepared native and DLDL before and after correction for cholesterol. Both cholesterol and micronutrient concentration were significantly lower in DLDL. However after correction of the data using cholesterol, the differences in micronutrient concentration between native and DLDL disappeared except for α-carotene and lycopene.

The micronutrient concentration of DLDL prepared in the presence and absence of EDTA in dialysis buffer is shown in Table 2. Values shown have been corrected for cholesterol. Results show that the loss in both vitamin E and carotenoids is greater when EDTA is not included in the dialysis buffer.

The results of experiments to compare dialysis with gel filtration are shown in Table 3. The data shown are corrected for cholesterol, and very similar changes in micronutrient content were obtained both following dialysis and gel filtration. The data suggest that the changes in carotenoids and tocopherol following dialysis or gel filtration in the presence of 10 μmol/L EDTA are more likely to be due to experimental error, since both positive and negative changes were obtained by both methods. Due to a smaller number of samples (LDL done in duplicate and results for GLDL and DLDL are an average of four measurements), it was not possible to do the statistical analysis of the data. Nevertheless, the results for dialyzed samples were similar to those obtained with other experiments. No difference was observed in the LDL lagphase between DLDL and GLDL samples. Freezing of plasma prior to isolating DLDL or GLDL did not affect the lagphase measurements.

**TABLE 1**
**Comparison of Micronutrient Concentrations in Native and Dialyzed LDL**

| | Micronutrient concentration of LDL | | | |
|---|---|---|---|---|
| | Before correction for cholesterol (μmol/L) | | After correction for cholesterol (μmol/mmol cholesterol) | |
| | Native Median (range) | Dialyzed Median (range) | Native Median (range) | Dialyzed Median (range) |
| α-Carotene | 0.11 (0.01–0.35) | 0.09 (0.01–0.37)*** | 0.03 (0.00–0.24) | 0.02 (0.00–0.10) * |
| β-Carotene | 0.50 (0.02–1.84) | 0.47 (0.02–2.00)*** | 0.09 (0.01–0.34) | 0.09 (0.01–0.38) |
| Lutein | 0.11 (0.02–0.30) | 0.10 (0.03–0.26) *** | 0.03 (0.01–0.06) | 0.02 (0.01–0.05) |
| Lycopene | 0.44 (0.09-0.89) | 0.33 (0.03–0.70) *** | 0.09 (0.02–0.22) | 0.08 (0.01–0.22)*** |
| α-Tocopherol | 15 (8–24) | 13 (8–50) *** | 3.09 (2.0–5.6) | 3.13 (1.8–10.1) * |
| γ-Tocopherol | 0.94 (0.45–2.46) | 0.88 (0.47–2.25)* | 0.21 (0.11–0.49) | 0.22 (0.12–0.54) |
| β-Cryptoxanthin | 0.09 (0.01–0.33) | 0.07 (0.01–0.31) *** | 0.02 (0.00–0.06) | 0.02 (0.00–0.06) |
| Cholesterol | 4.51 (2.8–8.7) | 4.19 (2.4–7.5) *** | — | — |

[a]Low density lipoprotein (LDL) (0.5 mL) from 35 healthy females (24–52 yr) were dialyzed in *ca.* 700 vol of degassed phosphate-buffered saline buffer pH 7.4 for 24 h at 4°C under nitrogen. Significance of difference was calculated using Wilcoxon paired rank sum test. * denotes $P < 0.05$, and *** $P < 0.001$.

## DISCUSSION

The effect of dialysis on LDL micronutrient concentration in freshly prepared LDL and DLDL samples was determined in samples obtained from 35 subjects. The results suggested that there was an apparent loss of micronutrients on dialysis. However, this effect was due to dilution since most of the differences were accounted for if the micronutrients were corrected using cholesterol. The exceptions were α-carotene and lycopene, but in both cases the difference between the mean values was only 0.01 μmol/mmol cholesterol. These small losses may be real, particularly for lycopene since in-house experience with lycopene standards has shown it to be far less stable than the other commonly measured carotenoids. However, the small (~1%) but statistically significant increase in α-tocopherol content of DLDL, which was observed, is probably within the precision of analysis of α-tocopherol.

The experiment in which dialysis was done with and without EDTA clearly shows the protective effect of EDTA during dialysis. When EDTA was not included in the buffer, concentration of all nutrients except γ-tocopherol was significantly lower than those in the native LDL in spite of correcting the data for cholesterol. However, both lutein and lycopene were significantly lower in the dialyzed samples even in the presence of EDTA in the dialysis buffer.

The results from our study are in disagreement with the reports of Scheek *et al.* (10), who found a 66% reduction of α-tocopherol, 56% in β-carotene, and 65% in lycopene following a 22-h dialysis in the presence of EDTA. However, our results are in agreement with reports by Esterbauer *et al.* (17)

**TABLE 2**
**Effect of the Presence of EDTA in the Dialysis Buffer on the Micronutrient Concentrations of LDL**

| | Micronutrient concentration in LDL (μmol/mmol cholesterol) | | |
|---|---|---|---|
| | Native Median (range) | Dialyzed with EDTA Median (range) | Dialyzed without EDTA Median (range) |
| α-Carotene | 0.026 (0.022–.027) | 0.023 (0.022–0.026) | 0.019* (0.015–0.021) |
| β-Carotene | 0.079 (0.066–0.082) | 0.081 (0.076–0.091) | 0.062* (0.054–0.066) |
| Lutein | 0.025 (0.024–0.026) | 0.022* (0.020–0.023) | 0.018* (0.018–0.019) |
| Lycopene | 0.105 (0.092–0.109) | 0.067* (0.060– 0.071) | 0.056* (0.049–0.058) |
| α-Tocopherol | 3.31 (3.21–3.43) | 3.25 (3.22–3.34) | 2.77* (2.71–2.93) |
| γ-Tocopherol | 0.204 (0.186–0.212) | 0.197 (0.179–0.209) | 0.175 (0.164–0.203) |
| β-Cryptoxanthin | 0.028 (0.028–0.029) | 0.026 (0.025–0.029) | 0.020* (0.019–0.025) |

[a]Plasma from five male volunteers was pooled, LDL prepared and separated into three fractions. Two fractions were dialyzed each in five separate aliquots with and without EDTA. Significance of decrease in micronutrient concentration in dialyzed compared to native LDL samples was calculated using a Wilcoxon paired rank test. *$P < 0.05$. See Table 1 for abbreviation.

**TABLE 3**
**Comparison of Effect of Dialysis and Gel Filtration on LDL Micronutrients[a]**

| | Native LDL $n = 2$ | Dialyzed (DLDL) $n = 4$ | Gel-filtered (GLDL) $n = 4$ | Change (%) After dialysis | Change (%) After filtration |
|---|---|---|---|---|---|
| LDL micronutrients (μmol/mmol cholesterol) | | | | | |
| α-Carotene | 0.030 ± 0.035 | 0.036 ± 0.003 | 0.035 ± 0.004 | +12 | +9 |
| β-Carotene | 0.078 ± 0.084 | 0.078 ± 0.006 | 0.085 ± 0.005 | +4 | +5 |
| Lutein | 0.022 ± 0.025 | 0.020 ± 0.002 | 0.025 ± 0.003 | –13 | +10 |
| Lycopene | 0.178 ± 0.190 | 0.166 ± 0.011 | 0.168 ± 0.011 | –10 | –9 |
| α-Tocopherol | 3.51 ± 3.90 | 3.27 ± 0.33 | 3.88 ± 0.22 | –11 | +5 |
| Lagphase (min) | | | | | |
| A | — | 41 ± 5 | 42 ± 1 | | |
| B | — | 46 ± 1 | 38 ± 6 | | |

[a]Native LDL was prepared from pooled plasma and split into three fractions. One fraction was dialyzed in duplicate and one treated using gel filtration in duplicate. All samples were then analyzed for micronutrients in duplicate. Lag phase measurements were done in triplicate on dialyzed and gel-filtered samples prepared from fresh (A) and stored plasma (B). See Table 1 for abbreviation.

and Carbonneau *et al.* (18). Esterbauer *et al.* (17) showed that when EDTA or BHT is present in the dialysis buffer, there is no loss in the antioxidant micronutrient or the polyunsaturated fatty acid content of LDL. Carbonneau *et al.* (18) did report a small loss in α-tocopherol (*ca.* 11%) following the dialysis step, but this change was much smaller than reported by Scheek *et al.* (10) Carbonneau and co-workers (18) suggested that such changes are unlikely to have an effect on the LDL oxidation as very large increases in the LDL antioxidant content are required to increase the resistance of LDL to oxidation. In the experiments done by Scheek *et al.* (10), the analysis of micronutrients in LDL and DLDL was done after the storage of samples (both native and DLDL) at –80°C. In the present study, we show that when the analysis is done on the freshly prepared native and dialyzed DLDL, only small changes in micronutrients are observed. However, in our preliminary experiments (data not shown), we found that the changes in micronutrient concentrations were greater when LDL samples (both native and dialyzed) were stored at –70°C, but the losses in carotenoids were smaller (20–40%) than that reported by Scheek *et al.* (10). We suggest that freezing of LDL and DLDL samples can cause greater losses of micronutrients, and in our experience freezing also leads to a greater inaccuracy in micronutrient determination due to the tendency for LDL to aggregate in thawed samples.

Gel filtration is another frequently used method for the removal of EDTA from LDL samples prior to *in vitro* oxidation (8). A comparison of gel filtration and dialysis was done using duplicate samples prepared from the same LDL preparation. In both cases the changes in micronutrient concentration observed were of a similar magnitude but slightly higher than the assay precision of micronutrients. The carotenoids α-carotene and lutein may be subject to a greater experimental error due to their lower concentrations in LDL. The β-carotene and lycopene are sensitive to degradation by light and oxidation (19,20); therefore, some variation in micronutrient concentrations can be expected between different experiments. Although precautions were taken for a minimal exposure of samples to the light and EDTA was included in most preparations, some losses are unavoidable during the experiment. LDL oxidizability as measured by the lag phase was not different between dialyzed and gel-filtered samples when either fresh or frozen plasma samples were used. The results shown in Table 3 from frozen plasma are in agreement with our previously published report in which between-assay variation of lag phase in LDL prepared from frozen plasma was found to be less than 5% (15).

In summary, dialysis or gel filtration of freshly isolated LDL does not cause a significant loss of micronutrients if the dilution occurring during dialysis or gel filtration is taken into account. Small differences in micronutrient concentration can, however, be attributed to losses occurring during the removal of EDTA as well as experimental errors in the HPLC procedure.

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# α-Linolenic Acid and Its Δ5-Desaturation Product, Coniferonic Acid, in the Seed Lipids of *Tsuga* and *Hesperopeuce* as a Taxonomic Means to Differentiate the Two Genera

Sir:

In our systematic study of Pinaceae seed fatty acid (SFA) compositions, we have analyzed *ca.* 150 species, subspecies, and varieties, which represent approximately one-half of total recognized taxa of this family (1). We additionally screened many geographical variants. Pinaceae seeds analyzed so far include: *Pinus*, 76 taxa; *Abies*, 36 taxa; *Picea*, 17 taxa; *Larix*, 8 taxa; *Cedrus*, 5 taxa; *Tsuga*, 4 taxa; *Keteleeria*, 2 taxa; *Pseudotsuga*, 1 taxon (2–5; Wolff, R.L., unpublished data). Recently, we had the opportunity to analyze the monospecific genus *Pseudolarix* (*P. amabilis*, golden larch; central and northeastern China) and *Tsuga mertensiana* (mountain hemlock; western North America, from southern Alaska to central California), with uncertain taxonomic position (1,6,7). With the exception of the monospecific genera *Cathaya* and *Nothotsuga*, all Pinaceae genera have now been screened for their SFA compositions. Unexpectedly, the two species studied here appear exceptional regarding their α-linolenic acid content as compared to all other Pinaceae analyzed so far, being placed at the extreme positions (lowest and highest contents in *T. mertensiana* and *P. amabilis*, respectively).

In all species from all Pinaceae genera analyzed so far, α-linolenic acid has always been present in the narrow range of 0.2–1.4% of total fatty acids, with one exception, *Pinus merkusii* (the only *Pinus* species that extends into the Southern Hemisphere), at 2.7% (5). The Δ5-desaturation product of α-linolenic acid, coniferonic (5,9,12,15-18:4) acid, reaches a maximum of 0.2% in Pinaceae seed lipids, being most often present at the limit of detection. However, in some Cupressaceae species, i.e., *Chamaecyparis lawsoniana* and *Fokienia hodginsii,* coniferonic acid can reach much higher levels [2.0 and 2.8%, respectively (8)], and these species are considerably richer in α-linolenic acid than any Pinaceae species.

Analyses of the species described here were made according to our standardized procedures (5), and their SFA compositions are given in Table 1. As can be seen from that table, the level of α-linolenic acid in *P. amabilis* is 5.2%, and this acid is accompanied by a fairly high level of coniferonic acid (1.8%), which is quite exceptional among Pinaceae species. On the other hand, *T. mertensiana* is devoid of these two fatty acids. Among Pinaceae species analyzed so far, *P. amabilis* and *T. mertensiana* thus appear as representing the two extreme species when considering their seed lipid α-linolenic acid content.

**TABLE 1**
**Fatty Acid Compositions (weight percentage of total fatty acids) of the Seed Lipids from *Pseudolarix amabilis* and *Tsuga mertensiana*. Comparison with Other *Tsuga* spp.**

| | | | *Tsuga* spp.[b] | |
|---|---|---|---|---|
| Fatty acid[a] | *P. amabilis* | *T. mertensiana* | Minimum | Maximum |
| 16:0 | 4.00 | 3.63 | 3.45 | 4.75 |
| 16:1[c] | 0.29 | 0.11 | 0.06 | 0.11 |
| aiso-17:0 | 0.37 | 0.51 | 0.23 | 0.57 |
| 17:0 | 0.07 | —[d] | — | — |
| 9-17:1 | 0.04 | 0.05 | — | — |
| 18:0 | 2.42 | 1.53 | 1.73 | 2.27 |
| 9-18:1 | 26.50 | 23.27 | 13.08 | 14.76 |
| 11-18:1 | 0.24 | 0.57 | 0.28 | 0.50 |
| 5,9-18:2 | 7.75 | 2.24 | 1.85 | 2.62 |
| 9,12-18:2 | 35.55 | 43.92 | 49.66 | 50.83 |
| 5,9,12-18:3 | 7.35 | 19.40 | 18.67 | 19.53 |
| 9,12,15-18:3 | 5.18 | — | 0.34 | 0.82 |
| 5,9,12,15-18:4 | 1.78 | — | — | 0.05 |
| 20:0 | 0.51 | 0.35 | 0.32 | 0.42 |
| 11-20:1 | 1.61 | 0.45 | 0.47 | 0.77 |
| 5,11-20:2 | 1.19 | 0.07 | 0.07 | 0.12 |
| 11,14-20:2 | 0.52 | 0.55 | 0.70 | 0.94 |
| 5,11,14-20:3 | 3.57 | 1.31 | 2.80 | 3.74 |
| 7,11,14-20:3 | 0.12 | 0.27 | 0.27 | 0.40 |
| Total Δ5[e] | 21.76 | 23.29 | 23.77 | 27.14 |
| Others[f] | 0.94 | 1.77 | 0.35 | 0.54 |

[a]Ethylenic bonds are in the *cis* configuration; aiso-17:0, anteiso-17:0 or 14-methylhexadecanoic acid.
[b]Minimum and maximum values determined in the seed lipids of *T. canadensis, T. caroliniana, T. chinensis,* and *T. heterophylla*.
[c]Sum of two isomers, 7- and 9-16:1 acids.
[d]Not detected.
[e]Sum of Δ5-olefinic acids and 7,11,14-20:3 acid.
[f]Include minor and unknown fatty acids.

The taxonomic ranking of *P. amabilis* has been recognized for a long time, but that of *T. mertensiana* has been and is still a matter of debate. It has been considered a hybrid, or even a monospecific genus, *Hesperopeuce* (1,6,7). Regarding the latter species, we also give in Table 1 the range of values encountered in the genus *Tsuga*, based on data established for *T. canadensis*, *T. heterophylla* (3), *T. caroliniana*, and *T. chinensis* (Wolff, R.L., unpublished data). This is intended to show the similarities and dissimilarities between *T. mertensiana*

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and other representatives of the same genus. The narrow ranges in which *Tsuga* spp. SFA compositions lie are noteworthy (see, e.g., 9,12-18:2 acid). Differences between *T. mertensiana* and *Tsuga* spp. appear for oleic, linoleic, and particularly α-linolenic acids. Oleic acid is considerably higher in *T. mertensiana* than in *Tsuga* spp., whereas linoleic acid is slightly lower. Δ5-Olefinic $C_{18}$ acid levels do not differ significantly, whereas it would appear that the elongation product of linoleic acid, 11,14-20:2 acid, and its desaturation product, sciadonic (5,11,14-20:3) acid, are definitely lower in *T. mertensiana* than in other *Tsuga* species. The most conspicuous feature, however, is the absence of α-linolenic acid and consequently of coniferonic acid in *T. mertensiana* seed lipids as compared to other *Tsuga* species.

Interestingly, *P. amabilis* and *T. mertensiana* SFA include the anteiso-17:0 acid found in all other Pinaceae genera and species (9). In the latter species, preliminary results obtained by mass spectrometry coupled to gas–liquid chromatography indicate that some fatty acids in *T. mertensiana* seeds, included here in the category "others" (Table 1), would be unsaturated branched $C_{19}$ acids, possibly similar to those characterized in *Picea abies* (Norway spruce) wood extracts by Ekman (10). However, this deserves further investigations.

In conclusion, as regards its SFA composition, *T. mertensiana* differs from all other *Tsuga* spp., which otherwise appear as a very homogeneous genus; and this observation, along with other botanical criteria, would support its ranking at the genus level (i.e., *Hesperopeuce mertensiana*) rather than at a *Tsuga* species level. On the other hand, *P. amabilis* is a genus quite distinct from all other genera of the Pinaceae family. Moreover, if one compares *P. amabilis* (Pinaceae) on the one hand, with *C. lawsoniana* and *F. hodginsii* (Cupressaceae) on the other hand, the Δ5-desaturation of α-linolenic acid appears more efficient in the former species. The two Cupressaceae species contain *ca.* 8–10 times more α-linolenic acid [40 and 50%, respectively (8)] than *P. amabilis*, although the levels of coniferonic acid are rather similar in the three species.

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# Overestimates of Oleic and Linoleic Acid Contents in Materials Containing *Trans* Fatty Acids and Analyzed with Short Packed Gas Chromatographic Columns

Sir:

*Trans* fatty acids (FA), mainly comprising *trans* 18:1 isomers, are found in milk and other dairy foods produced by ruminal biohydrogenation and in partially hydrogenated edible oils. Some *cis* isomers other than oleic acid (9*c*-18:1) are also found. These *cis* and *trans* isomers are not completely separable by a gas–liquid chromatography (GLC) analysis even with a 100-m polar column. Only thin-layer chromatography (TLC) separation of the methyl esters into bands of *trans* and *cis* isomers followed by further analyses of the separated esters with the column above allows an almost complete resolution of all isomers. This procedure is called the argentation TLC/GLC (Ag-TLC/GLC) method (1). Partial resolution can be attained by GLC alone, with polar capillary columns (1). However, as shown in Figure 1, the isomers cannot be resolved from, for example, 9*c*-18:1, by using the short packed GLC columns universally employed before the long, small-diameter capillary columns became available in the early 1980s. The single peak on the GLC chart designated as 18:1 could contain about 21 *cis* and *trans* isomers. The high-resolution Ag-TLC/GLC procedure was first employed by Wolff and Bayard (2) and Precht and Molkentin (3) in 1995. They used 100-m capillary columns to separate almost all individual *trans* and *cis* isomers in ruminant fats or partially hydrogenated oils. However, the overlaps with oleic acid cannot be quantified because, although they had high achievable resolution, they presented only the contents of the *trans* isomers (3). In the same year, Chen *et al.* (4) published results obtained by another method involving preseparation by Ag-TLC and further cleavage by oxidative ozonolysis and GLC of the resulting products. Also here, the overlaps cannot be quantified precisely because these authors presented the contents of the isomers only in bar graphs.

While preparing a review on *trans* unsaturated FA in human milk, one of us (RGJ) belatedly realized that the amounts of some unsaturated *cis* FA in materials which could

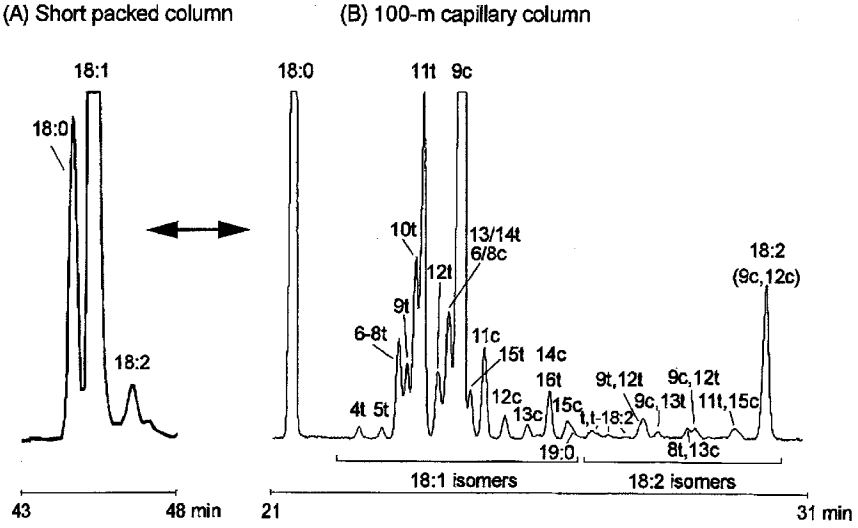


**FIG. 1.** Partial gas–liquid chromatograms of fatty acid methyl esters derived from a bovine milk fat sample obtained on different column types. (A) Packed column: 2 m, 15% DEGS; initial temperature 50°C; temperature increased at a rate of 4°C/min to 180°C; carrier gas: $N_2$; (B) capillary column: 100 m CP-Sil 88 (Chrompack, Middelburg, The Netherlands; 100% cyanopropyl polysiloxane), 172°C isothermal; carrier gas: $H_2$.

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Abbreviations: FA, fatty acids; Ag-TLC, argentation thin-layer chromatography; GLC, gas-liquid chromatography.

**TABLE 1**
**Derivation of Factors Needed to Calculate Overestimates of Oleic and Linoleic Acids (wt%) in Materials Containing *trans* Fatty Acids and Analyzed with Short Packed Gas—Liquid Chromatography (GLC) Columns**

| Isomers | Oleic acid (18:1) | | | | Isomers | Linoleic acid (18:2) | | |
|---|---|---|---|---|---|---|---|---|
| | Bovine milk[a] | Human milk[b] | Margarines | | | Bovine milk[d] | Human milk[b] | Margarines[d] |
| | | | 1994[a] | 1999[c] | | | | |
| *n* | 100 | 32 | 17 | 29 | | 100 | 27 | 46 |
| 9*c* | 19.09 | 28.89 | 21.06 | 25.78 | 9*c*,12*c* | 1.23 | 9.50 | 25.01 |
| 6-8, 11*c* | 0.64 | 2.04 | 3.08 | 2.55 | 16t-18:1 | 0.34 | 0.14 | 0.09 |
| 12-15*c* | 0.43 | 0.27 | 1.31 | 0.62 | All *trans*-18:2, no CLA | 0.99 | 1.07 | 0.61 |
| 4-15*t* | 3.49[e] | 2.27 | 16.41 | 11.05 | | | | |
| Old total | 23.65 | 33.47 | 41.86 | 40.00 | Old total | 2.56 | 10.71 | 25.71 |
| Correction factors | 0.81 | 0.86 | 0.50 | 0.64 | Correction factors | 0.48 | 0.89 | 0.97 |
| Seasonal | | | | | Seasonal[a] | | | |
| All | 0.81 ± 0.03 | | | | All | 0.49 | | |
| Pasture | 0.78 ± 0.02 | | | | Pasture | 0.43 | | |
| Transition | 0.80 ± 0.02 | | | | Transition | 0.47 | | |
| Barn | 0.84 ± 0.02 | | | | Barn | 0.55 | | |

[a]Precht, D., and Molkentin, J., unpublished data.
[b]From Reference 9.
[c]From Reference 10.
[d]From Reference 11.
[e]From Reference 12. CLA, conjugated linolenic acid.

contain *trans* FA, obtained with the packed columns and reported in older papers, e.g., (5–7), were all overestimated. The quantities in peaks identified as 18:1 and 18:2 reported in these papers are too high because the *trans* and some of the *cis* isomers coeluted with those of the major FA, oleic and linoleic acids. In this paper, we describe the calculation of factors needed to correct the overestimates of 9*c*-18:1 (oleic acid) and 9*c*,12*c*-18:2 (linoleic acid) obtained with short packed columns and present examples. On the other hand, *trans*-18:1 acid contents, even when determined by capillary column GLC, are mostly underestimated, and appropriate correction factors to obtain more precise data have been published (1,8).

As shown in Figure 1 and described below, the old GLC peaks identified as 18:1 and 18:2 contained numerous *cis* and *trans* isomers that were not 9*c*-18:1 or 9*c*,12*c*-18:2. From the chromatograms in Figure 1 obtained from the same sample of bovine milk fat and published identifications (1–3,9), we find that the 4–15 *cis* and *trans* isomers coelute with 9*c*-18:1 on the short packed GLC columns. These were identified and quantified as oleic acid (18:1) in the older publications. The old 18:2 peak contained 9*c*,12*c*-18:2, all of the *trans* 18:2 isomers with at least one *trans* double bond (except conjugated linoleic acids, and 16*t*-18:1. We have calculated correction factors by dividing the correct by the old or overestimated contents. The latter were obtained by adding the quantities of other *cis* and *trans* isomers from Ag-TLC/GLC analyses of German bovine and human milk lipids, and margarine FA to the exact contents of 9*c*-18:1 and 9*c*,12*c*-18:2 from these analyses. All of this information and references are given in Table 1.

In Table 1, we have provided seasonal (feed) correction factors for bovine milk. These should be generally applicable to most data since the FA contents of bovine milk lipids have stayed relatively stable for years. Further, Table 1 gives correction factors for margarines and human milk. Application of these factors to old U.S. data and the old German data on human milks from Harzer *et al.* (7) yields the corrected amounts of 18:1 and 18:2 in Table 2. Harzer *et al.* employed columns (4 m × 1/8″) that contained Silar 10C, a polar material. However, they did not detect *trans* 18:1. Thus, depending on the material, the contents of oleic and linoleic acids have been overestimated by 19-100% and 3–108%, respectively.

Our correction factors for human milk may not be so widely applicable since the consumption of foods containing partially hydrogenated oils varies considerably worldwide (13). The contents of fatty acids in human milk respond overnight to the quantities in the diet. The amounts of 18:1, 18:2, and *trans* isomers in margarines also vary depending upon the oils and processing methods used. Some manufacturers have reduced the *trans* FA content of their products. To summarize, the quantities of 18:1 and 18:2 in old data on FA contents of materials, which may have contained *trans* FA and which were analyzed with short packed GLC columns, are overestimated by up to more than 100%. The universally employed phases for packed columns were of medium polarity, e.g., diethylene glycol succinate (DEGS), with column lengths of 2 to 4 m. The major source of data on FA composition in the United States is available from the website of the U.S. Department of Agriculture (USDA): (http://www.nal.usda.gov/fnic/foodcomp). Many recommendations for changes in FA consumption are based on these data. However, these tables do not contain data on positional *cis* or *trans* FA isomers. Oleic and linoleic acids are assumed to be the 9*c*-18:1 and 9*c*,12*c*-18:2 FA, but are overlapped by the numerous other *cis/trans* isomers mentioned (9). However, in future

**TABLE 2**
**Old and Corrected Amounts (wt%) of 18:1 and 18:2 in Several Materials Containing *trans* Fatty Acids**

| | Oleic acid (18:1) | | | Linoleic acid (18:2) | | |
|---|---|---|---|---|---|---|
| Sample | Correction factors[a] | Old | Corrected | Correction factors[a] | Old | Corrected |
| Bovine milk, United States[b] | | | | | | |
| November, barn | 0.84 | 26.19 | 22.00 | 0.55 | 2.53 | 1.39 |
| May, transition | 0.80 | 28.10 | 22.48 | 0.47 | 3.05 | 1.43 |
| August, pasture | 0.78 | 29.00 | 22.62 | 0.43 | 2.86 | 1.23 |
| Total | 0.81 | 27.39 | 22.19 | 0.49 | 2.78 | 1.31 |
| Human milk[c] | 0.86 | 35.02 | 30.12 | 0.89 | 11.78 | 10.48 |
| Margarines, United States[d] | | | | | | |
| Soybean, stick | 0.50 | 45.80 | 22.90 | 0.97 | 13.40 | 13.00 |
| Corn, stick | 0.50 | 38.20 | 19.10 | 0.97 | 22.40 | 21.73 |

[a]Correction factors from Table 1.
[b]Data from Palmquist *et al.* (6). Samples from 50 cheese plants in 10 areas in the United States. Analyses done with short packed GLC columns.
[c]Data from Harzer *et al.* (7). Analyses done with short packed GLC column. German mothers, 36 d postpartum.
[d]Analyses done with short packed columns. For abbreviation see Table 1.

releases, the USDA will be publishing such values as these data become available from contract analyses and the literature. It is clear that all future analyses of materials containing positional and geometric isomers of unsaturated FA must be done with the Ag-TLC/GLC or a comparable method. Omission of the TLC step will result in an underestimate of *trans*-18:1 and total *trans* FA contents (1) and overestimates of 9*c*-18:1 and occasionally 9*c*,12*c*-18:2 even with capillary columns. However, linoleic acid (18:2) is not changed when columns 100 m in length are employed, with or without the TLC step. The contents of the essential linoleic acid and oleic acid in old 18:2 or 18:1 data are reduced by the correction factors. Are the old levels of 18:2 that were recommended to prevent deficiency symptoms incorrect? Possibly. This suggestion may also apply to the amounts of relevant FA in recent findings concerning premature atherosclerosis (3). This should be checked. Special effects of individual isomers on metabolism have already been found (e.g., Ref. 14), and more may exist but will not be detected until the contents of these isomers are determined. More relevant information is given in Reference 15.

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ARTICLE

# The Effect of Conjugated Linoleic Acid on Platelet Function, Platelet Fatty Acid Composition, and Blood Coagulation in Humans

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**ABSTRACT:** Despite extensive research on conjugated linoleic acid (CLA) showing multiple beneficial effects in animal models, little is known about the role of dietary CLA in human health. To investigate if the beneficial effects of CLA seen in animal models are relevant to humans, we conducted a study with 17 healthy female volunteers who lived in the Metabolic Research Unit of the Western Human Nutrition Research Center for 93 d. This paper reports only the results from this study that are related to the effects of CLA supplementation on blood coagulation, platelet function, and platelet fatty acid composition. Throughout the study, the subjects were fed a low-fat diet (30 en% fat, 19 en% protein, and 51 en% carbohydrate) consisting of natural foods with the recommended dietary allowances for all known nutrients. After a 30-d stabilization period, subjects were randomly assigned to either an intervention group ($n$ = 10) whose diet was supplemented with 3.9 g/d of CLA or a control group ($n$ = 7) who received an equivalent amount of sunflower oil consisting of 72.6% linoleic acid with no detectable CLA. Platelet aggregation was measured in platelet-rich plasma using adenosine diphosphate, collagen, and arachidonic acid agonists. No statistical difference was detected between the amount of agonist required to produce 50% aggregation of platelet-rich plasma before and after the subjects consumed the CLA, with the exception of a decrease in response to collagen. This decrease was found in both control and intervention groups with no significant difference between the groups, suggesting that both linoleic acid (sunflower oil) and CLA might have similar effects on platelet function. The prothrombin time, activated partial thromboplastin time, and the antithrombin III levels in the subjects were determined. Again, there was no statistically significant difference in these three parameters when pre- and post-CLA consumption values were compared. The *in vivo* bleeding times were also unaffected by CLA supplementation (10.4 + 2.8 min pre- and 10.2 + 1.6 min postconsumption). Platelet fatty acid composition was not markedly influenced by the consumption of dietary CLA, although there was a small increase in the amount of the 9 *cis*,11 *trans*-18:2 isomer normally present in platelets after feeding CLA for 63 days. In addition, small amounts of the 8 *trans*,10 *cis*-18:2 and the 10 *trans*,12 *cis*-18:2 isomers were detected in the platelets along with traces of some of the other isomers. Thus, when compared to sunflower oil, the blood-clotting parameters and *in vitro* platelet aggregation showed that adding 3.9 g/d of dietary CLA to a typical Western diet for 63 d produces no observable physiological change in blood coagulation and platelet function in healthy adult females. Short-term consumption of CLA does not seem to exhibit antithrombotic properties in humans.

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Atherosclerosis causes heart attack, gangrene, and stroke and is the cause of half of all mortalities in the United States (1). Platelets adhere to the wall of the blood vessel and contribute to atherogenesis by releasing growth factors (1). There are many studies on how dietary fatty acids could reduce platelet activation, thus minimizing the risk of cardiovascular disease. However, most of those studies have focused on the long-chain n-3 fatty acids and have consistently found a decrease in platelet activation after consuming fish oil diets or supplementation (2). In contrast, human studies on the effects of n-6 polyunsaturated fatty acids (PUFA) on platelet function are far less frequent, and the results are more controversial. On the one hand, linoleic acid, an n-6 PUFA, has been found to be antithrombotic in various *ex vivo* filtragometer human studies (3–5). On the other hand, there are intervention studies that have shown increased *in vitro* platelet aggregation in response to various agonists after consuming high-linoleic acid diets (6,7). However, the effect of dietary conjugated linoleic acid (CLA; a collective term for a mixture of positional and geometric isomers of linoleic acid) on platelet function in humans has not been studied.

CLA is a PUFA found naturally in the food supply. It is especially abundant in food lipids derived from ruminant animals such as beef and lamb and dairy products (8,9) due to increased conversion of linoleic acid to CLA by rumen microorganisms. Ever since Pariza and Hargraves (10) reported in 1985 that CLA exhibited anticarcinogenic activity in mouse skin and whose findings were later confirmed by other studies involving other types of tumors (stomach, mammary gland, and colon) (11–15), there has been great interest in these fatty acids. This interest intensified even more after numerous studies reported that CLA also exhibited other beneficial physiological effects such as protection against atherosclerosis (16–19), enhancement of immunological function (20–23), and reduction of body fat while increasing lean mass (24–28) and bone mass (29).

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Abbreviations: AA, arachidonic acid; APTT, activated partial thromboplastin times; CLA, conjugated linoleic acid; FAME, fatty acid methyl esters; GLC, gas–liquid chromatography; PRP, platelet-rich plasma; PT, prothrombin time; PUFA, polyunsaturated fatty acids; TX, thromboxane; WHNRC, Western Human Nutrition Research Center.

Since all of these effects have been observed in experiments with only human cell culture and animal models, we conducted a strictly controlled human feeding study to better understand the physiological and metabolic effects of dietary CLA in humans. Relevant to this paper is whether consumption of CLA may have thrombotic properties by affecting platelet function and blood coagulation. Here we report the effects of supplementing a natural food diet with 3.9 g/d of CLA in the form of a commercially available preparation, Tonalin® (Pharmanutrients, Inc., Lake Bluff, IL), on platelet function *in vitro*, bleeding times, coagulation factors, and platelet fatty acid composition. The study was conducted in a metabolic ward with healthy female subjects under the constant supervision of our dietary and nursing staffs.

*Study design.* The design was a randomized, placebo-controlled, single-blind feeding study in which healthy female volunteers ($n$ = 17) lived in the Metabolic Research Unit of the Western Human Nutrition Research Center (WHNRC) for 93 d. It was conducted with two cohorts since the Metabolic Research Unit can only accommodate 12 subjects.

From day 1 and throughout the study, subjects consumed a low-fat diet equivalent to the American Heart Association's Step II Diet with the RDA for all known nutrients and 30% of the calories from fat. During the stabilization period, the caloric intake of each subject was estimated, and the caloric value of the individual's menu adjusted accordingly to ensure that no significant gain or loss in body weight would occur. During the first 30 d (stabilization period), subjects received two placebo capsules (sunflower oil) with each meal every day to become accustomed to taking daily capsules. On day 31, subjects were randomly assigned to either the intervention group receiving supplemental CLA (3.9 g administered in six capsules daily, two with each meal) to the end of the study or the control group which continued taking the placebo capsules for all 93 d. Blood draws were taken at the start of the study (day 1), the end of the baseline period (day 30), midpoint of the supplementation period (day 63), and the end of the study (day 93).

## MATERIALS AND METHODS

*Subjects.* The protocol of this study was approved by the Institutional Review Boards of the University of California at Davis (Davis, CA) and the United States Department of Agriculture (Washington, DC). The recruitment and screening processes have been described previously in other papers from our Center (30–33).

The physical parameters of the 17 volunteers who completed the study were: age, 27.9 ± 6.0 yr; weight, 63.1 ± 8.5 kg; body mass index, 22.9 ± 2.4 kg/m$^2$; systolic blood pressure, 109.5 + 5.8 mm Hg; diastolic blood pressure, 67.4 + 7.8 mm Hg; menstrual cycle length, 28.6 + 1.9 d.

*Diet.* The nutrient composition of the diet was calculated from a computerized nutrient databank using the data from USDA Handbook No. 8 (34), the USDA Nutrient Database for Standard Reference (35), and the Food Processor for Windows (36). Diets were adjusted to provide at least the recommended daily intake for known essential nutrients (37). According to these databases, the nutrient composition of the diet was 30 en% fat, 19 en% protein, and 51 en% carbohydrate. The cholesterol content was 258 mg. All food was prepared in the WHNRC kitchen, and a 5-d menu cycle was used throughout the study. Specific menus were designed so that the fat would consist of saturated, monounsaturated, and polyunsaturated varieties at 10% each for both the placebo and intervention groups.

Sample menus were prepared before the study began to analyze the fatty acid composition of the diet so that the actual values would correspond to the theoretical values computed from the nutrient databank tables.

The fatty acid composition and a more detailed description of the diet are given in a companion paper from this study (38). A complete description of the diet, listing all major and minor nutrients, is available upon request.

*CLA supplements.* Subjects consumed a total of 3.9 g pure CLA daily (six Tonalin® capsules of 0.65 g of CLA each). This amount of CLA represents *ca.* 1.5% of the volunteers' total caloric intake. The placebo had the same daily amount of high-linoleic sunflower oil. All Tonalin® and placebo capsules were donated by Pharmanutrients, Inc.

The fatty acid compositions of the Tonalin® and placebo capsules are reported in a companion paper from this study (38). The CLA content of the Tonalin® capsules used for this study was *ca.* 65% of the total fatty acids. We identified 10 CLA isomers: the four major *cis/trans* (*c/t*) isomers (11.4% 9*c*,11*t*-18:2; 10.8% 8*t*,10*c*-18:2; 15.3% 11*c*,13*t*-18:2; 14.7% 10*t*,12*c*-18:2); their corresponding *c,c* varieties (1.38% 8*c*,10*c*-18:2; 1.59% 9*c*,11*c*-18:2; 2.45% 10*c*,12*c*-18:2; and 1.32% 11*c*,13*c*-18:2); and two *t,t* varieties (0.97% 11*t*,13*t*-plus 8*t*,10*t*-18:2 and 5.02% 9*t*,11*t*-plus 10*t*,12*t*-18:2).

*Materials.* Three individual CLA isomers, *c*-9,*t*-11 (catalog # 1248), *t*-9,*t*-11 (catalog # 1181), and *t*-10,*c*-12 (catalog # 1249) were obtained as free fatty acids from Matreya Inc. (Pleasant Gap, PA). A CLA reference standard containing a mixture of fatty acid methyl ester (FAME) isomers was used (catalog # UC-59-M; 99% pure; Nu-Chek-Prep, Inc., Elysian, MN), and the approximate isomeric composition of CLA according to the manufacturer's assay was: 41% *c,t/t,c*-9,11-18:2; 44% *c,t/t,c*-10,12-18:2; 10% *c,c*-10,12-18:2; others ~5% (*t,t*-9,11-18:2, *t,t*-10,12-18:2, *c,c*-9,11-18:2). Analyses in our laboratory indicated similar composition except that we also found the *c,t/t,c*-11,13 isomer (44% *c,t/t,c*-9,11-18:2; 35.5% *c,t/t,c*-10,12-18:2; 18% *c,t/t,c*-11,13-18:2; 2% others).

*Measurements.* Blood was drawn between 7:00 and 8:00 A.M. after an overnight fast or at least 12 h without food. Blood was drawn into either anticoagulant-coated vacutainers using Teflon catheters (Angiocath; Deseret Medical, Sandy, UT) for fatty acid analysis, or syringes containing 1 mL of citrate solution for platelet studies.

Platelet-rich plasma (PRP) was prepared by low-speed centrifugation for 15 min at 100 × *g*. Platelet-poor plasma was prepared by respinning the blood after the removal of PRP for

15 min at 400 × *g*. Red cells for fatty acid analysis (data not shown here) were prepared from the residue by resuspending the cells in 10 mL of phosphate-buffered saline and recentrifuging at 400 × *g* for 15 min three times. The supernatant was discarded. Platelets were isolated by centrifuging the PRP at 400 × *g* for 15 min, resuspending them in 7 mL of phosphate-buffered saline and recentrifuging at 400 × *g* for 15 min two more times. The supernatant was discarded. Samples were frozen at –70°C until extraction of the lipids.

*Platelet aggregation.* Platelet aggregation tests were performed as described previously (39,40). Aggregating agents were prepared as recommended by Chrono-log (Havertown, PA): ADP, 1 mM/mL; collagen, 0.5 mg/mL; arachidonic acid (AA), 0.05 mM. The *in vitro* aggregation characteristics of the subjects' platelets were measured in a dual channel aggregometer (model 560; Chrono-log Corp.). Briefly, 235 μL of PRP was added to an aggregation tube containing a Teflon-coated stirring bar. The blank was 250 μL of the individual platelet-poor plasma, unstirred. The instrument was zeroed to 10% transmission of the sample vs. the blank. An appropriate amount of aggregating reagent was added to the PRP, and aggregation was measured as the increase in transmission of light through the PRP. The threshold for aggregation was determined when the amount of a particular reagent produced at least 50% of maximum aggregation within 15 min of its addition to the PRP. Preparations of PRP were all adjusted to 300,000 platelets/mm$^3$. All aggregation tests were performed within 4 h of the initial blood draw.

*Platelet counts.* Platelet counts were determined with an electronic particle counter (model 9300, Differential Cell Counter; Baker-Serono Diagnostics, Inc., Allentown, PA) and a cytometer using the manufacturer's recommended procedures (Operations Manual No. DS-014A, 1988, Baker-Serono Diagnostics, Inc.).

*Bleeding times.* Bleeding times were measured on the left or right proximal forearm of each subject using a 1-mm deep by 1-cm long incision and a Simplate II bleeding time device (Organon Teknika, Durham, NC). A wipe of the incision was made every 30 s. Time was measured with a hand-held stopwatch (39).

*Clotting factors.* Prothrombin time (PT) and the activated partial thromboplastin times (APTT) were measured by the methods described by Houghie (41). Briefly, platelet-poor plasma was prepared as described elsewhere (37), and the PT and APTT were measured (MCA 110; Bio Data Corporation, Hatboro, PA) with coagulation reagents (Organon Teknika). Antithrombin III was measured by an automated centrifugal analyzer method (42). All tests for the clotting factors were performed within 1 h of separation of the plasma from the whole blood. Plasma samples were kept on ice until warmed to 37°C for the test.

*Lipid extraction and preparation of FAME.* The lipids were extracted as described by Nelson (43,44) using chloroform/methanol (2:1, vol/vol). The total lipid extracts and the free fatty acid CLA standards were transmethylated with sodium methylate for 10 min at 55°C followed by reaction with 1 N hydrochloric acid in methanol for 10 min at 80°C, as described by Carreau and Dubacq (45), in order to avoid isomerization of CLA isomers (46,47). The FAME were extracted with hexane and purified by thin-layer chromatography as described elsewhere (42) before dilution and injection into the gas chromatograph.

*Gas–liquid chromatography (GLC).* The FAME were analyzed by GLC (model 6893; Hewlett-Packard, Palo Alto, CA) with the Hewlett-Packard ChemStation III software running on an IBM-compatible desktop computer.

A SP-2380 column (100 m × 0.25mm i.d. × 0.2 mm film thickness; Supelco Inc., Bellefonte, PA) was used. The column was operated at 75°C for 4 min, then temperature-programmed at 13°C/min to 175°C, and held there for 27 min, followed by a second temperature program at 4°C/min to 215°C, then held there for 31 min. The total run time was 79.69 min.

Fatty acids were identified by comparison of their retention time with authentic standards. If a GLC peak was not clearly identified by its retention time, ion trap mass spectra were compared to mass spectra from NIST or mass spectra prepared in our laboratory.

*Statistical analysis.* Data from the start of the intervention diet (day 30) and the end of the study (day 93) were compared. The clotting data and aggregation data were analyzed using the paired *t*-test. A *P*-value of less than 0.05 was considered significant. The fatty acid data were using two-way analysis of variance (ANOVA) and Fisher post-ANOVA test. All statistical analyses were performed with the PC SAS Data System (Version 6.11) obtained under license from the SAS Institute (SAS PC Manual; SAS Institute, Chapel Hill, NC, 1993).

## RESULTS

No change in blood clotting and platelet function was detected in subjects who consumed CLA at 3.9 g/d for 63 d. The average bleeding time for the 10 subjects after consuming CLA for 63 d was 10.22 ± 1.64 min as compared to the baseline value of 10.40 ± 2.80 min after consuming a low-fat diet without supplement for 30 d. The difference between these values was not statistically significant. Similarly, blood pressure, measured daily in all subjects, did not change significantly from the corresponding baseline values in either the intervention or control groups throughout the study.

Table 1 shows the platelet aggregation *in vitro* and the platelet counts for both intervention and control groups after 63 d on either CLA or sunflower oil supplements, respectively. The platelet counts are the actual platelet numbers in the whole blood of the subjects, not the adjusted values used for the aggregation tests. Again, there was no statistically significant change in either the aggregation values or the platelet counts when the results from the two groups (intervention and control) were compared. Although there was a decrease in platelet aggregation in response to collagen for both groups after consuming CLA for 63 d, the difference between the

**TABLE 1**
**Platelet Aggregation and Cell Count[a]**

| | Study day 30 | Study day 93 | *P*-value[b] |
|---|---|---|---|
| Intervention group ($n$ = 10) | | | |
| ADP (μM) | 0.90 ± 0.65 | 0.84 ± 0.58 | 0.77 |
| Collagen (μg/mL) | 0.46 ± 0.13 | 0.30 ± 0.11 | 0.04 |
| AA (μM) | 0.28 ± 0.19 | 0.16 ± 0.12 | 0.19 |
| Count (1000/mm$^3$) | 292 ± 69 | 289 ± 59 | 0.58 |
| Control group ($n$ = 7) | | | |
| ADP (μM) | 1.13 ± 0.70 | 0.57 ± 0.35 | 0.16 |
| Collagen (μg/mL) | 0.49 ± 0.11 | 0.29 ± 0.11 | 0.04 |
| AA (μM) | 0.24 ± 0.13 | 0.14 ± 0.10 | 0.15 |
| Count (1000/mm$^3$) | 289 ± 116 | 288 ± 108 | 0.95 |

[a]Mean values ± SD. AA, arachidonic acid.
[b]Paired *t*-test (SD 30 vs. SD 93).

intervention and control groups was not statistically significant.

Table 2 lists values for the soluble blood coagulation parameters for these subjects before and after they consumed the CLA. Neither the PT nor the APTT value changed after CLA consumption. Antithrombin III values before and after CLA feeding were not significantly different either.

Table 3 compares the total fatty acid composition of the whole platelets after the subjects consumed CLA for 63 d. No major difference was detected in the normal fatty acids present in human platelets although the main CLA isomers present in the diet during the intervention phase of the study did show a slight increase after 63 d of CLA feeding. The CLA isomers present in significant amounts were the 9*c*,11*t* (perhaps some 8*t*,10*c*), and the 10*t*,12*c* varieties. Other minor CLA isomers were also detected but in amounts too small to quantify with the available samples. It should be noted that the quantities of these isomers present in platelets were minute, i.e., never more than 0.30 wt% of the total fatty acids in the platelets.

## DISCUSSION

While very little has been reported about the effects of CLA on platelet aggregation, the results of one *in vitro* study (48) suggest that CLA isomers may possess antithrombotic properties. Truitt *et al.* (48) found that 9*c*,11*t*-18:2 and 10*t*,12*c*-18:2 inhibited AA- and collagen-induced platelet aggregation more than their nonconjugated 9*c*,12*c* isomer (linoleic acid).

**TABLE 2**
**Blood Coagulation Properties[a]**

| | Study day 30 | Study day 93 | *P*-value[b] |
|---|---|---|---|
| Prothrombin time (s) | 12.73 ± 0.60 | 12.37 ± 0.60 | 0.13 |
| Activated partial thromboplastin time (s) | 31.30 ± 3.20 | 31.33 ± 3.20 | 0.93 |
| Antithrombin III (%) | 79.31 ± 16.78 | 88.19 ± 13.61 | 0.11 |

[a]Mean values ± SD. Intervention group ($n$ = 10).
[b]Paired *t*-test (SD 30 vs. SD 93).

Among the different aggregating agents used by Truitt *et al.* (48), the most pronounced inhibitory effect was found with collagen. In our study, we also found a decrease in platelet aggregation in response to collagen for both placebo and intervention groups. However, the difference between the groups was not significant, suggesting that both linoleic acid (the sunflower oil used as placebo contained 72.6% linoleic acid with no detectable CLA) and CLA have similar effects on platelet aggregation. This finding contradicts those from Truitt's study in which they found CLA to be more effective than linoleic acid in reducing platelet aggregation. We did not find any effect on platelet aggregation in response to either ADP or AA.

The CLA used by Truitt *et al.* (48) consisted of two individual isomers (9*c*,11*t* and 10*t*,12*c*) and a mixture consisting mainly of these two isomers (44% 9*c*,11*t*, 46% 10*t*,12*c*) plus a small amount of other CLA isomers (4%) and other fatty acids (4% linoleic acid; 2% oleic acid). The antithrombotic effect was found with both the individual isomers and CLA mixture. In our study, these two isomers made up about 40% of all isomers while in Truitt's CLA mix these two isomers were 90% of all the CLA isomers. Perhaps the discrepancy between our and Truitt's results is related to the difference in CLA composition since it is becoming apparent that different isomers appear to produce different effects (49). We used Tonalin® since purified isomers of CLA for human feeding trials were not available at the time we conducted this study. However, despite the fact that Tonalin® contains isomers other than the 9*c*,11*t* and the 10*t*,12*c* varieties, it should be noted that in the platelets of those volunteers who consumed the CLA we found mainly these two isomers.

It should also be noted that the study by Truitt *et al.* (48) consisted of a series of *in vitro* experiments where platelets obtained from human donors were isolated and platelet suspensions were preincubated with various concentrations of fatty acid followed by the addition of aggregating agents. However, in our study, platelet aggregation was measured in PRP from volunteers who had consumed a controlled diet supplemented with CLA for 63 d. To the best of our knowledge, our study is the first to examine the effects of CLA supplementation on platelet function and aggregation in humans. Further research is necessary to better understand the role of CLA on platelet function.

Linoleic acid can be converted to AA by humans and other animal species. Platelet aggregation involves the release of endogenous AA which is converted to the proaggregatory thromboxane $A_2$ ($TXA_2$). Our data on eicosanoids are not available yet, but the study by Truitt *et al.* (48) suggests that CLA may act as a competitive inhibitor of the AA substrate for the cyclooxygenase enzyme. When they compared the effects of CLA on the platelet cyclooxygenase and lipoxygenase activities (measured by $TXB_2$ and 12-hydroxyeicosatetraenoic acid formation, respectively) to linoleic acid, they found that the 9*c*,11*t*- and 10*t*,12*c*-isomers, as well as the CLA isomer mix, inhibited the formation of the proaggregatory cyclooxygenase-catalyzed product, $TXA_2$, as measured

**TABLE 3**
**Platelet Fatty Acid Composition (wt% of total fatty acids)[a]**

| | Intervention group ($n$ = 10) | | Control group ($n$ = 7) | |
|---|---|---|---|---|
| Fatty acid | Study day 30 | Study day 93 | Study day 30 | Study day 93 |
| | (Means ± SD) | | | |
| 16:0DMA | 2.50 ± 0.45 | 2.66 ± 0.31 | 2.21 ± 0.39 | 2.60 ± 0.51 |
| 16:0 | 14.98 ± 0.89 | 14.67 ± 1.50 | 14.62 ± 0.56 | 14.48 ± 0.88 |
| 18:0DMA | 3.47 ± 0.66 | 3.95 ± 0.40 | 3.05 ± 0.24 | 3.56 ± 0.79 |
| 18:1n-9DMA | 0.42 ± 0.09 | 0.51 ± 0.02 | 0.47 ± 0.06 | 0.50 ± 0.10 |
| 18:1n-7DMA | 0.88 ± 0.17 | 0.82 ± 0.05 | 0.67 ± 0.07 | 0.81 ± 0.18 |
| 18:0 | 17.08 ± 1.00 | 17.71 ± 0.94 | 15.81 ± 0.75 | 16.68 ± 1.09 |
| 18:1n-9*t* | 1.37 ± 0.21 | 1.46 ± 0.21 | 1.46 ± 0.33 | 1.41 ± 0.26 |
| 18:1n-9 | 13.14 ± 0.73 | 13.08 ± 0.77 | 13.32 ± 0.81 | 13.09 ± 0.73 |
| 18:1n-7 | 0.91 ± 0.07 | 0.79 ± 0.13 | 0.97 ± 0.13 | 0.88 ± 0.10 |
| 18:1n-5 | 0.50 ± 0.08 | 0.44 ± 0.07 | 0.62 ± 0.12 | 0.46 ± 0.11 |
| 18:2n-6 | 8.05[a] ± 0.87 | 6.77[b] ± 0.48 | 6.56 ± 0.41 | 7.74 ± 0.77 |
| 18:3n6+20:0 | 0.63 ± 0.10 | 0.60 ± 0.10 | 0.59 ± 0.10 | 0.63 ± 0.10 |
| 20:1n-9 | 0.40 ± 0.05 | 0.44 ± 0.08 | 0.39 ± 0.09 | 0.43 ± 0.09 |
| CLA1 + CLA2 | 0.12[a] ± 0.03 | 0.30[b] ± 0.08 | 0.14 ± 0.08 | 0.10 ± 0.02 |
| CLA3 | 0.08[a] ± 0.03 | 0.20[b] ± 0.06 | 0.05 ± 0.04 | 0.07 ± 0.03 |
| CLA4 | 0.00[a] ± 0.00 | 0.10b ± 0.06 | ND | ND |
| 20:2n-6 | 0.35 ± 0.06 | 0.36 ± 0.09 | 0.28 ± 0.03 | 0.37 ± 0.08 |
| 22:0 | 1.23 ± 0.33 | 1.06 ± 0.31 | 1.15 ± 0.09 | 1.10 ± 0.16 |
| 20:4n-6 | 24.45 ± 1.12 | 23.33 ± 2.26 | 27.12 ± 1.33 | 24.82 ± 1.20 |
| 22:2n-6 | 0.17 ± 0.04 | 0.17 ± 0.04 | 0.41 ± 0.22 | 0.17 ± 0.04 |
| 24:1n-9 | 2.22 ± 0.37 | 2.34 ± 0.52 | 2.39 ± 0.50 | 2.34 ± 0.25 |
| 22:5n-3 | 1.26 ± 0.17 | 1.27 ± 0.22 | 1.69 ± 0.31 | 1.41 ± 0.29 |
| 22:6n-3 | 1.43 ± 0.35 | 1.30 ± 0.30 | 1.56 ± 0.55 | 1.48 ± 0.30 |
| Sum of knowns | 96.98 ± 0.48 | 97.27 ± 0.50 | 98.00 ± 0.36 | 97.69 ± 0.64 |
| Sum of unknowns | 2.82 ± 0.48 | 2.73 ± 0.50 | 2.00 ± 0.36 | 2.31 ± 0.64 |

[a]ND, not detected. Values with different superscripts are significantly different at $P < 0.05$ using two-way analysis of variance (ANOVA) and Fisher post-ANOVA test. DMA stands for dimethyl acetal; *c* for *cis*; and *t* for *trans*. CLA1 is the 9*c*,11*t*-18:2 isomer; CLA2 is the 8*t*,10*c*-18:2 isomer; CLA3 is the 10*t*,12*c*-18:2 isomer; and CLA4 is the 11*c*,13*t*-18:2 isomer.

by decreased production of its inactive metabolite $TXB_2$ from exogenously added AA. However, none of the CLA isomers tested inhibited the production of the platelet lipoxygenase metabolite 12-HETE.

We did not find, after 63 d of CLA consumption at 3.9 g/d, any significant effect on *in vivo* bleeding times and soluble clotting factors as measured by PT, APTT, and antithrombin III (considered a measure of *in vivo* thrombotic tendency). We did find that CLA isomers, mainly the 9*c*,11*t*- and 10*t*,12*c*-isomers, were present in the platelets of those volunteers who consumed the CLA supplement for 63 d. However, the amount present in the platelet was at a much reduced level compared to the amount fed in the diet. It would appear that all *cis*, polyunsaturated, nonconjugated fatty acids are more readily incorporated into platelets than their conjugated analogs. While our supplement contained more than 20% of the 11*c*,13*t*-isomer of CLA, we did not find any trace of this compound in the volunteers' platelets. It is believed that the 11*c*,13*t*-isomer is an artifact of the synthetic process, thus not found in natural CLA present in food sources or produced by bacteria in the lumen of ruminants. The finding here suggests that this CLA isomer is not incorporated in tissues as readily as the CLA isomers normally found in the human food chain. We have reported the effects of dietary CLA on the fatty acid composition of plasma and adipose tissue, as well as on blood lipid concentrations, lipoproteins, and apolipoproteins, in a companion paper (38) using other data from this study.

The amount of CLA that subjects consumed in this study was three to four times that found in the Western diet. Daily consumption of CLA by the U.S. population is estimated to be from several hundred mg/person/d (11,50) to 1 g/person/d (13,51,52). We chose to provide *ca.* 1.5% of calories from CLA (3.9 g/d) because that concentration had been shown to affect mammary tumorigenesis and immune response in experimental animals, and no additional beneficial effect was observed when the dose exceeded that threshold (13,53,54). In our study, no short-term adverse health effect or discomfort was observed or reported when CLA was consumed continuously for 63 d at such dosage.

The absence of measurable effects of dietary CLA supplement in humans, as we have reported in other papers containing other results from this study (32,33,38,55), is in marked contrast to the effects of CLA in animal studies (16,20–23,56–59). Of course, one of the major differences between many of the animal studies and our study is that most of the animal studies were conducted in young animals undergoing rapid growth. Here we studied mature women with stable body weight. It could be that for CLA to exert its effects, the animal

must be in a growth phase. Another possible explanation for the differences between our study and most of the animal studies reported to date is that we fed our volunteers for only 63 d while many of the animal studies extended the feeding period to the equivalent of human years. In addition, ours was a metabolic ward study; thus the number of subjects was relatively small: 10 in the intervention group and 7 in the control group. With only 17 volunteers in this study, the statistical power was not necessarily sufficient to rule out the possibility of false negative results. The platelet function tests using ADP and AA were subject to a large standard deviation and hence cannot be considered definitive. The test with collagen was borderline at a power of 80% to detect a 25% change at the 0.05 significance level. The sample size was adequate for the fatty acid composition data and the bleeding parameters.

In summary, the results of this study suggest that dietary CLA consumed at the level used in this study has little, if any, effect on blood clotting and platelet function, despite the fact that CLA isomers are incorporated into platelets during the course of this study. CLA has no detectable adverse effect in human females at the level fed in this study for 63 d.

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# The Effect of Conjugated Linoleic Acid on Plasma Lipoproteins and Tissue Fatty Acid Composition in Humans

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**ABSTRACT:** Conjugated linoleic acid (CLA) has been suggested by some animal studies to possess antiatherogenic properties. To determine, in humans, the effect of dietary CLA on blood lipids, lipoproteins, and tissue fatty acid composition, we conducted a 93-d study with 17 healthy female volunteers at the Metabolic Research Unit of the Western Human Nutrition Research Center. Throughout the study, subjects were fed a low-fat diet [30 energy percent (en%) fat, 19 en% protein, and 51 en% carbohydrate] that consisted of natural foods with the recommended dietary allowances for all known nutrients. After a 30-d stabilization period, subjects were randomly assigned to either an intervention group ($n$ = 10) supplemented daily with capsules containing 3.9 g of CLA or a control group ($n$ = 7) that received an equivalent amount of sunflower oil. The CLA capsules (CLA 65%) contained four major *cis/trans* geometric isomers (11.4% 9 *cis*-,11 *trans*-18:2; 10.8% 8 *trans*-,10 *cis*-18:2; 15.3% 11 *cis*-,13 *trans*-18:2; and 14.7% 10 *trans*-,12 *cis*-18:2) and their corresponding *cis/cis* (6.74% total) and *trans/trans* (5.99% total) varieties in smaller amounts. Fasting blood was drawn on study days 30 (end of the stabilization period), 60 (midpoint of the intervention period), and 93 (end of the intervention period). Adipose tissue samples were taken on days 30 and 93. CLA supplementation for 63 d did not change the levels of plasma cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, and triglycerides. The weight percentage of CLA in plasma increased from 0.28 ± 0.06 to 1.09 ± 0.31 ($n$ = 10, $P < 0.05$) after the supplementation. The 9 *cis*-,11 *trans*-isomer was the most prominent variety followed by the 11 *cis*-,13 *trans*- and 10 *trans*-,12 *cis*-isomers in lesser amounts. CLA in adipose tissue was not influenced by the supplementation (0.79 ± 0.18 to 0.83 ± 0.19 wt%) ($n$ = 10) and the 9 *cis*-,11 *trans*-variety was the only isomer present. Thus, contrary to findings from some animal studies, CLA does not seem to offer health benefits, in the short term, regarding the prevention of atherosclerosis in humans. CLA supplementation for 2 mon did not alter the blood cholesterol or lipoprotein levels of healthy, normolipidemic subjects. The supplementation did increase CLA in the plasma but only 4.23% of the ingested CLA was present in the plasma at any given time. No adverse effect of CLA supplementation was detected in this study.

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Abbreviations: apo, apolipoprotein; CLA, conjugated linoleic acid; DMA, dimethyl acetal; FAME, fatty acid methyl ester; HDL, high density lipoprotein; LDL, low density lipoprotein; PRP, platelet-rich plasma; RDA, recommended daily allowance; TG, triglyceride; USDA, U.S. Department of Agriculture; WHNRC, Western Human Nutrition Research Center.

Conjugated linoleic acid (CLA) is a polyunsaturated fatty acid found naturally in the food supply. It is especially abundant in food lipids derived from ruminant animals such as beef, lamb, and dairy products (1,2) due to increased conversion of linoleic acid to CLA by rumen microorganisms.

Ever since Pariza and Hargraves reported in 1987 that CLA exhibited anticarcinogenic activity in mouse skin (3), which was subsequently confirmed by studies involving other types of tumors (stomach, mammary gland, and colon) (4–8), there has been great interest in these fatty acids. This interest intensified even more after numerous studies reported that CLA also exhibited other beneficial physiological effects such as protection against atherosclerosis (9,10), enhancement of immunological function (11,12), and reduction of body fat while increasing lean mass (13–16) and bone mass (17).

However, all of these effects have been observed in experiments with only human cell culture and animal models; little is known of the physiological effects of dietary CLA in humans. Therefore, we conducted a strictly controlled human feeding study in which healthy female subjects consumed 3.9 g CLA/d for 63 d while living in our metabolic ward under the constant supervision of our dietary and nursing staffs. Although the overall objective of the study was to evaluate, in humans, the potentially beneficial physiological effects attributed to dietary CLA in animal studies, relevant to the findings reported in this paper is whether consumption of CLA may have health benefits in the prevention of arteriosclerosis and other cardiovascular diseases by improving the blood lipid levels and lipoprotein profile. Here we report the effects of supplementing a natural food diet for 63 d with 3.9 g/d of CLA in the form of a commercially available triglyceride (TG), Tonalin®, on several lipid parameters. Specifically, we examined the blood lipid levels, fatty acid composition of the plasma and adipose tissue, and lipoprotein cholesterol levels.

## STUDY DESIGN

The design was a randomized, placebo-controlled, single-blind feeding study in which healthy female volunteers ($n$ = 17) lived in the Metabolic Research Unit of the Western Human Nutrition Research Center (WHNRC) for 93 d.

From day 1 and throughout the study, subjects consumed a low-fat diet equivalent to the American Heart Association's Step II Diet with the recommended dietary allowance (RDA) for all known nutrients and 30% of the calories from fat. Dur-

ing the first 30 d (baseline/stabilization period), subjects received two placebo capsules (sunflower oil) with each meal every day to become accustomed to taking daily capsules. On day 31, subjects were randomly assigned to either the intervention group receiving supplemental CLA (3.9 g administered in six capsules daily, two with each meal) to the end of the study or the control group that continued taking the placebo capsules for all 93 d. Blood samples were drawn at the start of the study (day 1), the end of the baseline period (day 30), in the middle of the supplementation period (day 60), and the end of the study (day 93). Details of the study design have also been reported in other papers (18–21).

## MATERIALS AND METHODS

*Subjects.* The protocol of this study was approved by the Institutional Review Boards of the University of California at Davis (Davis, CA) and the United States Department of Agriculture (USDA, Washington, DC). The recruitment and screening processes have been described in other papers (18–21). The physical characteristics of the subjects at entry into the study (day 0) are given in Table 1.

*CLA supplements.* All Tonalin® and placebo capsules were donated by Pharmanutrients, Inc. (Lake Bluff, IL). The Tonalin® oil produced by Pharmanutrients met all the requirements for human consumption. The fatty acid compositions of the Tonalin® and placebo capsules are shown in Table 2. The CLA content of Tonalin® used for this study was approximately 65% of all fatty acids in the capsule. We identified 10 CLA isomers consisting of the four major *cis/trans* isomers (11.4% 9 *cis*-,11 *trans*-18:2; 10.8% 8 *trans*-,10 *cis*-18:2; 15.3% 11 *cis*-,13-*trans* 18:2; 14.7% 10 *trans*-,12 *cis*-18:2), their corresponding *cis/cis* varieties (1.38% 8 *cis*-,10 *cis*-18:2; 1.59% 9 *cis*-,11 *cis*-18:2; 2.45% 10 *cis*-,12 *cis*-18:2; 1.32% 11 *cis*-,13 *cis*-18:2), and two *trans/trans* varieties (0.97% 11 *trans*-,13 *trans*- plus 8 *trans*-,10 *trans*-18:2 and 5.02% 9 *trans*-,11 *trans*- plus 10 *trans*-,12 *trans*-18:2).

The subjects consumed a total of 3.9 g CLA daily (six Tonalin® capsules containing 0.65 g of CLA each). We chose this amount of CLA to represent approximately 1.5% of the volunteers' total caloric intake. This CLA concentration was comparable to that used in many of the animal studies.

**TABLE 1**
**Characteristics of Subjects[a]**

| Parameter | Values | |
|---|---|---|
| | Intervention group | Control group |
| Number of subjects completing study | 10 | 7 |
| Age (yr, mean ± SD) | 27.0 ± 5.6 | 29.3 ± 6.8 |
| Weight (kg, mean ± SD) | 63.1 ± 6.5 | 63.2 ± 11.4 |
| Body mass index (kg/m$^2$, mean ± SD) | 23.6 ± 1.5 | 21.9 ± 3.1 |
| Menstrual cycle length (d, mean ± SD) | 27.4 ± 0.5 | 29.7 ± 2.0 |
| Blood pressure (systolic, mean ± SD) | 109.7 ± 4.3 | 109.1 ± 7.8 |
| Blood pressure (diastolic, mean ± SD) | 66.9 ± 7.1 | 68.0 ± 9.3 |

[a]All female; no smokers.

**TABLE 2**
**Fatty Acid Composition of Tonalin® Conjugated Linoleic Acid (CLA) and Placebo (sunflower oil) Capsules**

| FAME[a] | Tonalin® CLA[b] | Placebo[b] |
|---|---|---|
| 14:0 | 0.25 ± 0.01 | |
| 16:0 (palmitate) | 4.71 ± 0.03 | 6.23 ± 0.10 |
| 16:1n-9 | 0.12 ± 0.01 | |
| 18:0 (stearate) | 1.95 ± 0.02 | 4.09 ± 0.02 |
| 18:1n-9 (oleate) | 24.70 ± 0.20 | 16.39 ± 0.02 |
| 19:0 | 0.20 ± 0.01 | |
| 18:2n-6 (linoleate) | 2.29 ± 0.03 | 72.57 ± 0.03 |
| 20:1n-9 | 0.24 ± 0.08 | |
| 9*c*,11*t*-18:2 | 11.43 ± 0.07 | |
| 8*t*,10*c*-18:2 | 10.79 ± 0.12 | |
| 11*c*,13*t*-18:2 | 15.29 ± 0.31 | |
| 10*t*,12*c*-18:2 | 14.69 ± 0.09 | |
| 8*c*,10*c*-18:2 | 1.38 ± 0.05 | |
| 9*c*,11*c*-18:2 | 1.59 ± 0.05 | |
| 10*c*,12*c*-18:2 | 2.45 ± 0.05 | |
| 11*c*,13*c*-18:2 | 1.32 ± 0.10 | |
| 11*t*,13*t*- and 8*t*,10*t*-18:2 | 0.97 ± 0.04 | |
| 9*t*,11*t*- and 10*t*,12*t*-18:2 | 5.02 ± 0.04 | |
| 20:2n-6 | 0.11 ± 0.03 | |
| 20:3n-6 | 0.29 ± 0.02 | |
| 22:0 (behenate) | | 0.71 ± 0.06 |
| Total | 99.92 ± 0.08 | 100.00 ± 0.00 |
| Unknowns | 0.08 ± 0.08 | |

[a]FAME: fatty acid methyl ester.
[b]Values given as means ± SD ($n$ = 5); Tonalin® and placebo supplied by Pharmanutrients, Inc. (Lake Bluff, IL).

The placebo contained high-linoleic sunflower oil of equal quantity.

*Diet.* The diet was equivalent to the American Heart Association's Step II Diet containing the RDA for all known nutrients and 30% of the calories from fat. It consisted of natural foods except for the CLA supplement in the form of Tonalin® and sunflower oil capsules. In addition, a supplement of D-α-tocopherol (vitamin E, 100-mg capsules given every 5 d; Bronson Pharmaceutical, St. Louis, MO) was included in the diet to ensure adequate antioxidant levels in the volunteers during the study.

All food was prepared in the WHNRC kitchen and a 5-d menu cycle was used throughout the study. Specific menus were designed so that the fat would consist of saturated, monounsaturated, and polyunsaturated varieties at 10% each for both the placebo and intervention groups. No alcohol was included in these diets. Either two Tonalin® or placebo (sunflower oil) capsules were administered to the subjects before each meal (breakfast, lunch, and dinner) under the supervision of the kitchen staff. Consequently, compliance was 100%.

The nutrient composition of the diet was calculated from a computerized nutrient databank using the data from USDA Handbook No. 8 (22), the USDA Nutrient Database for Standard Reference (23), and the Food Processor for Windows (24). The diet was adjusted to provide at least the RDA for known essential nutrients (25). A complete description of the diet, including all major and minor nutrients, is available upon request. Based on these databases, the nutrient compo-

sition of the diet was 30 en% fat, 19 en% protein, and 51 en% carbohydrate. The cholesterol content was 258 mg.

Sample menus were prepared before the study began to analyze the fatty acid composition of the diet so that the actual values would correspond to the theoretical values computed from the nutrient databank tables. For these analyses, five individual diet composite samples were taken from each menu and the results for the five composite samples were averaged. Table 3 shows the measured fatty acid composition of the diet supplemented either with two CLA capsules (diet + CLA) or with two sunflower oil capsules (diet + placebo).

The caloric intake of each subject was estimated during the stabilization period and the caloric value of each individual's menu was adjusted accordingly to ensure that no significant gain or loss in body weight due to caloric intake would occur during the study. The intakes for each volunteer were averaged during the entire study and the mean energy intake was approximately 2,100 Kcal/d (2,116.51 ± 191).

*Materials*. A CLA reference standard containing a mixture of fatty acid methyl ester (FAME) isomers (99% pure) was obtained from Nu-Chek-Prep, Inc. (catalog # UC-59-M; Elysian, MN), and the approximate isomeric composition of CLA according to the manufacturer's assay was: 41% *c,t/t,c*-9,11-18:2; 44% *c,t/t,c*-10,12-18:2; 10% *c,c*-10,12-18:2; others approximately 5% (*t,t*-9,11-18;2, *t,t*-10,12-18:2, *c,c*-9,11-18:2). Analyses in our laboratory indicated similar compositions except that we found it also contained the *c,t/t,c*-11,13 isomer (44% *c,t/t,c*-9,11-18:2; 18% *c,t/t,c*-11,13-18:2; 35.5% *c,t/t,c*-10,12-18:2; 2% others). Three individual CLA isomers (*c*-9,*c*-11, catalog # 1248; *t*-9, *t*-11, catalog # 1181; and *t*-10,*c*-12, catalog # 1249) were obtained from Matreya Inc. (Pleasant Gap, PA) as free fatty acids.

**TABLE 3**
**Fatty Acid Composition (wt%) of Experimental Diets**

| FAME | Diet + CLA[a] | Diet + placebo[a] | Diet w/o suppl.[a] |
|---|---|---|---|
| 11:0 | 0.38 ± 0.15 | 0.39 ± 0.18 | 0.40 ± 0.23 |
| 12:0 (laurate) | 0.67 ± 0.40 | 0.70 ± 0.45 | 0.70 ± 0.43 |
| 14:0 | 3.29 ± 1.12 | 3.14 ± 1.25 | 3.11 ± 1.19 |
| 14:1n-7 | 0.18 ± 0.04 | 0.19 ± 0.03 | 0.18 ± 0.04 |
| 14:1n-5 | 0.32 ± 0.04 | 0.32 ± 0.05 | 0.30 ± 0.06 |
| 15:0 | 0.34 ± 0.13 | 0.33 ± 0.13 | 0.32 ± 0.13 |
| 16:0 (palmitate) | 19.04 ± 1.00 | 18.19 ± 1.23 | 17.99 ± 1.30 |
| 16:1*t* | 0.13 ± 0.01 | | |
| 16:1n-9 | 1.14 ± 0.25 | 1.07 ± 0.22 | 1.06 ± 0.23 |
| 17:0 | 0.38 ± 0.09 | 0.35 ± 0.08 | 0.35 ± 0.08 |
| 18:1n-7 DMA | 0.28 ± 0.00 | 0.26 ± 0.00 | 0.26 ± 0.00 |
| 18:0 (stearate) | 8.24 ± 0.99 | 7.91 ± 0.94 | 7.77 ± 0.93 |
| 18:1*t*, all isomers | 5.23 ± 2.28 | 4.96 ± 2.24 | 4.58 ± 1.86 |
| 18:1n-9 (oleate) | 25.13 ± 2.35 | 24.34 ± 2.40 | 25.23 ± 2.27 |
| 18:1n-7 | 1.24 ± 0.21 | 1.19 ± 0.20 | 1.21 ± 0.20 |
| 18:1n-5 | 0.98 ± 0.52 | 1.08 ± 0.35 | 1.06 ± 0.34 |
| 19:0 | 0.11 ± 0.00 | | |
| 18:2*tt* | 0.25 ± 0.01 | | |
| 18:2n-6 (linoleate) | 29.85 ± 2.85 | 32.97 ± 2.31 | 27.56 ± 2.30 |
| 20:0 (arachidate) | 0.14 ± 0.02 | 0.15 ± 0.03 | 0.14 ± 0.02 |
| 18:3n-3 (linolenate) | 1.83 ± 0.31 | 1.74 ± 0.27 | 1.71 ± 0.28 |
| 20:1n-9 | 0.26 ± 0.00 | 0.26 ± 0.00 | 0.26 ± 0.00 |
| 9*c*,11*t*- and 8*t*,10*c*-18:2 | 2.22 ± 0.62 | | 0.23 ± 0.00 |
| 11*c*,13*t*-18:2 | 1.34 ± 0.42 | | |
| 10*t*,12*c*-18:2 | 1.30 ± 0.40 | | |
| 9*t*,11*t*- and 10*t*,12*t*-18:2 | 0.44 ± 0.13 | | |
| 20:3n-6 | 0.25 ± 0.13 | 0.28 ± 0.15 | 0.30 ± 0.13 |
| 20:4n-6 (arachidonate) | 0.19 ± 0.04 | 0.20 ± 0.00 | 0.20 ± 0.04 |
| 24:0 (lignocerate) | 0.24 ± 0.00 | 0.25 ± 0.00 | 0.24 ± 0.00 |
| Total | 98.69 ± 0.52 | 98.71 ± 0.58 | 98.86 ± 0.39 |
| Unknowns | 1.31 ± 0.52 | 1.29 ± 0.59 | 1.14 ± 0.39 |

[a]Values given as means ± SD ($n$ = 5). Diet w/o suppl., diet without supplementation; DMA, dimethyl acetal; for other abbreviations see Table 2.

*Measurements*. Blood was drawn between 7:00 and 8:00 A.M. after an overnight fast or at least 12 h without food. Blood was drawn into anticoagulant-coated vacutainers using Teflon catheters (Angiocath; Deseret Medical, Sandy, UT) for fatty acid analysis. The blood was spun at 100 × *g* for 15 min, and the platelet-rich plasma (PRP) was removed. The PRP was spun again at 400 × *g* to remove the platelets and platelet-poor plasma. Samples were frozen at −70°C until extraction of the lipids.

Adipose tissue biopsy samples were taken from the buttocks using a hypodermic syringe equipped with a 13-gauge, stainless steel needle (26,27). The samples (10 to 50 mg) were immediately transferred to cold (0°C) isotonic saline, then placed in a −70°C freezer within an hour and stored until further processing.

*Lipid extraction and preparation of FAME.* The lipids were extracted as described by Nelson (28,29) using chloroform/methanol (2:1, vol/vol). The total lipid extracts and the free fatty acid CLA standards were transmethylated with sodium methylate (methoxide) for 10 min at 55°C followed by reaction with 1 N hydrochloric acid in methanol for 10 min at 80°C as described by Carreau and Dubacq (30) in order to avoid isomerization of CLA isomers (31,32). The FAME were extracted with hexane and purified by thin-layer chromatography as described elsewhere (29) before dilution and injection into the gas chromatograph.

*Gas–liquid chromatography (GLC).* The FAME were analyzed by GLC (model 6890; Hewlett-Packard, Palo Alto, CA) with the Hewlett-Packard ChemStation III software running on an IBM-compatible desktop computer. A SP-2380 column (100 m × 0.25 mm i.d. × 0.2 mm film thickness; Supelco Inc., Bellefonte, PA) was used. The column was operated at 75°C for 4 min, then temperature-programmed at 13°C/min to 175°C, and held there for 27 min, followed by a second temperature program at 4°C/min to 215°C, and finally held there for 31 min. The total run time was 79.69 min. Fatty acids were identified by comparison of their retention times with authentic standards. If a gas chromatograph peak was not clearly identified by its retention time, ion trap mass spectra were compared to mass spectra from NIST or mass spectra prepared in our laboratory.

*Plasma lipid analyses*. Plasma cholesterol, TG, low density lipoprotein (LDL) cholesterol, and high density lipoprotein (HDL) cholesterol were analyzed in a Cobas-Faras centrifugal analyzer (Hoffman-La Roche, Nutley, NJ) using automated enzymatic methods as described by McGowan *et al*. (33) and Allain *et al*. (34).

**TABLE 4**
**Plasma Cholesterol, Lipoprotein Cholesterol, and Triglyceride Concentrations[a]**

| | Intervention group[b] | | | Control group[c] | | |
|---|---|---|---|---|---|---|
| | Day 30 (mg/dL) | Day 93 (mg/dL) | *P*-value[d] | Day 30 (mg/dL) | Day 93 (mg/dL) | *P*-value[d] |
| Triglycerides | 75.0 ± 21.7 | 52.5 ± 18.0 | 0.01 | 68.6 ± 18.2 | 51.1 ± 11.5 | 0.10 |
| Total cholesterol | 191.2 ± 38.3 | 179.5 ± 27.3 | 0.17 | 193.5 ± 37.9 | 176.0 ± 33.4 | 0.21 |
| LDL-cholesterol | 109.7 ± 42.9 | 108.6 ± 24.0 | 0.93 | 109.3 ± 36.5 | 99.3 ± 37.1 | 0.21 |
| HDL-cholesterol | 52.1 ± 7.9 | 51.8 ± 5.7 | 0.93 | 57.9 ± 13.0 | 55.5 ± 12.0 | 0.22 |

[a]Mean values ± SD.
[b]$n = 10$.
[c]$n = 7$.
[d]Paired *t*-test (SD at day 30 vs. SD at day 93). LDL, low density lipoprotein; HDL, high density lipoprotein.

*Statistical analysis*. Data from the end of the stabilization period (day 30) and the end of the intervention period (day 93) were compared and analyzed using the paired *t*-test. The subjects acted as their own controls. A *P*-value of less than 0.05 was considered significant. The fatty acid data were analyzed with a single tailed *t*-test. All statistical analyses were performed with the PC SAS Data System, Version 6.11, obtained under license from the SAS Institute, PC-SAS (SAS PC Manual, SAS Institute, Chapel Hill, NC, 1993).

## RESULTS

*Blood lipids*. Table 4 shows the blood lipid values for both intervention and control groups on days 30 (end of baseline) and 93 (end of intervention). Consuming CLA for 63 d did not alter the plasma LDL cholesterol and HDL cholesterol levels of the volunteers in the intervention group. In fact, their levels did not differ significantly from the values of their counterparts in the control group. Although the total cholesterol values of both groups decreased somewhat, neither group's value reached statistical significance. Although plasma TG levels did decrease in both groups, most likely due to the consumption of a 30% fat diet, the difference between the intervention and control groups was not significant.

*Apolipoproteins (apo)*. Table 5 lists the values for apo A-1 and B before and after the subjects consumed the CLA supplement for 63 d. Whereas the intervention group did not exhibit any significant change in the apo A-1, the control group did show a significant change after 63 d on the placebo containing linoleic acid. This may be due to the added linoleic acid in the diet of the control group or an artifact of the paired *t*-test as the difference was small and the standard deviation large. However, all seven subjects showed a decrease in their level of apo-A-1—an effect that would have large impact on a paired *t*-test. Apo-B values differed significantly between the baseline and the end of intervention at the $P < 0.05$ level, but for the control group did not. The absolute change in apo-B was 2.7 mg/dL in the intervention group and 4.7 mg/dL in the control group. The difference in the intervention group, while statistically significant, is unlikely to have any major physiological impact. This apparent statistical anomaly is probably due to the use of a paired *t*-test. A paired *t*-test gives more significance to a group in which a particular change occurs in the same direction for each menber of the group than for a group which has both positive and negative changes as was true here for the intervention and control group, respectively.

*Plasma fatty acids*. After a 63-d intervention, the supplementation did increase CLA in plasma but we found only 4.23% of the ingested CLA in plasma on any given day. While the major increase in the CLA isomer was the 9 *cis*-,11 *trans*-variety, there were noticeable increases in several of the other CLA isomers as well. The weight percentage of CLA in plasma increased from 0.28 ± 0.06 to 1.09 ± 0.31 ($n = 10$, $P < 0.05$) after the supplementation. The 9 *cis*-,11 *trans*-isomer was the most prominent variety followed by the

**TABLE 5**
**Apolipoproteins (apo) Levels in Plasma of Subjects Before and After CLA Supplementation[a]**

| | Intervention group[b] Apo (mg/dL) | | Control group[c] Apo (mg/dL) | |
|---|---|---|---|---|
| | Study day 30 | Study day 93 | Study day 30 | Study day 93 |
| Apo-A-1 | 126.8 ± 15.9 | 124.5 ± 16.9 | 141.1 ± 26.1 | 129.08 ± 21.84 |
| *P*-value (SD 30 vs. SD 93)[d] | 0.25 | | 0.002 | |
| Apo-B | 53.1 ± 9.9 | 50.6 ± 8.9 | 49.9 ± 8.1 | 45.2 ± 10.2 |
| *P*-value (SD 30 vs. SD 93) | 0.05 | | 0.16 | |

[a]Mean value ± SD.
[b]$n = 10$.
[c]$n = 7$.
[d]SD 30, SD 93, study day 30, study day 93.

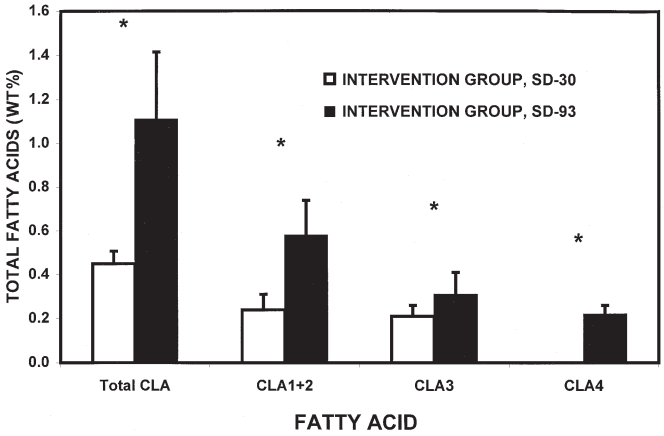


**FIG. 1.** The major conjugated linoleic acid (CLA) isomers found in plasma after supplementation with 3.9 g/d of CLA for 63 d. An asterisk indicates that the baseline and intervention values differed significantly at a $P < 0.05$ level using a paired *t*-test. CLA1 is the 9*c*,11*t*-18:2 isomer; CLA2 is the 8*t*,10*c*-18:2 isomer; CLA3 is the 11*c*,13*t*-18:2 isomer; and CLA4 is the 10*t*,12*c*-18:2 isomer. SD, study day.

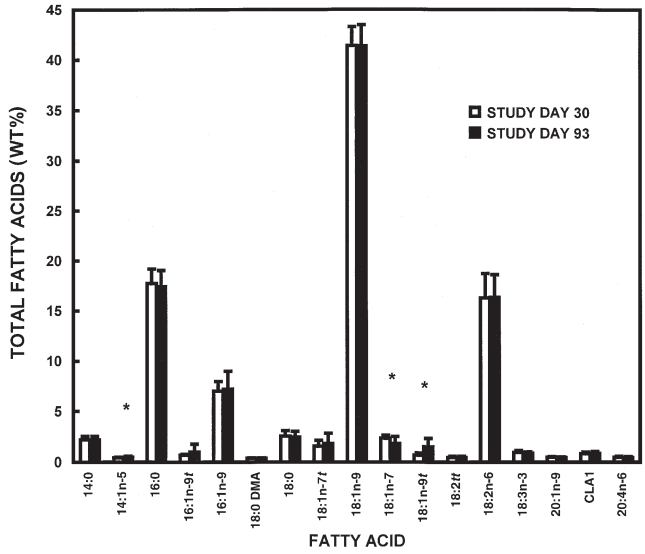


**FIG. 2.** The fatty acid composition of total lipid extract of adipose tissue before and after supplementation with 3.9 g/d of CLA for 63 d. An asterisk indicates that the baseline and intervention values differed significantly at a $P < 0.05$ level using a paired *t*-test. Fatty acids are designated with a shorthand where the number of carbon atoms in the chain is followed by the number of double bonds and the position of the first double bond from the methyl end of the molecule. DMA stands for dimethyl acetal; *c* for *cis*; and *t* for *trans*. CLA1 is the 9*c*,11*t*-18:2 isomer.

11 *cis*-,13 *trans*- and 10 *trans*-,12 *cis*-isomers in lesser amounts (see Fig. 1). Still, the total amount of CLA (all isomers) found in the plasma was rather small and well below the total amount consumed on a daily basis. Despite the presence of many CLA isomers in the diet, there was a bioconcentration of the 9 *cis*-,11 *trans*-18:2 variety followed by the 11 *cis*-,13 *trans*- and 10 *trans*-,12 *cis*-isomers. Also, the consumption of CLA appeared to have little or no effect on the major fatty acid composition of plasma, i.e., none of the major fatty acids such as linoleic, oleic, or palmitic acid showed significant changes in its percentage in plasma (data not shown). There was a slight rise in the plasma *trans*-18:1n-9 content in the intervention group, but we have no explanation for this change. It is also worth noting that the control group's plasma (data not shown) did not exhibit an increase in *trans*-18:1n-9 which did occur in the intervention group's plasma.

*Adipose tissue*. CLA in adipose tissue was not influenced by the supplementation (0.79 ± 0.18 to 0.83 ± 0.19 wt%) ($n$ = 10), and the 9 *cis*-,11 *trans*-variety was the only isomer present. This isomer is present in normal humans who are not taking CLA supplements and presumably is slowly incorporated into adipose tissue over many years. Short-term supplementation does not seem to have a detectable effect in 63 d. The adipose tissue fatty acid composition of the intervention group is shown in Figure 2.

## DISCUSSION

Antiatherogenic properties have been attributed to CLA (9,10) and are believed to be due, at least in part, to the changes in lipoprotein metabolism (9). Contrary to those hypocholesterolemic observations in animal studies, we did not observe any change in the levels of plasma cholesterol, LDL cholesterol, or HDL cholesterol in our study when the diet was supplemented with 3.9 g of CLA in an isocaloric substitution for linoleic acid for 63 d. Although at the end of the supplementation period we observed a decrease in the plasma TG levels in the intervention group, a similar reduction was also detected in the control group, indicating that this reduction was due to the consumption of the 30% fat diet by our normolipidemic subjects and not the CLA intervention. The fact that statistical difference was not achieved in the control group might have been due to the small sample size ($n$ = 7) or slightly higher TG values at baseline in the intervention group.

Lee *et al*. (9) reported that when rabbits were fed an atherogenic diet with or without CLA (0.5 g/d) for 22 wk, the CLA group showed markedly lower LDL cholesterol and TG levels in the blood by week 12 than did the control rabbits. The ratios of LDL to HDL cholesterol and total cholesterol to HDL cholesterol were significantly reduced by CLA feeding. The CLA-fed rabbits showed less atherosclerosis as estimated by examining the aorta of each animal at the end of the study. Similar effects in hamsters were reported by Nicolosi *et al*. (10). Those hamsters fed an atherogenic diet supplemented with CLA for 11 wk had lower plasma lipoprotein cholesterol and lipid levels and a significant reduction in aortic fatty streak formation when compared to controls fed an atherogenic diet alone or supplemented with linoleic acid.

Our intervention period lasted approximately 9 wk but the effects in the animal studies were detected after 11 or 12 wk. Whether a longer intervention period would have shown changes in the cholesterol and lipoprotein levels is not known. However, animals in those studies were fed a diet high in cholesterol and saturated fat while our human volunteers were all

healthy, normolipidemic subjects who consumed a low-fat diet. It is more difficult to achieve lipid reductions in normolipidemic individuals than in hyperlipidemic subjects (26,35,36). In our study, we fed the subjects a low-fat diet, instead of a hypercholesterolemic diet, to more accurately reflect the current eating habits of the U.S. population. Thus, our data indicate that, under the conditions of this study, consumption of dietary CLA at levels three to four times higher than those found in the normal diet does not have any effect on cholesterol and lipoproteins levels. Furthermore, there is no reason to expect a similar decrease in LDL cholesterol as total plasma cholesterol decreases between the control and intervention group. The placebo for the control group was high in linoleic acid, which is well known to selectively lower LDL cholesterol (LDL-C); thus, it is likely that we would observe different LDL-C values in the control and intervention group after 60 d on the two diets. Based on what is known about mono-*trans* fatty acids effects on LDL-C, there is no reason to believe that CLA would suppress LDL-C like linoleic acid. However, the decreases found here in the total cholesterol values of both groups did not reach statistical significance (see Table 4); thus, this issue does not seem to be a point worth discussing in any detail.

It is believed that humans do not have the inherent ability to synthesize CLA (37) and that most of the CLA in human tissues comes from dietary sources. Two studies of free-living persons showed that increased dietary intake of CLA increases plasma concentrations of CLA in men (38,39). Britton *et al*. (38) directed 14 subjects to consume self-selected diets, either high or low in CLA content, for 3 wk. Diet histories recorded by the subjects were used to estimate CLA intake. The serum phospholipid esterified CLA concentration and the molar ratio of CLA to linoleic acid increased significantly in the group consuming the high-CLA diet and decreased significantly in the group consuming the low-CLA diet. Huang *et al.* (39) reported that the amount of plasma phospholipid esterified CLA increased significantly (19–27%) after a dietary intervention in nine subjects who consumed approximately 100 g of Cheddar cheese (approximately 180 mg CLA) with their regular diets for 4 wk. As in Britton's study, the molar ratio of CLA to linoleic acid in the study of Huang *et al.* (39) also increased significantly after the dietary intervention.

In our controlled study, we found that plasma CLA isomers tended to reflect the CLA isomers in the supplement but were present in a smaller amount than would have been predicted from the amount fed to the volunteers. The weight percentage of plasma total CLA increased almost fourfold (from 0.28 to 1.09%) after the subjects consumed CLA for 63 d. In a typical, fasting, normolipidemic individual, there is a pool of 15 g of plasma fatty acid in the circulation based on a typical plasma total lipid level of 5 mg/dL and a 6-L blood volume. Normally, the plasma fatty acid profile reflects the fatty acid composition of dietary fat (40–43), but our calculation of the percentage of CLA in plasma (1.09%) after CLA supplementation indicated that only 4.23% of the ingested CLA was present on any given day. We observed that the 9 *cis*-,11 *trans*- and, perhaps, the 8 *trans*-,10 *cis*-isomers (since the chromatogram peaks were not separated at such low levels) were most abundant and that the 11 *cis*-,13 *trans*- and 10 *trans*-,12 *cis*-isomers existed in lesser quantities. It appears that, in humans, CLA is rapidly metabolized and eliminated from tissues. In fact, metabolites of CLA isomers were shown to occur in animal tissues (31,44). Data from Sébédio *et al*. (31) indicated that the 10 *trans*-,12 *cis*-18:2 isomer was metabolized to 8,12,14-20:3 and 5,8,12,14-20:4 in rats deficient in essential fatty acids.

It is interesting that we have found no increase in the level of CLA in the adipose tissue after feeding CLA for 63 d, yet the data on rats from Sugano *et al*. (45) indicate that adipose tissue and the lungs contained the highest proportion of the incorporated CLA. In humans, it appears that in the short term the increased dietary CLA metabolizes rapidly to other products and thus is not available for incorporation into adipose tissue. Despite the fact that Tonalin® contains isomers other than the 9 *cis*-,11 *trans*-variety, it is also interesting that this isomer was the only one present in the adipose tissue of the test subjects. As reported previously (21) in the platelets of those subjects who consumed CLA, we found mainly the 9 *cis*-,11 *trans*- and the 10 *trans*-,12 *cis*-isomers. Our supplement contained more than 20% of the 11 *cis*-,13 *trans*-isomer. It is believed that the 11 *cis*-,13 *trans*-isomer is an artifact of the synthetic process, and thus is not found in natural CLA present in food sources or produced by bacteria in the lumen of ruminants. The finding here suggests that this CLA isomer is not incorporated in tissues as readily as the CLA isomers normally found in the human food chain. We used the mixture of CLA isomers (Tonalin®) because purified isomers of CLA for human feeding trials were not available at the time we conducted this study. It should be noted that there was no obvious adverse effect on the health of the volunteers in this study.

Although no mechanism for CLA's beneficial effects in animal studies has been established, it is speculated that CLA may alter fatty acid composition and subsequent eicosanoid metabolism, perhaps by removing eicosanoid precursors such as arachidonic acid (46). In fact, arachidonic acid levels have been found to be reduced in the muscle and fat pad of CLA-fed rats (47). However, in our study the levels of arachidonate in adipose tissue did not change after the CLA feeding. Furthermore, we have not found any obvious beneficial effect of CLA supplementation. In other papers from this study (18–21), we have reported that CLA supplementation had little effect on platelet function and blood coagulation, indices of immune response, body composition, and appetite.

In summary, our study shows that, in normolipidemic subjects, CLA supplementation for 2 mon at a level three to four times that found in a typical Western diet offers no obvious benefit in terms of blood lipid and lipoprotein profile. Dietary CLA at this level caused a fourfold increase in the plasma CLA levels of the volunteers but only 4.23% of the ingested dietary CLA on any given day was found in the plasma.

Among all the isomers in the plasma, there was a bioconcentration of the 9 *cis-*,11 *trans*-18:2 isomer followed by the 11 *cis*-,13 *trans*- and 10 *trans*-,12 *cis*- varieties. Dietary CLA was not incorporated into adipose tissue at significant levels. In humans, CLA appears to metabolize rapidly to other products.

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# Peripheral Differential Leukocyte Counts in Humans Vary with Hyperlipidemia, Smoking, and Body Mass Index

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**ABSTRACT:** Reports of diverse relationships between blood concentrations of different lipids and peripheral total leukocyte count, and a unique lower peripheral monocyte count in hypercholesterolemia, have driven us to think that in humans, peripheral differential leukocyte counts may be influenced differently by different types of hyperlipidemia. Our subjects were Taipei residents who attended a regular health check program in our hospital in 1998. A total of 3,282 subjects was enrolled, including 1,677 normolipidemic, 960 untreated borderline hyperlipidemic, and 645 untreated hyperlipidemic subjects. By one-way analysis of variance (ANOVA), we found that different types of hyperlipidemia were associated with significant differences in differential leukocyte counts. In hypertriglyceridemia, the total leukocyte count and counts of all leukocyte subtypes were significantly higher than those in normolipidemia. Pure hypercholesterolemia, by contrast, was associated with a significantly lower monocyte count and no significant difference in other leukocyte counts. By two-way ANOVA adjusted for presence and degree of hyperlipidemia, we found significantly higher counts of total leukocytes and of all leukocyte subtypes in smokers, and significantly positive trends in relationships between body mass index (BMI) and counts of all leukocytes, neutrophils, lymphocytes, and monocytes. By multivariate regression analysis including all subjects, the serum triglyceride (TG) level was positively correlated with total leukocyte count and counts of all subtypes except eosinophils. On the contrary, serum high density lipoprotein-cholesterol had a negative correlation with total leukocyte count and with counts of neutrophils, monocytes, and basophils. In these multivariate regression analyses, there was no significant correlation between lipid levels and eosinophil count, whereas smoking was consistently associated with significantly higher counts of all leukocyte subtypes, including eosinophils. BMI had a significantly positive correlation with counts of all leukocytes, neutrophils, lymphocytes, and monocytes.

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Reports of a positive association between human peripheral total leukocyte counts and serum triglyceride (TG) levels (1,2), a negative association between peripheral total leukocyte count and serum high density lipoprotein (HDL)-cholesterol level (1–3), and a lower peripheral monocyte count in hypercholesterolemia (4) suggest that different types of hyperlipidemia may influence peripheral differential leukocyte counts differently. There is little information concerning this issue so far in the literature. An in-depth study on this point may help in understanding the pathophysiology of hyperlipidemia-induced atherosclerosis, since accumulating evidence from human studies has shown that hyperlipidemia is a major cause of atherosclerosis and cardiovascular disease (CVD) (5–7), that different types of leukocytes are likely to play important but different roles in atherosclerosis (8–10), and that a higher peripheral total leukocyte count is associated with an increased incidence of CVD (3,11–13). In addition, because cigarette smoking and obesity are frequently found in association with both lipid disorders (14–16) and a higher peripheral leukocyte count (1,3,13,17–19), we included the factors of smoking and body mass index (BMI) in the study.

## MATERIALS AND METHODS

*Study subjects.* Our subjects were selected from participants of a regular health check program in our hospital in 1998. The first condition for enrollment was to be a resident of the Taipei area including Taipei City and neighboring Taipei County. This condition was to ensure the feasibility of telephone interviews of hyperlipidemic and borderline hyperlipidemic subjects to see if they had received medication for hyperlipidemia before undergoing their health check in 1998. Among Taipei residents in 1998 who attended our health check program, 4,461 subjects were selected initially because they fit the following two conditions: (i) they had received serum lipid analysis with the same biochemical analyzer (Automatic Multichannel Biochemical Analyzer, Hitachi-7450; Hitachi, Tokyo, Japan); and (ii) they had received peripheral leukocyte analysis with the same blood cell counter (Sysmex Cell Counter NE-8000; TOA Medical Electronics, Kobe, Japan). Among the 4,461 subjects, 67 cases with serum TG level ≥ 450 mg/dL were separated as a special group because of the profound influence of their very high TG levels on the laboratory measurements of serum low density lipoprotein (LDL)-cholesterol levels. In the remaining 4,394 subjects, diagnoses of normolipidemia, untreated hyperlipidemia, untreated bor-

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Abbreviations: ANOVA, analysis of variance; BMI, body mass index; CVD, cardiovascular diseases; HDL, high density lipoprotein; LDL, low density lipoprotein; SE, standard error; TC, total cholesterol; TG, triglyceride.

derline hyperlipidemia, and others were made according to the criteria listed in the following *Diagnostic criteria for hyperlipidemia* subsection, based on health records and results of telephone interviews for the history of and treatment for hyperlipidemia. Telephone interviews were conducted as follows. For each subject, at least seven calls were made, each call on a separate day, with the aim of having verbal communication with the enrolled subject personally over the telephone. Information from the family was considered unreliable and was not taken. Only information taken from the enrolled subjects themselves was used to conclude whether they had received medication for hyperlipidemia before undergoing the health check in 1998. Ultimately, 3,282 subjects were enrolled in the study, including 1,677 normolipidemic subjects, 645 untreated hyperlipidemic subjects, and 960 untreated borderline hyperlipidemic subjects. The 645 untreated hyperlipidemic subjects were subclassified into 102 cases of pure hypercholesterolemia, 187 cases of pure hypertriglyceridemia, 111 cases of combined hyperlipidemia, and 245 cases of probable combined hyperlipidemia.

*Laboratory data.* The following data were collected from the health records: (i) age, gender, weight, height, existence of a smoking habit, and history of and treatment for hyperlipidemia; and (ii) results of serum lipid study and leukocyte analysis. BMI was calculated as body weight/body height$^2$ (in kg/m$^2$). The items of the lipid study included serum levels of total cholesterol (TC), HDL-cholesterol, LDL-cholesterol, and TG (all in mg/dL). The leukocyte analyses included total leukocyte count (in $10^9$/L) and differential percentages (%) of neutrophils, lymphocytes, monocytes, eosinophils, and basophils, as analyzed by the same Sysmex Cell Counter NE-8000 as mentioned above. Any specimen with abnormal or atypical leukocytes that had been re-analyzed by using another blood cell counter was excluded from the study. The differential percentages of the above five kinds of leukocytes add up to 100%. The absolute count of a leukocyte subtype (in $10^9$/L) was calculated as the product of its respective differential percentage and total leukocyte count.

*Diagnostic criteria for hyperlipidemia.* (i) Hypercholesterolemia: The diagnosis of normocholesterolemia was made when serum TC level was <200 mg/dL and there was no history of treatment for hyperlipidemia. Untreated hypercholesterolemia was diagnosed in a subject with serum TC level ≥240 mg/dL and no previous treatment for hyperlipidemia. Untreated borderline hypercholesterolemia was diagnosed in a subject with serum TC level between 199 and 240 mg/dL and no previous treatment for hyperlipidemia. Those subjects with unknown or uncertain history of and/or treatment for hypercholesterolemia or who did not fit any of the above criteria for normocholesterolemia, untreated hypercholesterolemia, and untreated borderline hypercholesterolemia were classified as "others." (ii) Hypertriglyceridemia: The diagnosis of normotriglyceridemia was made when serum TG level was <130 mg/dL and there was no history of treatment for hyperlipidemia. Untreated hypertriglyceridemia was diagnosed in a subject with serum TG level ≥200 mg/dL and no previous treatment for hyperlipidemia. Untreated borderline hypertriglyceridemia was diagnosed in a subject with serum TG level between 129 and 200 mg/dL and no previous treatment for hyperlipidemia. Those subjects with unknown or uncertain history of and/or treatment for hypertriglyceridemia or who did not fit any of above criteria for normotriglyceridemia, untreated hypertriglyceridemia, and untreated borderline hypertriglyceridemia were classified as "others." (iii) Pure or combined hyperlipidemia: In untreated hyperlipidemic subjects, the presence of hypercholesterolemia with normotriglyceridemia was diagnosed to have "pure hypercholesterolemia," and the presence of hypertriglyceridemia with normocholesterolemia was diagnosed to have "pure hypertriglyceridemia." Subjects with both hypercholesterolemia and hypertriglyceridemia were diagnosed as having "combined hyperlipidemia." Those who had hypercholesterolemia with borderline hypertriglyceridemia or had hypertriglyceridemia with borderline hypercholesterolemia were diagnosed as having "probable combined hyperlipidemia."

*Statistical methods.* The data in the study were analyzed by using the SAS statistical program (SAS, Cary, NC) as follows: (i) The following comparisons were analyzed by one-way analysis of variance (ANOVA) using Bonferroni method to compare pairs of means: various laboratory measurements in normolipidemic and different hyperlipidemic groups (Tables 1 and 2), and differential leukocyte counts in negative, borderline, and positive groups for different types of hyperlipidemia separately (Table 3); (ii) the following comparisons were analyzed by two-way ANOVA: differential leukocyte counts between nonsmokers and smokers adjusted for the presence and degree of hyperlipidemia (Table 4) and differential leukocyte counts by three levels of BMI adjusted for the presence and degree of hyperlipidemia (Table 5); and (iii) multivariate regression analyses of differential leukocyte counts on age, gender, BMI, smoking, and serum lipid levels in all study subjects ($n$ = 3,282) were estimated by the ordinary least squares method (Tables 6 and 7).

## RESULTS

Table 1 shows the results of comparisons of age, various serum lipid levels, and differential leukocyte counts in normolipidemic, untreated borderline hyperlipidemic, and untreated hyperlipidemic groups by one-way ANOVA. The serum levels of TC, LDL-cholesterol, and TG and the counts of all leukocytes, neutrophils, lymphocytes, and basophils all had a significantly positive association with the presence and degree of hyperlipidemia ($P < 0.0001$). The serum HDL-cholesterol level, on the contrary, had a significantly negative correlation with the presence and degree of hyperlipidemia ($P < 0.0001$). The monocyte count in the hyperlipidemic group, but not in the borderline hyperlipidemic group, was significantly higher than that in the normolipidemic group ($P < 0.05$). There was no significant difference in eosinophil count in these three groups.

The results of comparisons of various laboratory measure-

**TABLE 1**
**Comparisons of Age, Serum Lipid Levels, and Peripheral Differential Leukocyte Counts in Normolipidemic, Untreated Borderline Hyperlipidemic, and Untreated Hyperlipidemic Groups by One-way ANOVA[a]**

| | | Serum lipid levels (mg/dL) | | | |
|---|---|---|---|---|---|
| | Age (yr) | TC | HDL-cholesterol | LDL-cholesterol | TG |
| Normolipidemic group ($n$ = 1,677) | 51.77 ± 0.32 | 169.1 ± 0.507 | 53.50 ± 0.331 | 99.31 ± 0.476 | 81.54 ± 0.606 |
| Borderline hyperlipidemic group ($n$ = 960) | 55.23 ± 0.37 | 201.9 ± 0.773 | 50.84 ± 0.478 | 124.4 ± 0.744 | 133.2 ± 1.271 |
| Hyperlipidemic group ($n$ = 645) | 54.67 ± 0.42 | 224.7 ± 1.526 | 46.08 ± 0.577 | 134.1 ± 1.553 | 223.6 ± 3.242 |
| $P$ value | <0.0001[b] | <0.0001[c] | <0.0001[c] | <0.0001[c] | <0.0001[c] |

| | Leukocyte count ($10^9$/L) | | | | | |
|---|---|---|---|---|---|---|
| | All leukocytes | Neutrophils | Lymphocytes | Monocytes | Eosinophils | Basophils |
| Normolipidemic group ($n$ = 1,677) | 6.055 ± 0.038 | 3.309 ± 0.029 | 2.126 ± 0.015 | 0.386 ± 0.003 | 0.197 ± 0.004 | 0.037 ± 0.001 |
| Borderline hyperlipidemic group ($n$ = 960) | 6.398 ± 0.050 | 3.500 ± 0.039 | 2.262 ± 0.020 | 0.392 ± 0.004 | 0.204 ± 0.004 | 0.039 ± 0.001 |
| Hyperlipidemic group ($n$ = 645) | 6.727 ± 0.063 | 3.657 ± 0.045 | 2.418 ± 0.027 | 0.403 ± 0.006 | 0.206 ± 0.006 | 0.043 ± 0.001 |
| $P$ value | <0.0001[c] | <0.0001[c] | <0.0001[c] | <0.05[d] | NS | <0.0001[c] |

[a]Values are means ± SE. NS, no significant difference.
[b]The mean age in the normolipidemic group was significantly lower than those in other two groups.
[c]For the specified lipid level or leukocyte count, there was a significant trend of relationship, either positively or negatively, in the normolipidemic, borderline hyperlipidemic, and hyperlipidemic groups.
[d]For the monocyte count, a significant difference exists only between the normolipidemic group and the hyperlipidemic group. ANOVA, analysis of variance; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglyceride.

ments in normolipidemic, pure hypercholesterolemic, pure hypertriglyceridemic, combined hyperlipidemic, and probable combined hyperlipidemic groups by one-way ANOVA are shown in Table 2. Major findings include the following: (i) In the pure hypertriglyceridemic group, the total leukocyte count and counts of all leukocyte subtypes were significantly higher than those in the normolipidemic group. (ii) In contrast, in the pure hypercholesterolemic group, the total leukocyte count and counts of all leukocyte subtypes were not significantly different from those in the normolipidemic group. The monocyte count in the pure hypercholesterolemic group was significantly lower than that in normolipidemic group when compared by Student's $t$ test [$(0.350 \pm 0.015) \times 10^9$/L, $n$ = 102, and $(0.386 \pm 0.003) \times 10^9$/L, $n$ = 1,677, respectively, $P < 0.05$]. (iii) In the combined and the probable combined hyperlipidemic groups, the total leukocyte count and counts of neutrophils, lymphocytes, and basophils were significantly higher than those in the normolipidemic group. The obviously lower monocyte count in the patients with pure hypercholesterolemia was not seen in these combined hyperlipidemic groups.

The results of comparisons of differential leukocyte counts in negative, borderline, and positive groups for hypercholesterolemia, pure hypercholesterolemia, hypertriglyceridemia, and pure hypertriglyceridemia, made separately by one-way ANOVA, are listed in Table 3. The results confirmed several interesting findings in Tables 1 and 2. (i) In both the hypertriglyceridemic and the pure hypertriglyceridemic groups, the total leukocyte count and counts of all leukocyte subtypes were significantly higher than those in the normolipidemic group and showed significantly positive trends of relationships with the presence and degree of hypertriglyceridemia. (ii) In contrast, in the pure hypercholesterolemic group, only the monocyte count was significantly different from that in the normolipidemic group. It showed a negative relationship with the presence and degree of hypercholesterolemia. The significantly lower monocyte count in the pure hypercholesterolemic group, as compared with the normolipidemic group, did not exist in the hypercholesterolemic group that contained subjects with hypertriglyceridemia.

Because of well-documented positive associations between smoking and lipid disorders (14,15) and higher peripheral total leukocyte counts (1,3,13,17,18), we compared differential leukocyte counts between smokers and nonsmokers in normolipidemic, borderline hyperlipidemic, and hyperlipidemic groups by two-way ANOVA, as shown in Table 4. This analysis confirmed a higher total leukocyte count ($P < 0.0001$), resulting from higher counts of all leukocyte subtypes ($P$ values all <0.0001), in smokers after adjustments for presence and degree of hyperlipidemia. Obesity has been also associated with both lipid disorders (16) and higher peripheral total leukocyte counts (18,19). Comparisons of differential leukocyte counts by three levels of BMI in normolipidemic, borderline hyperlipidemic, and hyperlipidemic groups by two-way ANOVA showed a significantly positive relationship between BMI and counts of all leukocytes, neutrophils,

**TABLE 2**
**Comparisons of Age, Serum Lipid Levels, and Peripheral Differential Leukocyte Counts Among Normolipidemic Group and Subgroups of Untreated Hyperlipidemia by One-way ANOVA[a]**

| | | Serum lipid levels (mg/dL) | | | |
|---|---|---|---|---|---|
| | Age[b] (yr) | TC[c] | HDL-cholesterol[d] | LDL-cholesterol[c] | TG[e] |
| Normolipidemia ($n$ = 1,677) | 51.77 ± 0.32 | 169.1 ± 0.507 | 53.50 ± 0.331 | 99.31 ± 0.476 | 81.54 ± 0.606 |
| Pure hypercholesterolemia ($n$ = 102) | 55.45 ± 0.85 | 256.7 ± 1.345 | 64.96 ± 1.527 | 172.6 ± 1.804 | 95.60 ± 2.144 |
| Pure hypertriglyceridemia ($n$ = 187) | 53.97 ± 0.88 | 178.0 ± 1.276 | 35.75 ± 0.576 | 89.85 ± 1.466 | 262.2 ± 3.771 |
| Combined hyperlipidemia ($n$ = 111) | 56.05 ± 1.01 | 262.5 ± 2.688 | 47.73 ± 0.914 | 164.7 ± 2.686 | 248.6 ± 6.502 |
| Probable combined hyperlipidemia ($n$ = 245) | 54.25 ± 0.66 | 223.0 ± 1.431 | 45.36 ± 0.776 | 138.0 ± 1.735 | 236.3 ± 4.573 |
| $P$ value | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

| | Leukocyte count ($10^9$/L) | | | | | |
|---|---|---|---|---|---|---|
| | All leukocytes[f] | Neutrophils[f] | Lymphocytes[f] | Monocytes[g] | Eosinophils[h] | Basophils[f] |
| Normolipidemia ($n$ = 1,677) | 6.055 ± 0.038 | 3.309 ± 0.029 | 2.126 ± 0.015 | 0.386 ± 0.003 | 0.197 ± 0.004 | 0.037 ± 0.001 |
| Pure hypercholesterolemia ($n$ = 102) | 6.085 ± 0.139 | 3.338 ± 0.117 | 2.197 ± 0.050 | 0.350 ± 0.015 | 0.163 ± 0.011 | 0.038 ± 0.002 |
| Pure hypertriglyceridemia ($n$ = 187) | 6.713 ± 0.107 | 3.671 ± 0.082 | 2.349 ± 0.045 | 0.419 ± 0.010 | 0.230 ± 0.012 | 0.043 ± 0.002 |
| Combined hyperlipidemia ($n$ = 111) | 6.911 ± 0.170 | 3.770 ± 0.115 | 2.488 ± 0.068 | 0.414 ± 0.017 | 0.194 ± 0.011 | 0.044 ± 0.002 |
| Probable combined hyperlipidemia ($n$ = 245) | 6.923 ± 0.103 | 3.729 ± 0.071 | 2.530 ± 0.047 | 0.408 ± 0.010 | 0.212 ± 0.010 | 0.044 ± 0.002 |
| $P$ value | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.005 | <0.0001 |

[a]Values are means ± SE.
[b]For age, significant difference exists only between the normolipidemic and the combined hyperlipidemic groups.
[c]For TC and LDL-cholesterol levels, there was no significant difference between the pure hypercholesterolemic and the combined hyperlipidemic groups.
[d]For HDL-cholesterol level, there was no significant difference between the combined hyperlipidemic and the probable combined hyperlipidemic groups.
[e]For TG level, significant difference exists between any two study groups.
[f]For all leukocytes, neutrophils, lymphocytes, and basophils, the counts from the normolipidemic group were significantly lower than those in other study groups except for the pure hypercholesterolemic group.
[g]For monocytes, the count for the normolipidemic group was only significantly lower than that in the pure hypertriglyceridemic group, and the pure hypercholesterolemic group was significantly lower than those in other three hyperlipidemic groups.
[h]For eosinophils, the count for the normolipidemic group was only significantly lower than that in the pure hypertriglyceridemic group, and the pure hypercholesterolemic group was significantly lower than those in pure hypertriglyceridemic and probable combined hyperlipidemic groups. For abbreviations see Table 1.

lymphocytes, and monocytes after adjustments for presence and degree of hyperlipidemia (Table 5).

In all subjects taken together ($n$ = 3,282), there were universal significant linear correlations between any two levels of serum TC, HDL-cholesterol, LDL-cholesterol, and TG. The correlation coefficients and probability values estimated by Pearson's analysis were as follows (data not shown in tables): TC and HDL-cholesterol, $R = 0.1904$, $P < 0.0001$; TC and LDL-cholesterol, $R = 0.9041$, $P < 0.0001$; TC and TG, $R = 0.3473$, $P < 0.0001$; HDL-cholesterol and LDL-cholesterol, $R = -0.0474$, $P < 0.01$; HDL-cholesterol and TG, $R = -0.4596$, $P < 0.0001$; and LDL-cholesterol and TG, $R = 0.1570$, $P < 0.0001$. These significantly linear correlations are likely to have a great influence on the demonstration of true correlations between each lipid level and the differential leukocyte counts in a multivariate regression analysis including all lipid levels. Therefore, we performed the multivariate regression analysis repeatedly using one kind of lipid level each time, adjusted for age, gender, BMI, and smoking. The results, as listed in Table 6, revealed the following: (i) Smoking was associated independently with higher total leukocyte count and counts of all leukocyte subtypes; (ii) age had independently negative correlations with total leukocyte count and counts of all leukocyte subtypes except neutrophils; (iii) BMI had independently positive correlations with total leukocyte count and counts of neutrophils, lymphocytes, and monocytes; (iv) men had significantly higher counts of monocytes and eosinophils; (v) serum levels of TC, LDL-cholesterol, and TG all had independently positive correlations with total leukocyte count ($P$ values all <0.0001), neutrophil count ($P < 0.01$, <0.01, and <0.0001, respectively), and lymphocyte count ($P$ values all <0.0001); (vi) serum TC and TG levels also had independently positive correlations with basophil count ($P < 0.01$ and <0.0001, respectively). Serum TC level

**TABLE 3**
**Comparisons of Differential Leukocyte Counts in Negative, Borderline, and Positive Groups of Different Hyperlipidemia Separately by One-way ANOVA[a]**

| | Leukocyte count ($10^9$/L) | | | | | |
|---|---|---|---|---|---|---|
| | All leukocytes | Neutrophils | Lymphocytes | Monocytes | Eosinophils | Basophils |
| Hypercholesterolemia[b] | | | | | | |
| No ($n$ = 1,677) | 6.055 ± 0.038 | 3.309 ± 0.029 | 2.126 ± 0.015 | 0.386 ± 0.003 | 0.197 ± 0.004 | 0.037 ± 0.001 |
| Borderline ($n$ = 789) | 6.511 ± 0.057 | 3.542 ± 0.042 | 2.323 ± 0.024 | 0.395 ± 0.005 | 0.211 ± 0.005 | 0.041 ± 0.001 |
| Yes ($n$ = 269) | 6.536 ± 0.097 | 3.567 ± 0.070 | 2.364 ± 0.039 | 0.380 ± 0.010 | 0.183 ± 0.007 | 0.041 ± 0.001 |
| $P$ value | <0.0001[c] | <0.0001[c] | <0.0001[c] | NS | <0.05[d] | <0.0001[d] |
| Pure hypercholesterolemia[b] | | | | | | |
| No ($n$ = 1,677) | 6.055 ± 0.038 | 3.309 ± 0.029 | 2.126 ± 0.015 | 0.386 ± 0.003 | 0.197 ± 0.004 | 0.037 ± 0.001 |
| Borderline ($n$ = 381) | 6.129 ± 0.076 | 3.323 ± 0.059 | 2.194 ± 0.031 | 0.370 ± 0.007 | 0.203 ± 0.008 | 0.038 ± 0.001 |
| Yes ($n$ = 102) | 6.085 ± 0.139 | 3.338 ± 0.117 | 2.197 ± 0.050 | 0.350 ± 0.015 | 0.163 ± 0.011 | 0.038 ± 0.002 |
| $P$ value | NS | NS | NS | <0.05[d] | <0.05[d] | NS |
| Hypertriglyceridemia[b] | | | | | | |
| No ($n$ = 1,677) | 6.055 ± 0.038 | 3.309 ± 0.029 | 2.126 ± 0.015 | 0.386 ± 0.003 | 0.197 ± 0.004 | 0.037 ± 0.001 |
| Borderline (n = 366) | 6.550 ± 0.076 | 3.609 ± 0.059 | 2.307 ± 0.031 | 0.393 ± 0.007 | 0.201 ± 0.006 | 0.040 ± 0.001 |
| Yes ($n$ = 487) | 6.875 ± 0.074 | 3.733 ± 0.053 | 2.463 ± 0.032 | 0.418 ± 0.007 | 0.216 ± 0.007 | 0.044 ± 0.001 |
| $P$ value | <0.0001[c] | <0.0001[c] | <0.0001[c] | <0.0001[c] | <0.05[d] | <0.0001[c] |
| Pure hypertriglyceridemia[b] | | | | | | |
| No ($n$ = 1,677) | 6.055 ± 0.038 | 3.309 ± 0.029 | 2.126 ± 0.015 | 0.386 ± 0.003 | 0.197 ± 0.004 | 0.037 ± 0.001 |
| Borderline ($n$ = 208) | 6.462 ± 0.102 | 3.552 ± 0.080 | 2.275 ± 0.041 | 0.398 ± 0.010 | 0.197 ± 0.008 | 0.040 ± 0.002 |
| Yes ($n$ = 187) | 6.713 ± 0.107 | 3.671 ± 0.082 | 2.349 ± 0.045 | 0.419 ± 0.010 | 0.230 ± 0.012 | 0.043 ± 0.002 |
| $P$ value | <0.0001[c] | <0.0001[c] | <0.0001[c] | <0.01[d] | <0.05[d] | <0.0005[d] |

[a]Values are means ± SE.
[b]The diagnosis of hypercholesterolemia did not exclude coexisting hypertriglyceridemia or borderline hypertriglyceridemia, whereas a diagnosis of pure hypercholesterolemia was made only when serum TG level was normal. The difference between hypertriglyceridemia and pure hypertriglyceridemia is similarly defined.
[c]For the specified leukocyte count, there was a significant positive trend of relationship in the negative, borderline, and positive groups.
[d]Significant difference exists only between the group with the largest mean value and the group with the smallest mean value. For abbreviations see Table 1.

had an independently negative correlation with monocyte count ($P < 0.05$); (vii) serum HDL-cholesterol level had independently negative correlations with total leukocyte count ($P < 0.0001$), neutrophil count ($P < 0.0001$), monocyte count ($P < 0.0001$), and basophil count ($P < 0.05$); and (viii) interestingly, there was no significant correlation between the studied lipid levels and eosinophil count in these multivariate regression analyses.

To see which lipid levels were most closely associated with each type of leukocyte, we performed the multivariate regression analyses (Table 6) a second time including the serum levels of HDL-cholesterol, LDL-cholesterol, and TG simultaneously. The serum TC level was excluded because of its extremely strong positive linear correlation with serum LDL-cholesterol level ($R = 0.9041$). The major findings, as shown in Table 7, are as follows: (i) Serum TG level was an independently positive determinant for total leukocyte count and for counts of all leukocyte subtypes except eosinophils.

**TABLE 4**
**Comparisons of Differential Leukocyte Counts Between Smokers and Nonsmokers in Different Hyperlipidemic Groups by Two-way ANOVA[a]**

| | Leukocyte count ($10^9$/L) | | | | | |
|---|---|---|---|---|---|---|
| | All leukocytes | Neutrophils | Lymphocytes | Monocytes | Eosinophils | Basophils |
| Normolipidemic group | | | | | | |
| Nonsmokers ($n$ = 1,287) | 5.890 ± 0.040 | 3.225 ± 0.031 | 2.069 ± 0.016 | 0.373 ± 0.004 | 0.187 ± 0.004 | 0.036 ± 0.001 |
| Smokers ($n$ = 390) | 6.598 ± 0.089 | 3.583 ± 0.072 | 2.317 ± 0.036 | 0.429 ± 0.008 | 0.229 ± 0.008 | 0.040 ± 0.001 |
| Borderline hyperlipidemic group | | | | | | |
| Nonsmokers ($n$ = 698) | 6.251 ± 0.057 | 3.423 ± 0.043 | 2.210 ± 0.022 | 0.381 ± 0.005 | 0.198 ± 0.005 | 0.038 ± 0.001 |
| Smokers ($n$ = 262) | 6.791 ± 0.104 | 3.704 ± 0.085 | 2.402 ± 0.041 | 0.422 ± 0.009 | 0.221 ± 0.009 | 0.042 ± 0.001 |
| Hyperlipidemic group | | | | | | |
| Nonsmokers ($n$ = 473) | 6.430 ± 0.063 | 3.519 ± 0.048 | 2.303 ± 0.027 | 0.379 ± 0.007 | 0.187 ± 0.006 | 0.041 ± 0.001 |
| Smokers ($n$ = 172) | 7.547 ± 0.142 | 4.037 ± 0.102 | 2.732 ± 0.060 | 0.468 ± 0.014 | 0.260 ± 0.014 | 0.048 ± 0.002 |
| $P$ value for lipid group[b] | <0.0001 | <0.0001 | <0.0001 | <0.05 | NS | <0.0001 |
| $P$ value for smoking[c] | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

[a]Values are means ± SE.
[b]Probability values were tested for different lipid groups and adjusted for smoking groups.
[c]Probability values were tested for smoking groups and adjusted for different lipid groups. For abbreviations see Table 1.

**TABLE 5**
**Comparisons of Differential Leukocyte Counts by Three Levels of BMI in Different Hyperlipidemic Groups by Two-way ANOVA[a]**

| | Leukocyte count ($10^9$/L) | | | | | |
|---|---|---|---|---|---|---|
| | All leukocytes | Neutrophils | Lymphocytes | Monocytes | Eosinophils | Basophils |
| Normolipidemic group | | | | | | |
| BMI < 22 ($n$ = 605) | 5.855 ± 0.061 | 3.160 ± 0.047 | 2.095 ± 0.025 | 0.370 ± 0.005 | 0.193 ± 0.006 | 0.036 ± 0.001 |
| BMI = 22–25 ($n$ = 615) | 6.035 ± 0.059 | 3.282 ± 0.045 | 2.132 ± 0.024 | 0.383 ± 0.006 | 0.201 ± 0.006 | 0.037 ± 0.001 |
| BMI > 25 ($n$ = 457) | 6.346 ± 0.077 | 3.542 ± 0.061 | 2.159 ± 0.030 | 0.411 ± 0.007 | 0.197 ± 0.007 | 0.037 ± 0.001 |
| Borderline hyperlipidemic group | | | | | | |
| BMI < 22 ($n$ = 178) | 6.117 ± 0.123 | 3.364 ± 0.091 | 2.155 ± 0.047 | 0.362 ± 0.010 | 0.199 ± 0.012 | 0.038 ± 0.002 |
| BMI = 22–25 ($n$ = 363) | 6.309 ± 0.076 | 3.433 ± 0.060 | 2.258 ± 0.031 | 0.383 ± 0.007 | 0.197 ± 0.007 | 0.038 ± 0.001 |
| BMI > 25 ($n$ = 419) | 6.595 ± 0.078 | 3.616 ± 0.062 | 2.312 ± 0.031 | 0.413 ± 0.007 | 0.213 ± 0.006 | 0.041 ± 0.001 |
| Hyperlipidemic group | | | | | | |
| BMI < 22 ($n$ = 66) | 6.556 ± 0.191 | 3.545 ± 0.127 | 2.402 ± 0.085 | 0.383 ± 0.019 | 0.184 ± 0.017 | 0.043 ± 0.003 |
| BMI = 22–25 ($n$ = 251) | 6.594 ± 0.102 | 3.604 ± 0.076 | 2.345 ± 0.039 | 0.392 ± 0.010 | 0.211 ± 0.008 | 0.042 ± 0.002 |
| BMI > 25 ($n$ = 328) | 6.864 ± 0.087 | 3.721 ± 0.062 | 2.477 ± 0.039 | 0.415 ± 0.009 | 0.207 ± 0.009 | 0.043 ± 0.001 |
| $P$ value for lipid group[b] | <0.0001 | <0.0001 | <0.0001 | NS | NS | <0.0001 |
| $P$ value for BMI level[c] | <0.0001 | <0.0001 | <0.001 | <0.0001 | NS | NS |

[a]Values are means ± SE. In the statistical analysis, the normolipidemic, borderline hyperlipidemic, and hyperlipidemic groups were represented digitally by 0, 1, and 2, respectively, and the groups of BMI < 22, BMI = 22–25, and BMI > 25 were represented by 0, 1, and 2, respectively.
[b]Probability values were tested for different lipid groups and adjusted for BMI levels.
[c]Probability values tested for BMI levels and adjusted for different lipid groups. BMI, weight (kg)/height$^2$ (m$^2$); for other abbreviations see Table 1.

It was the only independent determinant for basophil count among the studied lipid levels; (ii) serum HDL-cholesterol level was an independently negative determinant for total leukocyte count and for counts of neutrophils and monocytes, and it was an independently positive determinant for lymphocyte count; and (iii) serum LDL-cholesterol level had independently positive correlations with total leukocyte count and counts of neutrophils and lymphocytes. The same multivariate regression analyses in Table 7 were performed once again after replacing the serum LDL-cholesterol level with serum TC level. The results were very similar to those in Table 7, probably because of the strong positive linear correlation between LDL-cholesterol level and TC level.

Among the 67 subjects not included in the above analyses owing to a very high TG level (≥450 mg/dL) that interfered with the laboratory calculation of serum LDL-cholesterol level, 33 patients had never received medication for hyperlipidemia before their health check in 1998. The mean ± SE of age, serum lipid levels, and differential leukocyte counts in these 33 untreated extremely hypertriglyceridemic subjects were as follows: age, 49.73 ± 2.06 yr; TC level, 220.1 ± 6.598 mg/dL; HDL-cholesterol level, 35.15 ± 1.346 mg/dL; TG level, 561.8 ± 19.32 mg/dL; total leukocyte count, (7.391 ± 0.330) × $10^9$/L; neutrophil count, (4.134 ± 0.269) × $10^9$/L; lymphocyte count, (2.501 ± 0.099) × $10^9$/L; monocyte count, (0.458 ± 0.031) × $10^9$/L; eosinophil count, (0.247 ± 0.027) × $10^9$/L; and basophil count, (0.051 ± 0.005) × $10^9$/L. The means of these leukocyte counts were all obviously higher than those in the normolipidemic group, the untreated borderline hyperlipidemic group, and the untreated hyperlipidemic group (see data in Table 1), but probably because of a small sample size with a large SE in the extreme hypertriglyceridemic group, only the differences in total leukocyte count and counts of neutrophils and monocytes reach statistical significances estimated by using Student's $t$ test. When compared with the pure hypertriglyceridemic group (with serum TG level between 199 and 450 mg/dL, see data in Table 2), the means of all leukocyte counts in the extreme hypertriglyceridemic group were also all higher, but only the differences in total leukocyte count and neutrophil count reach statistical significances by Student's $t$ test [(6.713 ± 0.107 vs. 7.391 ± 0.330) × $10^9$/L and (3.671 ± 0.082 vs. 4.134 ± 0.269) × $10^9$/L, respectively, both $P < 0.05$].

## DISCUSSION

Although several human studies have found different associations between peripheral total leukocyte count and different serum lipid levels (1–3), the actual relationships between counts of different leukocyte types and different patterns of hyperlipidemia in humans have been unclear. Our study has shown that different types of hyperlipidemia are associated with very different peripheral differential leukocyte counts (Tables 2 and 3). A higher peripheral total leukocyte count in human hyperlipidemia (Table 1) was associated with pure hypertriglyceridemia but not pure hypercholesterolemia (Table 2), and the higher total leukocyte count in hypertriglyceridemia seemed secondary to higher counts of all leukocyte subtypes (Tables 2 and 3). In multivariate regression analyses, we demonstrated that serum TG level had independent and positive correlations with total leukocyte count and counts of all leukocyte subtypes except eosinophils (Tables 6 and 7) and serum TG was the only lipid level having an independent correlation with basophil count (Table 7). The means of total leukocyte count and counts of all leukocyte subtypes in the extreme hypertriglyceridemic group were all higher than those in the pure hypertriglyceridemic group, although only the differences in total leukocyte count and neu-

**TABLE 6**
**Multivariate Regression Analyses of Differential Leukocyte Counts on Age, Gender, BMI, Smoking, and One of Serum Levels of TC, HDL-cholesterol, LDL-cholesterol, and TG in All Study Subjects ($n$ = 3,282)**

| | Total leukocyte count ($10^9$/L) | | | Neutrophil count ($10^9$/L) | | | Lymphocyte count ($10^9$/L) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Regression coefficient[a] | SE | $P$[a] | Regression coefficient | SE | $P$ | Regression coefficient | SE | $P$ |
| Age (yr) | –0.01764 | 0.00218 | 0.0001 | –0.00275 | 0.00171 | 0.1072 | –0.01327 | 0.00086 | 0.0001 |
| Gender[b] | 0.06366 | 0.05971 | 0.2864 | –0.01433 | 0.04668 | 0.7589 | 0.00103 | 0.02364 | 0.9653 |
| BMI (kg/m$^2$) | 0.29306 | 0.03507 | 0.0001 | 0.17683 | 0.02742 | 0.0001 | 0.09269 | 0.01389 | 0.0001 |
| Smoking[b] | 0.71519 | 0.06643 | 0.0001 | 0.36947 | 0.05194 | 0.0001 | 0.27702 | 0.02631 | 0.0001 |
| TC (mg/dL) | 0.00460 | 0.00078 | 0.0001 | 0.00191 | 0.00061 | 0.0019 | 0.00279 | 0.00031 | 0.0001 |
| Age (yr) | –0.01551 | 0.00217 | 0.0001 | –0.00164 | 0.00169 | 0.3321 | –0.01228 | 0.00087 | 0.0001 |
| Gender[b] | –0.04620 | 0.06200 | 0.4563 | –0.09478 | 0.04829 | 0.0498 | –0.01956 | 0.02483 | 0.4309 |
| BMI (kg/m$^2$) | 0.25719 | 0.03658 | 0.0001 | 0.13811 | 0.02849 | 0.0001 | 0.10243 | 0.01465 | 0.0001 |
| Smoking[b] | 0.67621 | 0.06663 | 0.0001 | 0.34268 | 0.05190 | 0.0001 | 0.26738 | 0.02669 | 0.0001 |
| HDL-cholesterol (mg/dL) | –0.01171 | 0.00207 | 0.0001 | –0.00915 | 0.00161 | 0.0001 | –0.00144 | 0.00083 | 0.0816 |
| Age (yr) | –0.01717 | 0.00218 | 0.0001 | –0.00266 | 0.00170 | 0.1190 | –0.01290 | 0.00087 | 0.0001 |
| Gender[b] | 0.03691 | 0.05982 | 0.5373 | –0.02660 | 0.04669 | 0.5689 | –0.01417 | 0.02380 | 0.5517 |
| BMI (kg/m$^2$) | 0.29518 | 0.03525 | 0.0001 | 0.17508 | 0.02751 | 0.0001 | 0.09634 | 0.01402 | 0.0001 |
| Smoking[b] | 0.71287 | 0.06655 | 0.0001 | 0.36925 | 0.05194 | 0.0001 | 0.27494 | 0.02647 | 0.0001 |
| LDL-cholesterol (mg/dL) | 0.00432 | 0.00091 | 0.0001 | 0.00221 | 0.00071 | 0.0020 | 0.00225 | 0.00036 | 0.0001 |
| Age (yr) | –0.01678 | 0.00214 | 0.0001 | –0.00246 | 0.00169 | 0.1448 | –0.01263 | 0.00085 | 0.0001 |
| Gender[b] | 0.00415 | 0.05912 | 0.9441 | –0.04346 | 0.04650 | 0.3500 | –0.02722 | 0.02351 | 0.2470 |
| BMI (kg/m$^2$) | 0.20297 | 0.03612 | 0.0001 | 0.12765 | 0.02841 | 0.0001 | 0.05897 | 0.01436 | 0.0001 |
| Smoking[b] | 0.65881 | 0.06579 | 0.0001 | 0.34150 | 0.05174 | 0.0001 | 0.25094 | 0.02616 | 0.0001 |
| TG (mg/dL) | 0.00418 | 0.00040 | 0.0001 | 0.00215 | 0.00031 | 0.0001 | 0.00180 | 0.00016 | 0.0001 |

| | Monocyte count ($10^9$/L) | | | Eosinophil count ($10^9$/L) | | | Basophil count ($10^9$/L) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Regression coefficient[a] | SE | $P$[a] | Regression coefficient | SE | $P$ | Regression coefficient | SE | $P$ |
| Age (yr) | –0.00072 | 0.00020 | 0.0004 | –0.00079 | 0.00021 | 0.0001 | –0.00011 | 0.00003 | 0.0007 |
| Gender[b] | 0.03321 | 0.00550 | 0.0001 | 0.04145 | 0.00564 | 0.0001 | 0.00229 | 0.00090 | 0.0111 |
| BMI (kg/m$^2$) | 0.02002 | 0.00323 | 0.0001 | 0.00231 | 0.00331 | 0.4865 | 0.00121 | 0.00053 | 0.0225 |
| Smoking[b] | 0.04105 | 0.00612 | 0.0001 | 0.02408 | 0.00627 | 0.0001 | 0.00358 | 0.00100 | 0.0004 |
| TC (mg/dL) | –0.00015 | 0.00007 | 0.0427 | 0.000011 | 0.000074 | 0.8790 | 0.000032 | 0.000012 | 0.0065 |
| Age (yr) | –0.00072 | 0.00020 | 0.0004 | –0.00078 | 0.00021 | 0.0001 | –0.00010 | 0.00003 | 0.0030 |
| Gender[b] | 0.02508 | 0.00569 | 0.0001 | 0.04151 | 0.00585 | 0.0001 | 0.00154 | 0.00094 | 0.1005 |
| BMI (kg/m$^2$) | 0.01322 | 0.00335 | 0.0001 | 0.00245 | 0.00345 | 0.4789 | 0.00097 | 0.00055 | 0.0796 |
| Smoking[b] | 0.03876 | 0.00611 | 0.0001 | 0.02408 | 0.00629 | 0.0001 | 0.00331 | 0.00101 | 0.0010 |
| HDL-cholesterol (mg/dL) | –0.00106 | 0.00019 | 0.0001 | 0.000012 | 0.000195 | 0.9496 | –0.000080 | 0.000031 | 0.0103 |
| Age (yr) | –0.00074 | 0.00020 | 0.0002 | –0.00077 | 0.00021 | 0.0002 | –0.00011 | 0.00003 | 0.0011 |
| Gender[b] | 0.03402 | 0.00550 | 0.0001 | 0.04153 | 0.00564 | 0.0001 | 0.00212 | 0.00090 | 0.0192 |
| BMI (kg/m$^2$) | 0.01986 | 0.00324 | 0.0001 | 0.00264 | 0.00332 | 0.4272 | 0.00125 | 0.00053 | 0.0193 |
| Smoking[b] | 0.04115 | 0.00612 | 0.0001 | 0.02398 | 0.00627 | 0.0001 | 0.00355 | 0.00100 | 0.0004 |
| LDL-cholesterol (mg/dL) | –0.00012 | 0.00008 | 0.1445 | –0.000042 | 0.000086 | 0.6281 | 0.000027 | 0.000014 | 0.0524 |
| Age (yr) | –0.00079 | 0.00020 | 0.0001 | –0.00079 | 0.00020 | 0.0001 | –0.00011 | 0.00003 | 0.0011 |
| Gender[b] | 0.03215 | 0.00550 | 0.0001 | 0.04083 | 0.00565 | 0.0001 | 0.00184 | 0.00090 | 0.0417 |
| BMI (kg/m$^2$) | 0.01505 | 0.00336 | 0.0001 | 0.00082 | 0.00345 | 0.8121 | 0.00047 | 0.00055 | 0.3939 |
| Smoking[b] | 0.03980 | 0.00612 | 0.0001 | 0.02345 | 0.00628 | 0.0002 | 0.00314 | 0.00100 | 0.0017 |
| TG (mg/dL) | 0.00014 | 0.00004 | 0.0001 | 0.000055 | 0.000038 | 0.1483 | 0.000033 | 0.000006 | 0.0001 |

[a]Regression coefficients were estimated by ordinary least squares method, and probability values were calculated by $t$-test.
[b]In the statistical analysis, female, male, nonsmoker, and smoker were represented digitally by 0, 1, 0, and 1, respectively. For abbreviations see Tables 1 and 5.

trophil count reached statistical significances (see data in the last paragraph of the Results section). There is accumulating evidence from human studies that hypertriglyceridemia is a risk factor for atherosclerosis and CVD (6,7), and it seems likely that different leukocytes play different roles in atherogenesis (8–10,20). A higher peripheral total leukocyte count is associated with a higher risk of CVD (3,11–13) and the association of hypertriglyceridemia with a higher CVD risk

**TABLE 7**
**Multivariate Regression Analyses of Differential Leukocyte Counts on Age, Gender, BMI, Smoking, and Serum Levels of HDL-cholesterol, LDL-cholesterol, and TG in All Study Subjects (*n* = 3,282)**

| | Total leukocyte count ($10^9$/L) | | | Neutrophil count ($10^9$/L) | | | Lymphocyte count ($10^9$/L) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Regression coefficient[a] | SE | *P*[a] | Regression coefficient | SE | *P* | Regression coefficient | SE | *P* |
| Age (yr) | –0.01731 | 0.00215 | 0.0001 | –0.00252 | 0.00169 | 0.1368 | –0.01319 | 0.00085 | 0.0001 |
| Gender[b] | –0.03993 | 0.06123 | 0.5144 | –0.09321 | 0.04816 | 0.0530 | –0.01633 | 0.02430 | 0.5016 |
| BMI (kg/m$^2$) | 0.16790 | 0.03719 | 0.0001 | 0.09639 | 0.02925 | 0.0010 | 0.05699 | 0.01476 | 0.0001 |
| Smoking[b] | 0.65873 | 0.06574 | 0.0001 | 0.33555 | 0.05171 | 0.0001 | 0.25845 | 0.02609 | 0.0001 |
| HDL-cholesterol (mg/dL) | –0.00484 | 0.00220 | 0.0280 | –0.00614 | 0.00173 | 0.0004 | 0.00206 | 0.00087 | 0.0182 |
| LDL-cholesterol (mg/dL) | 0.00358 | 0.00091 | 0.0001 | 0.00193 | 0.00071 | 0.0067 | 0.00181 | 0.00036 | 0.0001 |
| TG (mg/dL) | 0.00368 | 0.00043 | 0.0001 | 0.00162 | 0.00034 | 0.0001 | 0.00188 | 0.00017 | 0.0001 |

| | Monocyte count ($10^9$/L) | | | Eosinophil count ($10^9$/L) | | | Basophil count ($10^9$/L) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Regression coefficient[a] | SE | *P*[a] | Regression coefficient | SE | *P* | Regression coefficient | SE | *P* |
| Age (yr) | –0.00070 | 0.00020 | 0.0005 | –0.00079 | 0.00021 | 0.0001 | –0.00011 | 0.00003 | 0.0008 |
| Gender[b] | 0.02593 | 0.00569 | 0.0001 | 0.04204 | 0.00587 | 0.0001 | 0.00162 | 0.00094 | 0.0828 |
| BMI (kg/m$^2$) | 0.01261 | 0.00346 | 0.0003 | 0.00163 | 0.00356 | 0.6475 | 0.00029 | 0.00057 | 0.6121 |
| Smoking[b] | 0.03802 | 0.00611 | 0.0001 | 0.02356 | 0.00630 | 0.0002 | 0.00315 | 0.00100 | 0.0017 |
| HDL-cholesterol (mg/dL) | –0.00089 | 0.00020 | 0.0001 | 0.00015 | 0.00021 | 0.4868 | –0.000022 | 0.000034 | 0.5078 |
| LDL-cholesterol (mg/dL) | –0.00013 | 0.00008 | 0.1243 | –0.000058 | 0.000087 | 0.5011 | 0.000020 | 0.000014 | 0.1414 |
| TG (mg/dL) | 0.000083 | 0.000040 | 0.0373 | 0.000068 | 0.000041 | 0.0989 | 0.000031 | 0.000007 | 0.0001 |

[a]Regression coefficients were estimated by ordinary least squares method, and probability values were calculated by *t*-test.
[b]In the statistical analysis, female, male, nonsmoker, and smoker were represented digitally by 0, 1, 0, and 1, respectively. For abbreviations see Tables 1 and 5.

may relate in part to its coexistence with higher peripheral counts of all leukocyte subtypes (Tables 2 and 3), probably except eosinophils (Tables 6 and 7).

For pure hypercholesterolemia, the results were quite different from those in pure hypertriglyceridemia. Compared with the normolipidemic group, pure hypercholesterolemia was associated with a significantly lower monocyte count and no significant difference in other leukocyte counts (Tables 2 and 3). Such a result may be partly related to the following complex relationships: (i) Both serum HDL-cholesterol and LDL-cholesterol levels were significantly higher in the pure hypercholesterolemic group (Table 2); (ii) there were independent and negative correlations between serum HDL-cholesterol level and total leukocyte count and counts of neutrophils and monocytes (Tables 6 and 7); and (iii) there were independent and positive correlations between serum LDL-cholesterol level and total leukocyte count and counts of neutrophils and lymphocytes (Tables 6 and 7). A finding in Table 3 worth mentioning here is that the significantly lower monocyte count in the pure hypercholesterolemic group, as compared with the normolipidemic group, did not exist in the hypercholesterolemic group. The reason may be simply that the hypercholesterolemic group contained subjects with hypertriglyceridemia that was associated with a significantly higher monocyte count which had masked the presence of a significantly lower monocyte count associated with hypercholesterolemia.

The significantly lower monocyte count in pure hypercholesterolemia (Tables 2 and 3) and the independent and negative correlations between serum HDL-cholesterol level and counts of both monocytes and neutrophils (Tables 6 and 7) may be related to an anti-inflammatory effect of HDL particles. Several studies of humans have shown the abilities of HDL to bind inflammatory mediators, neutralize their leukocyte chemotactic activities, and thus attenuate the inflammation- or infection-induced increase in the count or recruitment of leukocytes, especially the monocytes and neutrophils (21, 22). In this way, HDL may ultimately reduce the peripheral monocyte and neutrophil counts. This may be one of the reasons that a high circulating HDL-cholesterol level is antiatherogenic (23,24) in view of the possible underlying immunoinflammatory nature of atherosclerosis (8,10).

Smoking has been well documented to have a positive effect on peripheral total leukocyte count (17,18). Our study not only confirms such an independent and positive link between smoking and total leukocyte count but also reveals that such a link is related to independent and positive links between smoking and counts of all leukocyte subtypes (Tables 4, 6, and 7). These results strongly indicate that whenever peripheral leukocyte counts are the subjects of study, the factor of smoking should always be considered and included. Obesity has also been found to be associated with a higher peripheral total leukocyte count (18,19). Our study confirms the existence of an independent and positive correlation between BMI and total leukocyte count and, furthermore, demonstrates independent and positive correlations between BMI and counts of neutrophils, lymphocytes, and monocytes (Tables 5, 6, and 7). These findings, together with other related results in the study and a positive association between obesity and lipid disorders (16), imply a close and complex inter-

relationship among obesity, hyperlipidemia, and counts of neutrophils and monocytes.

In summary, our study revealed that an increase in peripheral total leukocyte count in hyperlipidemia is mainly associated with a higher serum TG level and the presence of hypertriglyceridemia. The higher total leukocyte count in hypertriglyceridemia is apparently due to increases in counts of all leukocyte subtypes except eosinophils. In contrast, pure hypercholesterolemia is associated with a lower monocyte count, possibly related to both a higher serum HDL-cholesterol level in pure hypercholesterolemia and a negative link between serum HDL-cholesterol level and monocyte count. Our study also reveals that smoking is independently associated with higher total leukocyte count and counts of all leukocyte subtypes, and BMI has independent and positive correlations with total leukocyte count and counts of neutrophils, monocytes, and lymphocytes.

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# Unique Phospholipid Metabolism in Mouse Heart in Response to Dietary Docosahexaenoic or α-Linolenic Acids

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**ABSTRACT:** Diet and fatty acid metabolism interact in yet unknown ways to modulate membrane fatty acid composition and certain cellular functions. For example, dietary precursors or metabolic products of n-3 fatty acid metabolism differ in their ability to modify specific membrane components. In the present study, the effect of dietary 22:6n-3 or its metabolic precursor, 18:3n-3, on the selective accumulation of 22:6n-3 by heart was investigated. The mass and fatty acid compositions of individual phospholipids (PL) in heart and liver were quantified in mice fed either 22:6n-3 (from crocodile oil) or 18:3n-3 (from soybean oil) for 13 wk. This study was conducted to determine if the selective accumulation of 22:6n-3 in heart was due to the incorporation of 22:6n-3 into cardiolipin (CL), a PL most prevalent in heart and known to accumulate 22:6n-3. Although heart was significantly enriched with 22:6n-3 relative to liver, the accumulation of 22:6n-3 by CL in heart could not quantitatively account for this difference. CL from heart did accumulate 22:6n-3, but only in mice fed preformed 22:6n-3. Diets rich in non-22:6n-3 fatty acids result in a fatty acid composition of phosphatidylcholine (PC) in heart that is unusually enriched with 22:6n-3. In this study, the mass of PC in heart was positively correlated with the enrichment of 22:6n-3 into PC. The increased mass of PC was coincident with a decrease in the mass of phosphatidylethanolamine, suggesting that 22:6n-3 induced PC synthesis by increasing phosphatidylethanolamine-*N*-methyltransferase activity in the heart.

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Dietary oils rich in n-3 fatty acids have cardioprotective effects including the inhibition of ischemia-reperfusion induced cardiac dysfunction in rats (1–3), reduced arrhythmia in normal or ischemic marmoset monkeys (4) and rats (5), and the prevention of ischemia-induced ventricular fibrillation in dogs (6). Dietary 22:6n-3 is at least in part responsible for these effects. Docosahexaenoic acid given as a dietary supplement in the absence of other n-3 fatty acids increased the survival of isolated rat cardiomyocytes subjected to hypoxia/reoxygenation (7), altered cardiomyocyte beta-adrenergic function (8), inhibited ischemia-induced arrhythmias in rats (9), and lowered the heart rate of humans (10,11). The mechanisms underlying these cardioprotective actions are not yet understood, nor is it known how 22:6n-3 accumulates in specific cells and membrane components.

Heart phospholipids (PL) rapidly accumulate 22:6n-3 in response to dietary 22:6n-3 (12–14). Although 22:6n-3 is usually consumed in the form of marine oils that also contain 20:5n-3, the accumulation of 22:6n-3 in heart PL occurs without a similar increase in heart 20:5n-3 content (12,14–16), suggesting a specific mechanism for the uptake and/or retention of 22:6n-3. One possible mechanism for the accumulation of 22:6n-3 in heart would be a highly selective incorporation into PL classes that are notably rich in heart. Heart tissue is mitochondria-dense and as a result, the mitochondrial PL cardiolipin (CL) comprises as much as 15% of the PL mass of rat heart (17,18). Since dietary 22:6n-3 is known to enrich CL with 22:6n-3 to as much as 50 mol% of its fatty acid composition (12,19), the high concentration of CL in heart tissue may explain the selective incorporation of 22:6n-3 in heart.

Considerable information can be obtained from the relative fatty acid compositions of individual lipid classes. The present study was designed to couple highly quantitative data on fatty acid and lipid class mass with precise compositional data. Combined, accurate mass and compositional data provide a powerful tool for making metabolic inference. To determine the effect of dietary n-3 fatty acids on the mass of individual lipid classes in heart, dietary 22:6n-3 [from crocodile oil (CRO), which contained 1.1 and 2.9 mol% 20:5n-3 and 22:6n-3, respectively] was compared with 18:3n-3 [from soybean oil (SO), which contained 7.4 mol% 18:3n-3 as the only source of n-3 fatty acid] for its influence on the mass and fatty acid composition of liver and heart PL classes.

## MATERIALS AND METHODS

*Reagents.* All chromatography solvents were obtained from Fisher Scientific (Pittsburgh, PA). Silica Gel 60 thin-layer chromatography (TLC) plates (10 × 20 cm) were obtained

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Abbreviations: CE, cholesterol ester; CL, cardiolipin; CRO, crocodile oil; FFA, free fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine-*N*-methyltransferase; PL, phospholipids; SO, soybean oil; TG, triacylglyceride; TLC, thin-layer chromatography.

from E. Merck (Darmstadt, Germany). EDTA and butylated hydroxytoluene were obtained from Sigma Chemical Co. (St. Louis, MO). Fatty acid methyl ester standards were obtained from Nu-Chek-Prep (Elysian, MN), and internal standard PL were obtained from Avanti Polar Lipids (Alabaster, AL).

*Animals*. Litters of mice were obtained from a breeding colony of HTLV-1 mice derived from an original founder line (20) maintained at the University of California, Davis. Weanling, 21-d-old mice were housed either individually or as same-sex pairs from identical liters in stainless steel wire-bottomed cages in a room with a 12-h light and 12-h dark cycle at 20–23°C and 50% relative humidity. Mice were assigned to each diet group and fed until 112 d of age. At that time, the mice were killed and the tissues harvested for fatty acid analyses.

*Diets*. Mice were fed a nutritionally adequate, amino acid-based diet (21) (Dyets, Bethlehem, PA), without added succinyl sulfathiazole, in which essential fatty acid requirements were met by addition of 0.5% SO by weight. Dietary CRO or SO were added at 6.5% by weight; thus the total dietary fat content was 7% by weight. CRO was chosen because it is a novel food oil with a low concentration of 20:5n-3 and 22:6n-3. Seven mice fed SO and four mice fed CRO were used in these analyses. The CRO was a gift (Madagascar CrocFarm, Ivato, Madagascar). Diets were fed to the mice for 13 wk before necropsy and tissue harvest following $CO_2$ anesthesia. Animal care and use protocols were approved by the University of California, Davis, Animal Welfare committee. The fatty acid composition of each dietary oil (Table 1) was determined by gas chromatographic analysis. Preparation of fatty acid methyl esters and chromatographic conditions are described below. Mice had unrestricted access to food and water and were provided fresh food daily.

*Extraction and TLC*. TLC plates were impregnated with 1 mM EDTA, pH 5.5, and washed by ascending development using the method of Ruiz and Ochoa (22). Just before use, each plate was activated by heating at 110°C for 10 min and allowed to cool to room temperature. Lipids were extracted from *ca.* 100 mg of tissue by homogenization in 6 mL chloroform/methanol, (2:1, vol/vol) (23). Phosphatidylcholine (PC) and CL internal standards (Avanti Polar Lipids) were added to facilitate quantification. Sample extracts were dried under nitrogen and spotted onto EDTA-impregnated TLC plates. Two TLC standard lanes consisting of authentic PC, phosphatidylethanolamine (PE), CL, and free fatty acid (FFA) were spotted on the outside lanes of the TLC plate as reference samples. The mobile phase employed for the separation of PL classes was a modification of the solvent system described by Holub and Skeaf (24) that consisted of chloroform/methanol/acetic acid/water (100:67:7:4, by vol). For the separation of lipid classes [total PL, FFA, triacylglycerides (TG), and cholesterol esters (CE)], a solvent system consisting of petroleum ether/diethyl ether/acetic acid (80:20:1, by vol) (25) was employed.

*Isolation and methylation of lipid classes*. Lipid classes and individual PL classes were identified by comparison with the authentic standards chromatographed in the reference lanes. Reference samples were visualized by cutting the reference lanes from the plate, dipping the reference lanes in 10% cupric sulfate/8% phosphoric acid, and charring them at 200°C. The reference lanes were then compared to the uncharred plate to determine the location of individual lipid classes in the sample. Each lipid fraction was scraped from the plate and methylated by incubating the silica in 3 N methanolic-HCl in sealed vials under a nitrogen atmosphere at 100°C for 45 min. Fatty acid methyl esters were extracted with hexane containing 0.05% butylated hydroxytoluene and prepared for gas chromatography by sealing the hexane extracts under nitrogen.

*Fatty acid analysis.* Fatty acid methyl esters were separated and quantified by capillary gas chromatography using a Hewlett-Packard (Wilmington, DE) gas chromatograph (model 6890) equipped with a 60 m DB-23 capillary column (J&W Scientific, Folsom, CA), a flame-ionization detector, and Hewlett-Packard ChemStation software.

*Data analysis*. The mass of total fatty acids, PL fatty acids, PC fatty acids, and CL fatty acids were quantified per gram of tissue using the internal standards described above. TG, FFA, and CE were quantified by adding 5 μg of tritridecanoin as an internal standard to each fraction after TLC separation. The ratio of the sum of areas from fatty acids to the area of tridecanoic acid in each chromatogram was used to calculate the mass fraction of each lipid class. PE was calculated as the difference between the mass of total PL and the sum of the masses of PC and CL. Therefore, mass data for PE include the masses of other minor PL including phosphatidylserine and sphingomyelin. However, PC, PE, and CL represent 90 wt% of the PL mass of heart tissue (26,27), and thus the majority of this fraction is composed of PE. The statistics were performed in the Statistical Support Laboratory at the University of California at Davis. Differences between tissues,

**TABLE 1**
**Fatty Acid Composition of the Dietary Oils[a]**

| | Soybean oil | Crocodile oil |
|---|---|---|
| 14:0 | 0.1 | 3.3 |
| 16:0 | 11.5 | 28.4 |
| 16:1n-7 | 0.1 | 5.7 |
| 18:0 | 3.8 | 11.8 |
| 18:1n-9 | 20.9 | 27.9 |
| 18:1n-7 | 1.2 | 6.4 |
| 18:2n-6 | 54.1 | 9.7 |
| 18:3n-6 | 0.0 | 0.1 |
| 18:3n-3 | 7.4 | 0.5 |
| 20:0 | 0.3 | 0.2 |
| 20:1n-9 | 0.2 | 0.3 |
| 20:2n-6 | 0.1 | 0.2 |
| 20:3n-6 | 0.0 | 0.2 |
| 20:4n-6 | 0.0 | 1.1 |
| 20:5n-3 | 0.0 | 1.1 |
| 22:0 | 0.3 | 0.0 |
| 22:1n-9 | 0.0 | 0.1 |
| 22:5n-3 | 0.0 | 0.0 |
| 22:6n-3 | 0.0 | 2.9 |

[a]Data are expressed in mol% of total fatty acids.

lipid classes, and diets were determined by a multivariate GLM/ANOVA using SAS/STAT (Cary, NC). Correlation analyses were performed in Excel '97 (Microsoft Co., Redmond, WA), and significance was assigned to a correlation if the absolute value of the Pearson's sample correlation coefficient ($r$) was greater than the critical value for significance. Linear regressions were also performed in Excel '97.

## RESULTS

*Fatty acid composition*. SO contained 18:3n-3 as the only source of n-3 fatty acid, whereas CRO contained 22:6n-3, 20:5n-3, and 18:3n-3 (Table 1). The fatty acid compositions of total PL, PC, PE, and CL in heart from mice fed SO or CRO are shown in Table 2. The fatty acid compositions of total PL, PC, PE, and CL from liver in mice fed SO or CRO are shown in Table 3. Only trace amounts of dietary 18:3n-3 accumulated as 18:3n-3 in liver or heart PL, indicating that the fatty acid was rapidly catabolized, exported, or converted to 20:5n-3, 22:5n-3, or 22:6n-3. Eicosapentaenoic acid was also poorly accumulated by heart PL. However, PC and PE from liver of mice fed CRO each contained 1.7 mol% 20:5n-3. In stark contrast to the low concentrations of 18:3n-3 and 20:5n-3 found in tissues, PL from the hearts of mice fed SO or CRO contained 23.4 and 32.3 mol% 22:6n-3, respectively. Heart PL, therefore, were rich in 22:6n-3, regardless of whether 22:6n-3 was fed intact (as a component of CRO) or as its precursor 18:3n-3 (as a component of SO). With respect to 22:6n-3, tissue-specific incorporation of the fatty acid was clearly more important than the dietary source of n-3 fatty acid. Table 4 shows the differential enrichment of heart and liver with 22:6n-3. The percentage difference in the 22:6n-3 of heart relative to liver was consistent in both PC and PE, regardless of the type of dietary n-3 fatty acid. Surprisingly, the 22:6n-3 content of CL was similar for heart and liver from mice fed SO. However, the CL in heart from mice fed CRO contained 104% more 22:6n-3 than did the CL in liver. Table 5 shows the percentage difference in the amount of 22:6n-3 in PL classes between mice fed CRO and mice fed SO. The total PL, PC, and PE from heart and liver incorporated more 22:6n-3 when mice were fed CRO than when the mice were fed SO. The percentage increase in the amount of 22:6n-3 in total PL, PC, and PE of mice fed CRO relative to mice fed SO was consistent between heart and liver. However, the CL in heart from mice fed CRO contained 318% more 22:6n-3 than the CL in heart of mice fed SO. The magnitude of this difference was not mirrored in liver, where mice fed CRO had only 82% more 22:6n-3 than mice fed SO. Therefore, unlike PC and PE, the 22:6n-3 content of CL was dependent on the form of dietary n-3 fatty acid.

*Lipid mass analysis.* The mass of total lipids, total PL, FFA, TG, CE, PC, PE, and CL from heart or liver were measured by quantifying the mass of individual fatty acids present in each fraction. Diet did not significantly affect the mass of total lipid per gram of heart or liver. Heart tissue from mice fed SO or CRO contained 33.9 or 36.1 mg of fatty acid per

**TABLE 2**
**Fatty Acid Composition[a] of Phospholipids from the Hearts of Mice Fed Soybean Oil (SO) or Crocodile Oil (CRO)**

| | Total phospholipid | | Phosphatidylcholine | | Phosphatidylethanolamine | | Cardiolipin | |
|---|---|---|---|---|---|---|---|---|
| | SO | CRO | SO | CRO | SO | CRO | SO | CRO |
| 14:0 | 0.1 ± 0.1 | 0.3 ± 0.2[b] | 0.2 ± 0.1 | 0.2 ± 0.0 | 0.3 ± 0.2 | 0.2 ± 0.1 | 0.8 ± 0.5 | 1.0 ± 0.6 |
| 15:0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.0 | 0.2 ± 0.1 | 0.1 ± 0.0 | 0.3 ± 0.3 | 0.3 ± 0.1 |
| 16:0 | 16.1 ± 0.6 | 18.8 ± 1.0[b] | 29.2 ± 2.0 | 27.2 ± 0.8 | 12.7 ± 2.6 | 11.5 ± 0.3 | 4.5 ± 2.3 | 4.0 ± 1.3 |
| 16:1n-7 | 0.2 ± 0.1 | 0.7 ± 0.1[b] | 0.3 ± 0.1 | 0.4 ± 0.1[b] | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.8 ± 0.2 | 3.3 ± 0.7[b] |
| 18:0 | 21.6 ± 0.5 | 22.1 ± 1.2 | 21.7 ± 1.0 | 21.7 ± 1.4 | 29.3 ± 1.3 | 27.1 ± 0.4[b] | 5.3 ± 5.4 | 2.0 ± 0.7 |
| 18:1n-9 | 5.3 ± 0.6 | 8.1 ± 0.5[b] | 5.0 ± 0.6 | 5.8 ± 0.6 | 4.4 ± 0.2 | 4.5 ± 0.5 | 4.4 ± 0.7 | 19.8 ± 0.5[b] |
| 18:1n-7 | 1.9 ± 0.2 | 2.8 ± 0.2[b] | 1.6 ± 0.2 | 1.5 ± 0.1 | 1.0 ± 0.1 | 1.5 ± 0.0[b] | 3.5 ± 1.0 | 10.1 ± 1.8[b] |
| 18:2n-6 | 19.0 ± 1.1 | 6.1 ± 0.8[b] | 9.9 ± 1.3 | 2.5 ± 0.4[b] | 4.6 ± 0.6 | 1.3 ± 0.3[b] | 68.0 ± 11.7 | 30.1 ± 5.0[b] |
| 18:3n-6 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.0[b] | 0.1 ± 0.1 | 0.1 ± 0.1 |
| 18:3n-3 | 0.2 ± 0.0 | 0.1 ± 0.0[b] | 0.2 ± 0.0 | 0.0 ± 0.0[b] | 0.1 ± 0.1 | 0.0 ± 0.0[b] | 0.5 ± 0.2 | 0.2 ± 0.1[b] |
| 20:0 | 0.4 ± 0.0 | 0.3 ± 0.0[b] | 0.2 ± 0.1 | 0.1 ± 0.0[b] | 0.2 ± 0.0 | 0.1 ± 0.0[b] | 0.3 ± 0.2 | 0.1 ± 0.1[b] |
| 20:1n-9 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0[b] | 0.2 ± 0.0 | 0.1 ± 0.0[b] | 0.2 ± 0.1 | 0.3 ± 0.1[b] |
| 20:2n-6 | 0.5 ± 0.1 | 0.3 ± 0.0[b] | 0.3 ± 0.1 | 0.1 ± 0.0[b] | 0.2 ± 0.0 | 0.1 ± 0.0[b] | 1.4 ± 0.3 | 1.1 ± 0.1 |
| 20:3n-6 | 0.6 ± 0.1 | 0.5 ± 0.1 | 0.4 ± 0.0 | 0.2 ± 0.0[b] | 0.2 ± 0.0 | 0.1 ± 0.0[b] | 1.1 ± 0.4 | 1.9 ± 0.2[b] |
| 20:4n-6 | 7.4 ± 0.4 | 4.8 ± 0.1[b] | 7.6 ± 0.6 | 4.5 ± 0.3[b] | 8.2 ± 0.7 | 5.6 ± 0.3[b] | 1.0 ± 0.6 | 1.5 ± 0.2 |
| 20:5n-3 | 0.1 ± 0.0 | 0.2 ± 0.0[b] | 0.0 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.1 | 0.2 ± 0.1[b] | 0.3 ± 0.4 | 0.1 ± 0.1 |
| 22:0 | 0.4 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.1 | 0.0 ± 0.0 | 0.1 ± 0.1 | 0.0 ± 0.1 | 0.3 ± 0.2 | 0.1 ± 0.1 |
| 22:1n-9 | 0.2 ± 0.1 | 0.3 ± 0.2 | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.3 ± 0.3 | 0.1 ± 0.1 |
| 22:4n-6 | 0.4 ± 0.0 | 0.2 ± 0.0[b] | 0.4 ± 0.1 | 0.2 ± 0.0[b] | 0.5 ± 0.0 | 0.2 ± 0.0[b] | 0.3 ± 0.2 | 0.1 ± 0.1[b] |
| 22:5n-3 | 1.5 ± 0.4 | 1.2 ± 0.1 | 1.7 ± 0.5 | 1.7 ± 0.0 | 1.5 ± 0.4 | 1.2 ± 0.1 | 0.4 ± 0.2 | 0.6 ± 0.2[b] |
| 24:0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 22:6n-3 | 23.4 ± 1.7 | 32.3 ± 1.5[b] | 20.2 ± 2.6 | 32.9 ± 0.5[b] | 35.3 ± 4.2 | 45.2 ± 0.8[b] | 5.5 ± 4.8 | 23.0 ± 4.5[b] |
| 24:1n-9 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.3 ± 0.1 | 0.0 ± 0.1[b] | 0.3 ± 0.1 | 0.1 ± 0.1 | 0.7 ± 0.3 | 0.3 ± 0.1[b] |

[a]Data are expressed in mol% of total fatty acids ± SD. Results indicate the average of four independent analyses of CRO-fed mice and seven independent analyses of SO-fed mice.
[b]Significantly different from data for mice fed SO.

**TABLE 3**
**Fatty Acid Composition of Phospholipids from the Livers of Mice Fed SO or CRO[a]**

| | Total phospholipids | | Phosphatidylcholine | | Phosphatidylethanolamine | | Cardiolipin | |
|---|---|---|---|---|---|---|---|---|
| | SO | CRO | SO | CRO | SO | CRO | SO | CRO |
| 14:0 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.2 ± 0.0 | 1.0 ± 0.8 | 1.2 ± 0.6 |
| 15:0 | 0.1 ± 0.1 | 0.2 ± 0.1[b] | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.4 ± 0.3 | 0.3 ± 0.3 | 0.4 ± 0.5 | 0.4 ± 0.9 |
| 16:0 | 27.2 ± 1.1 | 29.5 ± 1.6[b] | 36.3 ± 1.6 | 40.1 ± 1.5[b] | 27.0 ± 1.9 | 31.6 ± 2.8[b] | 10.0 ± 1.9 | 12.4 ± 1.5 |
| 16:1n-7 | 0.4 ± 0.1 | 1.2 ± 0.5[b] | 0.6 ± 0.2 | 1.4 ± 0.6 | 0.6 ± 0.3 | 0.8 ± 0.4 | 1.6 ± 0.9 | 3.4 ± 0.9[b] |
| 18:0 | 19.6 ± 0.7 | 18.2 ± 1.5 | 15.2 ± 1.6 | 12.6 ± 1.5 | 21.1 ± 1.9 | 18.2 ± 2.3 | 12.0 ± 5.3 | 16.6 ± 1.4 |
| 18:1n-9 | 5.5 ± 0.6 | 9.4 ± 0.8[b] | 5.8 ± 0.8 | 11.5 ± 1.4[b] | 4.0 ± 0.7 | 4.0 ± 0.1 | 5.6 ± 1.0 | 10.2 ± 0.3[b] |
| 18:1n-7 | 1.2 ± 0.1 | 1.6 ± 0.3[b] | 0.9 ± 0.2 | 1.4 ± 0.3[b] | 0.7 ± 0.1 | 0.8 ± 0.3 | 4.1 ± 0.9 | 4.8 ± 0.2[b] |
| 18:2n-6 | 16.6 ± 1.2 | 8.6 ± 0.5[b] | 18.1 ± 2.0 | 7.8 ± 0.8[b] | 6.5 ± 1.1 | 2.9 ± 0.3[b] | 49.0 ± 9.7 | 30.7 ± 2.6[b] |
| 18:3n-6 | 0.2 ± 0.0 | 0.1 ± 0.0[b] | 0.6 ± 0.2 | 0.5 ± 0.6 | 0.4 ± 0.3 | 0.4 ± 0.5 | 0.4 ± 0.6 | 0.4 ± 0.5 |
| 18:3n-3 | 0.2 ± 0.0 | 0.1 ± 0.0[b] | 0.1 ± 0.1 | 0.0 ± 0.0[b] | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.3 ± 0.4 | 0.0 ± 0.0[b] |
| 20:0 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.2 | 0.1 ± 0.1 | 0.2 ± 0.2 | 0.3 ± 0.4 |
| 20:1n-9 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.2 | 0.1 ± 0.1 |
| 20:2n-6 | 0.4 ± 0.1 | 0.0 ± 0.0[b] | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.1 ± 0.1 | 1.2 ± 0.6 | 0.3 ± 0.2[b] |
| 20:3n-6 | 1.3 ± 0.2 | 1.1 ± 0.3 | 1.4 ± 0.2 | 1.2 ± 0.3 | 0.5 ± 0.1 | 0.4 ± 0.1 | 0.5 ± 0.6 | 1.2 ± 0.1 |
| 20:4n-6 | 14.2 ± 0.8 | 11.6 ± 1.1[b] | 11.4 ± 0.9 | 8.8 ± 0.7[b] | 18.3 ± 1.0 | 14.7 ± 1.8[b] | 4.4 ± 2.1 | 4.4 ± 1.1 |
| 20:5n-3 | 0.3 ± 0.1 | 0.6 ± 0.1[b] | 0.4 ± 0.1 | 1.7 ± 0.6[b] | 0.4 ± 0.1 | 1.7 ± 0.4[b] | 0.0 ± 0.1 | 0.4 ± 0.3 |
| 22:0 | 0.6 ± 0.1 | 0.6 ± 0.1 | 0.1 ± 0.1 | 0.0 ± 0.1 | 0.1 ± 0.2 | 0.0 ± 0.0 | 0.0 ± 0.1 | 0.0 ± 0.0 |
| 22:1n-9 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.2 | 0.2 ± 0.2 | 0.4 ± 0.4 | 0.2 ± 0.4 | 1.5 ± 1.6 | 0.6 ± 1.2 |
| 22:4n-6 | 0.2 ± 0.1 | 0.6 ± 0.2[b] | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.5 ± 0.2 | 0.2 ± 0.1[b] | 0.1 ± 0.1 | 0.0 ± 0.0 |
| 22:5n-3 | 0.5 ± 0.1 | 0.6 ± 0.2 | 0.4 ± 0.1 | 0.5 ± 0.2 | 0.8 ± 0.1 | 0.9 ± 0.3 | 0.4 ± 0.5 | 0.4 ± 0.5 |
| 24:0 | 0.3 ± 0.1 | 0.2 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 22:6n-3 | 9.7 ± 1.0 | 13.6 ± 1.1[b] | 7.0 ± 0.7 | 10.9 ± 1.6[b] | 16.5 ± 1.7 | 21.7 ± 2.3[b] | 6.2 ± 3.9 | 11.3 ± 2.4[b] |
| 24:1n-9 | 0.3 ± 0.1 | 0.4 ± 0.2[b] | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.4 ± 0.2 | 0.4 ± 0.2 | 0.8 ± 0.7 | 0.9 ± 0.8 |

[a]Data are expressed in mol% of total fatty acids ± SD. Results indicate the average of four independent analyses of CRO-fed mice and seven independent analyses of SO-fed mice.
[b]Significantly different from data for mice fed SO. See Table 2 for abbreviations.

gram of tissue, respectively (data not shown). Liver tissue from mice fed SO or CRO contained 45.5 or 48.1 mg of fatty acid per gram of tissue, respectively (data not shown). Mass fractions for each lipid class were calculated by dividing the mass of PL, FFA, TG, or CE by the mass of total lipid (measured per gram of tissue). Mass fractions for PC and CL were calculated by dividing the mass of PC or CL by the mass of total PL. The mass fraction of PE was calculated by difference as described in the Materials and Methods section. Mass fraction data are shown in Table 6. The mass fractions of PL, FFA, TG, CE, PC, PE, and CL in liver were not different between mice fed SO or CRO. The mass fractions of PL, FFA, TG, CE, and CL in heart were not different between mice fed SO and mice fed CRO. However, the mass fraction of PC in heart from mice fed CRO was significantly greater than the mass fraction of PC in heart from mice fed SO. Therefore, although the amount of total PL per gram of heart was constant, there was a significant shift in the composition of total PL toward a greater amount of PC and a lesser amount of PE in mice fed CRO relative to mice fed SO. Heart contained similar amounts of total PL, but significantly more CL per gram than liver.

*Interaction between PL mass and fatty acid composition.* Because the mass fraction of PC was greater in mice fed CRO than in mice fed SO, regressions were performed to determine if specific fatty acids were associated with the increased mass of PC. All mice, regardless of dietary group, were included in the regressions. The mass fraction of PC in heart was significantly and positively correlated with the mol% of 22:6n-3 found in PC. Figure 1A shows a positive relationship between the amount of 22:6n-3 in the PC of heart and the mass frac-

**TABLE 4**
**Percentage Difference[a] in the 22:6n-3 Content Between Heart and Liver Phospholipids from Mice Fed SO or CRO**

| | Dietary oil (%) | |
|---|---|---|
| | SO | CRO |
| Total phospholipids | +141 | +138 |
| Phosphatidylcholine | +189 | +202 |
| Phosphatidylethanolamine | +114 | +108 |
| Cardiolipin | −11 | +104 |

[a]Data were calculated using the equation [(mol% 22:6n-3 in heart/mol% 22:6n-3 in liver) − 1] and expressed as a percentage difference. See Table 2 for abbreviations.

**TABLE 5**
**Percentage Difference[a] in the 22:6n-3 Content of Phospholipids Between Mice Fed CRO and Mice Fed SO**

| | Heart (%) | Liver (%) |
|---|---|---|
| Total phospholipids | +38 | +40 |
| Phosphatidylcholine | +63 | +56 |
| Phosphatidylethanolamine | +28 | +32 |
| Cardiolipin | +318 | +82 |

[a]Data were calculated using the equation: [(mol% 22:6n-3 in mice fed CRO/mol% 22:6n-3 in mice fed SO) − 1] and expressed as a percentage difference. See Table 2 for abbreviations.

**TABLE 6**
**Lipid Class Percentage Composition of Heart and Liver from Mice Fed SO or CRO**

| | Heart | | Liver | |
|---|---|---|---|---|
| | SO | CRO | SO | CRO |
| Lipid classes[a] | | | | |
| Total phospholipids (PL) | 0.80 ± 0.09 | 0.81 ± 0.02 | 0.81 ± 0.11 | 0.75 ± 0.16 |
| Free fatty acids | 0.10 ± 0.02 | 0.08 ± 0.02 | 0.06 ± 0.02 | 0.08 ± 0.04 |
| Triacylglycerides | 0.10 ± 0.08 | 0.10 ± 0.03 | 0.12 ± 0.11 | 0.15 ± 0.11 |
| Cholesterol esters | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.01 | 0.02 ± 0.01 |
| PL classes[b] | | | | |
| Phosphatidylcholine | 0.38 ± 0.09 | 0.61 ± 0.09[c] | 0.40 ± 0.06 | 0.36 ± 0.04 |
| Phosphatidylethanolamine[d] | 0.50 ± 0.13 | 0.24 ± 0.12[c] | 0.53 ± 0.08 | 0.57 ± 0.06 |
| Cardiolipin | 0.14 ± 0.04 | 0.15 ± 0.04 | 0.06 ± 0.03 | 0.06 ± 0.02 |

[a]Data were calculated by dividing the mass of each lipid class by the total lipid mass of the tissue and expressed as an average percentage ± SD. Results indicate the average of four independent analyses of CRO-fed mice and seven independent analyses of SO-fed mice. See Table 2 for abbreviations.
[b]Data were calculated by dividing the mass of each PL class by the total PL mass of the tissue and expressed as an average percentage ± SD.
[c]Significantly different from data for mice fed SO.
[d]The mass of phosphatidylethanolamine contains the mass of other minor PL classes including phosphatidylserine and sphingomyelin.

tion of PC in heart. Ten other fatty acids (14:0, 16:0, 18:2n-6, 18:3n-3, 20:0, 20:2n-6, 20:3n-6, 20:4n-6, 22:0, and 24:1n-9) were significantly but negatively correlated with the mass fraction of PC in heart. Because these fatty acids were negatively correlated with heart PC content, they were likely co-variables of the incorporation of 22:6n-3 into PC. Indeed, the increase in 22:6n-3 mass observed in all mice accounted for 96% of the combined mass decrements in the other responsive fatty acids. A linear regression of the sum of the fatty acids negatively correlated with the PC content of heart, in mol%, plotted against the mol% of 22:6n-3 in the PC of heart produced a line with a slope of −1.04 and an $r^2$ of 0.96 (data not shown). This strongly indicated co-variation and that 22:6n-3, not the negatively correlated fatty acids, was linked to the greater PC content of heart. No fatty acid was significantly related to the mass fraction of any other lipid in heart. No fatty acid was significantly related to the mass fraction of any lipid in liver tissue. Figure 1B shows the absence of a relationship between the mol% of 22:6n-3 in the PC of liver and the mass fraction of PC in liver.

*Distribution of fatty acids among lipid classes*. Hearts from SO-fed mice contained 7.24 µg of 22:6n-3 per gram, whereas CRO-fed mice had 9.77 µg 22:6n-3 per gram of heart. The distribution of the mass of 22:6n-3 among lipid classes in heart and liver is shown in Table 7. Heart from mice fed SO contained only 0.19 µg of 18:3n-3 and less than 0.01 µg of 20:5n-3 per gram despite the fact that the mice consumed a diet containing 7.4 mol% 18:3n-3. Mice fed CRO had 0.03 µg of 18:3n-3 and 0.08 µg of 20:5n-3 per gram of heart, demonstrating a clear selection against uptake and/or

**TABLE 7**
**Fraction of Total 22:6n-3 Present in Each Lipid Class from Mice Fed SO or CRO**

| | Heart | | Liver | |
|---|---|---|---|---|
| | SO | CRO | SO | CRO |
| Lipid classes[a] | | | | |
| Total PL | 0.98 ± 0.01 | 0.98 ± 0.01 | 0.95 ± 0.02 | 0.93 ± 0.05 |
| Free fatty acids | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.04 ± 0.01 | 0.04 ± 0.03 |
| Triacylglycerides | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.01 | 0.02 ± 0.03 |
| Cholesterol esters | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| PL classes[b] | | | | |
| Phosphatidylcholine | 0.34 ± 0.07 | 0.60 ± 0.11[c] | 0.24 ± 0.06 | 0.24 ± 0.03 |
| Phosphatidylethanolamine[d] | 0.62 ± 0.06 | 0.30 ± 0.15[c] | 0.72 ± 0.09 | 0.72 ± 0.05 |
| Cardiolipin | 0.04 ± 0.04 | 0.10 ± 0.04[c] | 0.04 ± 0.04 | 0.04 ± 0.02 |

[a]Data were calculated by dividing the mass of 22:6n-3 in each lipid class by the total mass of 22:6n-3 in the tissue and expressed as an average percentage ± SD. Results indicate the average of four independent analyses of CRO-fed mice and seven independent analyses of SO-fed mice.
[b]Data were calculated by dividing the mass of 22:6n-3 in each PL class by the mass of 22:6n-3 in the total PL fraction of the tissue and expressed as an average percentage ± SD.
[c]Significantly different from data for mice fed SO.
[d]The mass of phosphatidylethanolamine contains the mass other minor PL classes including phosphatidylserine and sphingomyelin. See Tables 2 and 6 for abbreviations.

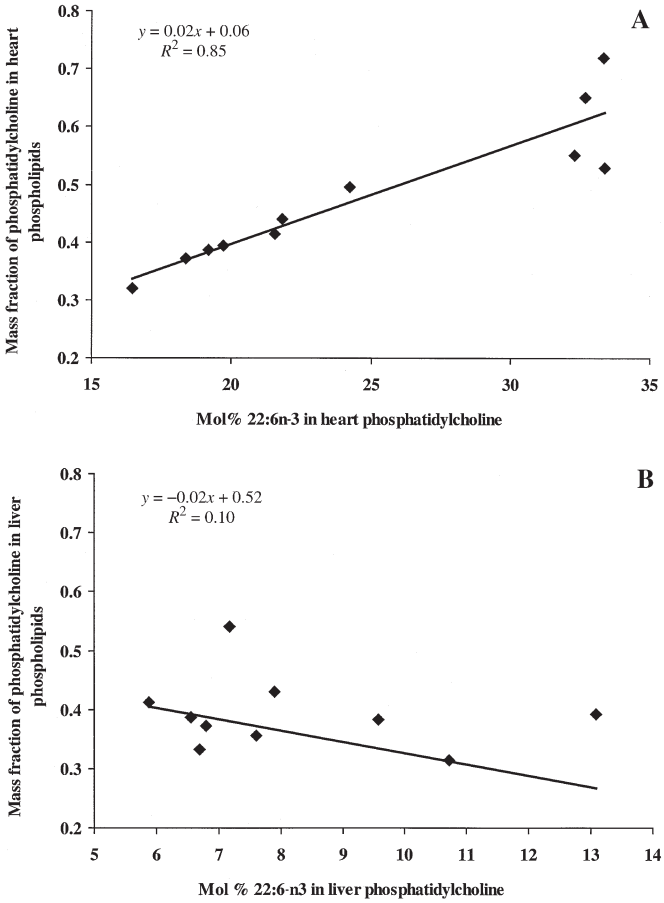


**FIG. 1.** (A) Relationship between 22:6n-3 and the mass of phosphatidylcholine in heart. The mass of phosphatidylcholine is expressed as a mass fraction of total phospholipids. Mass fractions were calculated for each mouse by dividing the mass of phosphatidylcholine per gram of heart by the mass of total phospholipids per gram of heart. The mass fraction of phosphatidylcholine was significantly and positively correlated with the 22:6n-3 content of phosphatidylcholine. (B) Relationship between 22:6n-3 and the mass of phosphatidylcholine in liver. The mass of phosphatidylcholine is expressed as a mass fraction of total phospholipids. Mass fractions were calculated for each mouse by dividing the mass of phosphatidylcholine per gram of liver by the mass of total phospholipids per gram of liver. Liver phosphatidylcholine content did not correlate with 22:6n-3 content.

retention of these fatty acids by the heart. The increased contribution of 22:6n-3 in the PC of mice fed CRO to the total 22:6n-3 in heart was directly proportional to an increase in the mass of PC (Tables 6 and 7). Likewise, the contribution of 22:6n-3 in PE to the total 22:6n-3 in heart was decreased proportionally with the decreased mass of PE in heart from mice fed CRO. However, 22:6n-3 from CL, which accumulated 22:6n-3 only when 22:6n-3 was fed intact, contributed a larger fraction of the total 22:6n-3 in heart from mice fed CRO without a proportional increase in the mass of CL.

## DISCUSSION

We applied a comprehensive lipid analysis technique encompassing both mass and compositional analyses to elucidate metabolic processes occurring in heart from mice fed 18:3n-3 or 22:6n-3. Whereas the retention of 22:6n-3 by all tissues may be due to the fact that 22:6n-3 is an extremely poor substrate for β-oxidation (28), the preferential accumulation of 22:6n-3 by heart remains unexplained. The basis for the preferential incorporation of 22:6n-3 into heart may be its accumulation in PL that are especially prevalent in heart. Several investigators have reported the rapid incorporation of 22:6n-3 into the CL of heart upon feeding 22:6n-3 or dietary oils containing 22:6n-3 (12,19,29). In the present study, heart from mice fed SO or CRO accumulated significantly more 22:6n-3 than did liver. However, CL did not accumulate more 22:6n-3 than other PL classes, and the mass of CL per gram of heart did not change in response to diet. Heart from mice fed SO and CRO contained 3.1 and 4.3 μg more 22:6n-3 per gram, respectively, than liver. Mass analysis demonstrated that 22:6n-3 in the CL of heart accounted for only 4 or 17 wt% of this difference in mice fed SO or CRO, respectively. Therefore, incorporation of 22:6n-3 into CL cannot quantitatively account for the preference of heart for 22:6n-3. Rather, 22:6n-3 accumulated preferentially in each of the PL classes of heart relative to liver. Thus, an explanation for the selective accumulation of 22:6n-3 in the heart cannot be based on the argument that heart is a CL-rich tissue.

With the notable exception of CL, the PL in heart were enriched with metabolic products of 18:3n-3 in mice fed SO. The abundance of 22:6n-3 in the hearts of animals fed SO suggested that either 18:3n-3 was converted to 22:6n-3 within the heart itself or that the heart preferentially accumulated fatty acids from a plasma lipid pool rich in 22:6n-3. It is not clear whether heart is capable of desaturating/elongating 18:3n-3 to 22:6n-3. However, production of 22:6n-3 from 18:3n-3 in heart could explain both why 22:6n-3 accumulates preferentially in heart and why 22:6n-3 accumulates in CL only when the dietary source of n-3 fatty acid is 22:6n-3.

When 22:6n-3 enters a cell intact, it is available for acylation into phosphatidic acid, a precursor to all PL, or for acylation into lyso-PL. Therefore, it is available for the biosynthesis or remodeling of all PL classes. However, when the n-3 fatty acid entering a cell is a precursor to 22:6n-3, there are two possible metabolic fates for the fatty acid apart from catabolism. The fatty acid is either acylated into glycerolipids or metabolized *via* elongation/desaturation to 22:6n-3. The data demonstrate that 18:3n-3 and 20:5n-3 are not accumulated by any glycerolipid in heart, but that mice fed 18:3n-3 had a substantial quantity of 22:6n-3 in the total PL of heart. The final step in the conversion of 18:3n-3 or any other n-3 fatty acid to 22:6n-3 requires a two-carbon chain-shortening reaction that takes place in the peroxisome (30–32). When the product of peroxisomal chain shortening contains a delta-four unsaturated bond, the fatty acid is preferentially moved to the endoplasmic reticulum for acylation into lyso-PL (32–34). Therefore, any 22:6n-3 produced from n-3 precursor by a cell is preferentially incorporated into PL that have synthesis or remodeling pathways present at the endoplasmic reticulum. If this metabolic pathway is active in heart, it would explain

the absence of an accumulation of 22:6n-3 in CL from mice fed 18:3n-3 because CL and the enzymes required for its synthesis and remodeling are present only in the inner-mitochondrial membrane (35–37). The conversion of 18:3n-3 to 22:6n-3 within the heart would also explain the preferential accumulation of 22:6n-3 by the heart.

In investigating the accumulation of 22:6n-3 by PL classes in heart, it was observed that the mass of 22:6n-3 in PC accounted for 26% more of the total mass of 22:6n-3 in heart in mice fed CRO than in mice fed SO (Table 7). The increased contribution of 22:6n-3 from PC to the total amount of 22:6n-3 in heart resulted from an increased mass of PC per gram of total PL. Regression and correlation analyses demonstrated that the mass of PC in heart was significantly and positively associated with the amount of 22:6n-3 in PC (Fig. 1A). This relationship remained significant even when mice fed CRO were removed from the regression, suggesting that dietary 18:3n-3 was also capable of increasing heart PC content. No relationship was found for liver PC content and 22:6n-3 (Fig. 1B). The data suggest that 22:6n-3 induced an increase in the PC content of heart. Because the increased mass of PC was not accompanied by an increase in the mass of total PL (Table 6), the mechanism for increasing PC was likely to be increased conversion from PE *via* phosphatidylethanolamine-methyl transferase (PEMT) activity (38), rather than *de novo* synthesis *via* the Kennedy pathway (39). Quantitative measurements of lipid and PL support this hypothesis (Table 6). Previously, PEMT activity was thought to contribute significantly to PL metabolism only in the liver, with nonliver organs expressing less than 2% of liver PEMT activity (38). However, the present study suggested that heart PEMT activity was induced by dietary n-3 fatty acids and that 22:6n-3 was responsible for this induction in heart. There is some precedent for an increase in PEMT activity in response to dietary lipid, as brain synapses and liver microsomes (40) contained increased PEMT activity in rats fed polyunsaturated fatty acids and coconut oil, respectively. There is also evidence for increased di-saturated PC synthesis in lung in response to 22:6n-3 (41,42). However, an increase in the activity of CTP:phosphocholine cytidylyltransferase, and therefore an increase in CDP-choline mediated PC synthesis, was responsible for these observations.

Insignificance of extrahepatic PEMT activity was based on a view toward evaluating the ability of PEMT to maintain whole-body PC content during choline deficiency. However, PEMT may serve a different and specific function in the heart. In light of the current findings, it is interesting to note that data from isolated hepatocytes indicated that only newly synthesized PE was a substrate for PC synthesis *via* PEMT (43) and that 16:0-22:6 PE was PEMT's preferred substrate (44).

The present study was designed to couple mass and compositional data to make metabolic inference about heart tissue n-3 fatty acid metabolism. Fatty acid composition and quantitative PL mass measurements suggested that 22:6n-3 induced PEMT activity in the heart. Further, the data suggested that dietary 18:3n-3 was capable of inducing this activity, presumably because heart rapidly accumulated 22:6n-3 in response to dietary 18:3n-3. The induction of PEMT in heart by 22:6n-3 or 18:3n-3 may be partially responsible for the beneficial effects of n-3 fatty acids on heart function. In contrast to the induction of PEMT activity, dietary 22:6n-3 was required for the enrichment of CL with 22:6n-3. The difference between the metabolic fates of various n-3 fatty acids in heart is important to nutrition and health and should be investigated. CL containing 22:6n-3 has been shown to induce a decrease in mitochondrial oxygen consumption (29) and an increase in cellular oxidant production (19). Based on the findings of this study, the selective accumulation of 22:6n-3 by heart may be linked to the conversion of n-3 precursors to 22:6n-3 within the heart itself. An increased mass of 22:6n-3-rich PC also partially accounts for this phenomenon.

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# 1-$^{14}$C-Linoleic Acid Distribution in Various Tissue Lipids of Guinea Pigs Following an Oral Dose

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**ABSTRACT:** A recent study on the metabolism of 1-$^{14}$C-α-linolenic acid in the guinea pig revealed that the fur had the highest specific activity of all tissues examined, 48 h after dosing. The present study investigated the pattern of tissue lipid labeling following an oral dose of 1-$^{14}$C-linoleic acid after the animals had been dosed for the same time as above. Guinea pigs were fed one of two diets with a constant linoleic acid content (18% total fatty acids) and a different content of α-linolenic acid (0.3 or 17.3%) from weaning for 3 wk and 1-$^{14}$C-linoleic acid was given orally to each animal for 48 h prior to sacrifice. The most highly labeled tissues (dpm/mg of linoleic acid) were liver, followed by brain, lung and spleen, heart, kidney and adrenal and intestines, in both diet groups. The liver had almost a threefold higher specific activity than skin and fur which was more extensively labeled than the adipose and carcass. Approximately two-thirds of the label in skin plus fur was found in the fur which, because of a low lipid mass, would indicate that the fur was highly labeled. All tissues derived from animals on the diet with the low α-linolenic acid level were significantly more labeled than the tissues from the animals on the high α-linolenic acid diet, by a factor of 1.5 to 3. The phospholipid fraction was the most highly labeled fraction in the liver, free fatty acids were the most labeled fraction in skin & fur, while triacyglycerols were the most labeled in the carcass and adipose tissue. In these tissues, more than 90% of the radioactivity was found in fatty acids with 2-double bonds in the tissue lipids. These data indicate that the majority of label found in guinea pig tissues 48 h after dosing was still associated with a fatty acid fraction with 2-double bonds, which suggests there was little metabolism of linoleic acid to more highly unsaturated fatty acids in this time frame. In this study, the labeling of guinea pig tissues with linoleic acid, 48 h after dosing, was quite different from the labeling with α-linolenic acid reported previously. The retention of the administered radioactivity from $^{14}$C-linoleic acid in the whole body lipids was 1.6 times higher in the group fed the low α-linolenic acid diet (diet contained a total of 1.8 g PUFA/100 g diet) compared with the group fed the high α-linolenic acid diet (diet contained 3.6 g PUFA /100 g diet). The lack of retention of $^{14}$C-labeled lipids in the whole body would be consistent with an increased rate of β-oxidation of the labeled fatty acid on the diet rich in PUFA, a result supported by other studies using direct measurement of labeled carbon dioxide.

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The essential fatty acids (EFA) linoleic and α-linolenic acid (18:3n-3) were discovered in 1930 (1). It is now known that each EFA is the parent member of a family of fatty acids (n-6 family and n-3 family) that have different biological functions. For the n-6 family, linoleic acid prevents water loss through the skin (2), while arachidonic acid is the precursor of all the main eicosanoids. In the case of the n-3 family, eicosapentaenoic acid and docosahexaenoic acid (DHA) are effective in reducing plasma triacylglycerol levels (3) while DHA plays an important role in membranes in excitable tissues such as the brain, retina and heart (4–6).

The classical symptoms of EFA deficiency in mammals are alterations in skin and fur integrity, including scaly skin, dandruff, and hair loss in rats (7). Other studies have shown that linoleic acid plays an important role in skin in preventing water loss and in maintaining epidermal integrity (2,8). Linoleic acid is included in commercial dog foods to maintain "shiny coats" (9). The effects of EFA deficiency on the skin and fur of other species, including man, have been widely reported as reviewed by Holman (7).

The effect of α-linolenic acid deficiency on mammals is less obvious than combined EFA deficiency, with generally subtle effects being reported in various species on the electroretinogram (5) and other membrane-related events in tissues such as the brain and heart (4,10,11). Some studies have indicated that α-linolenic acid might also play a role in skin and fur (12–14).

We have recently reported that guinea pig skin contains a substantial quantity of α-linolenic acid (15), an observation that supports data also reported recently in rats (16,17). Furthermore, we showed that skin & fur of guinea pigs were extensively labeled following oral dosing with 1-$^{14}$C-linolenic acid. It was speculated that in the guinea pig α-linolenic acid may have a function in relation to fur, perhaps as a secreted lipid from sebaceous glands to protect the fur from damage by water, light or other agents.

Because the extensive labeling of fur with 1-$^{14}$C-α-linolenic acid was unexpected, the present study investigated the distribution of radioactivity in all tissue lipids following an oral dose of 1-$^{14}$C-linoleic acid in guinea pigs using the same single time point (48 h).

## MATERIALS AND METHODS

The experimental protocol was approved by the Animal Ethics Committee of RMIT University, Melbourne, Australia. Male pigmented guinea pigs, 4 wk old, were purchased from Monash University in Melbourne, Australia. After having been fed on a guinea pig chow diet for 10 d, they were randomly divided into two groups ($n$ = 4/group), fed with one of two semisynthetic diets mixed with normal chow for 1 wk, and then fed with the semisynthetic diet only for the next 3 wk. The major ingredients, including the vitamin and mineral composition, have been reported previously (18). The fat, pro-

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Abbreviations: 18:3n-3, α-linolenic acid; DHA, docosahexaenoic acid; EFA, essential fatty acids; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

tein, and carbohydrate content of the diets were 10, 30, and 47% (by weight), respectively, with the remainder being made up of vitamins, minerals, and fiber. The oils used in the diet consisted of mixtures of coconut, linseed, palm stearin, safflower, sunola, and canola oils in order to provide two diets, each with a constant proportion of linoleic acid and variable α-linolenic acid content. The fatty acid compositions of these two different diets are shown below. For the high α-linolenic acid diet, the percentages of the following fatty acids were determined (in duplicate, as described below): caprylic + myristic 10.9%, palmitic 13.7%, stearic 4.3%, oleic 35.3%, linoleic 18.2%, and α-linolenic 17.3%. For the low α-linolenic acid diet, the percentages of the following fatty acids were determined: caprylic + myristic 11.4%, palmitic 31.7%, stearic 4.1%, oleic 34.5%, linoleic 17.5%, and α-linolenic 0.3%. The ratio of 18:2n-6/18:3n-3 was 1.05 and 58 in the high and low α-linolenic acid diets, respectively. After 19 days on the diet, each guinea pig was given an oral dose of 1.95 μCi of 9,12-[1-$^{14}$C-]linoleic acid (NEN Life Sciences Product Inc., Boston, MA; specific activity 1.961 gbq/mmol) mixed in 0.4 mL of olive oil; the dose was given using a 1-mL syringe fitted with a blunt, wide-gauge needle and while the oil was dripped into their mouths, the guinea pigs were tightly held in a vertical position. The guinea pigs were then returned to their cages for 48 h, where they had normal access to food and water.

At the conclusion of the study, the guinea pigs were asphyxiated in $CO_2$ gas, the head was severed from the body, and then the skin and fur were removed from the skull and carcass of the body, respectively. About 2–5 g of skin was cut into slices for lipid analysis. Part of the fur from the carcass was shaved for lipid analysis. The other tissues were collected, washed free from blood in ice-cold normal saline, and dried with blotting paper; the total weight of the organs was recorded, and then the tissues were stored at −20°C. The stomach and intestines were opened and washed free of digesta/feces and stored as above. Lipids were extracted from approximately 2 g of each tissue using chloroform/methanol as described previously (18). One aliquot of each tissue lipid was counted in a scintillation counter, another aliquot was converted to fatty acid methyl esters for determination of the tissue fatty acid content by capillary gas–liquid chromatography using 23:0 as an internal standard on a BPX-70 column (SGE, Melbourne, Australia) (15), and a third aliquot was subjected to thin-layer chromatography (TLC) on silica gel G to separate the neutral lipids into fractions, which were then scraped into vials and counted in a scintillation counter. Fatty acid methyl esters from some tissues were examined by silver nitrate TLC to separate trienoic from dienoic and tetraenoic fractions with petroleum ether/diethyl ether (40:60, vol/vol). Part of the fur was shaved from the carcass for further radioactivity counting. The lipids on the surface of the fur were extracted separately from the lipids contained within the hairs based on a procedure devised for wool fiber (19). Briefly the surface lipids were extracted by refluxing with *t*-butanol for 1 h and then with *n*-heptane for another hour. The extracts were combined and considered to be the surface lipids (19). For the internal fur lipids, the lipids were extracted overnight at 4°C from the residue of the above treatment with chloroform/methanol (1:1, vol/vol).

## RESULTS

The weights of the animals after the 3-wk feeding study were 587 ± 55 and 570 ± 55 g (mean ± SD) in the high and low α-linolenic acid diet groups, respectively. The animals appeared healthy in each group.

The tissue n-6 and n-3 fatty acid contents (mg/total weight of tissue) and the fatty acid composition (%) in tissue data have been published previously (18). The proportional distribution of individual fatty acids in each tissue as a percentage of whole body fatty acid content (sum of fatty acids in all tissues) is shown in Table 1. In both diet groups, the major sites for linoleic acid, α-linolenic acid, and arachidonic acid accumulation were adipose, carcass (muscle plus bone), and skin & fur, with approximately 90% of these PUFA in the whole body being found in these tissue sites. In the case of DHA, the brain and carcass were the major sites of deposition in both groups, and adipose was also a site of DHA accumulation in the high α-linolenate group.

Table 2 shows the $^{14}$C-label per total tissue (dpm/total weight of tissue) in each diet group. All tissue lipids derived from animals on the diet with low α-linolenic acid level contained more $^{14}$C-label than the tissues from animals on the high α-linolenic acid. The total of $^{14}$C-label found in the whole body was $1.7 \times 10^6$ dpm on the high α-linolenic acid diet and $2.7 \times 10^6$ dpm on the low α-linolenic acid diet. For both diet groups, the most highly labeled tissues were carcass, followed by adipose, skin & fur, liver, stomach and intestine, and kidney plus adrenal. When the data were expressed as a specific activity (dpm/mg linoleic acid), the most highly labeled tissues in the high and low α-linolenic acid diets were (in descending order): liver, brain, lung and spleen, hearts, guts, and skin & fur (head). For both diet groups the liver had a higher specific activity than the skin & fur by approximately four- to sevenfold. There was a significantly higher specific activity for all tissues on the low α-linolenic acid diet compared with the high α-linolenic acid diet group.

The mean recovery of the oral dose in the body as carbon $^{14}$C-labeled lipids for the high-α-linolenic acid diet group was 39%, which was significantly lower than the recovery for the animals in the low α-linolenic acid diet group of 62% ($P < 0.05$). In both diet groups, the phospholipids were the most labeled fraction in the liver (approximately 90%), while the label was mostly in the triacylglycerol fraction in adipose tissue and carcass (>95%), and free fatty acids were the most labeled fraction in skin & fur of the carcass and the head (>66%) (Table 3). For both diet groups, more than 92% of the label in the fatty acids separated by silver nitrate TLC was found in the 2-double bond fraction in the liver, adipose, carcass, and skin & fur of the head & carcass (data not shown).

Of the combined counts in the skin and fur, more than 65% of the counts were in the fur compared with the skin lipids.

**TABLE 1**
**Proportional Distribution of Major Polyunsaturated Fatty Acids in Carcass, Adipose, Skin & Fur, Liver and Brain (% of whole body fatty acid content)**

| Tissue | Dietary 18:3n-3 content[a] | 18:2n-6 | 20:4n-6 | 18:3n-3 | 20:5n-3 | 22:6n-3 |
|---|---|---|---|---|---|---|
| Carcass (muscle + bone) | High | 37[b,c] ± 1 | 47 ± 2 | 33 ± 0 | 44 ± 5 | 47 ± 4 |
| | Low | 39 ± 3 | 56 ± 6 | 47 ± 3 | ND | 62 ± 3 |
| Adipose | High | 46 ± 2 | 16 ± 1 | 54 ± 1 | 25 ± 5 | 10 ± 2 |
| | Low | 39 ± 2 | 17 ± 2 | 34 ± 1 | <0.1 | ND |
| Skin + fur, head | High | 3 ± 0 | 5 ± 1 | 2 ± 0 | 8 ± 2 | 3 ± 1 |
| | Low | 3 ± 1 | 3 ± 1 | 3 ± 1 | 24 ± 1 | 3 ± 1 |
| Skin + fur, carcass | High | 10 ± 1 | 7 ± 1 | 7 ± 1 | 10 ± 1 | 5 ± 1 |
| | Low | 15 ± 2 | 10 ± 2 | 15 ± 2 | 68 ± 1 | 9 ± 2 |
| Liver | High | 1 ± 0 | 6 ± 0 | 1 ± 0 | 6 ± 2 | 6 ± 0 |
| | Low | 2 ± 0 | 4 ± 1 | 0.4 ± 0 | ND | 3 ± 1 |
| Brain | High | <0.1 | 4 ± 0 | ND | 1 ± 0 | 25 ± 2 |
| | Low | <0.1 | 3 ± 1 | ND | 3 ± 1 | 23 ± 2 |

[a]High 18:3n-3 diet contained 1.73 g 18:3n-3 per 100 g diet, whereas the low 18:3n-3 diet had 0.03 g/100 g of 18:3n-3.
[b]Represents the mass of linoleic acid in the carcass relative to the mass of linoleic acid in the whole body, expressed as a percentage. The whole body value was obtained by summing the values for each tissue.
[c]Results are shown as mean ± SD, $n = 4$ per diet group. ND, not detected (<0.05% fatty acids).

About 80% of the counts was recovered in the surface lipids of the fur. The $^{14}C$ distribution in the surface and internal fur was not different between the two diet groups. In both diet groups, more than 95% of the counts was in the free fatty acid fraction for the surface fur lipids, and more than 92% of all the counts was in the 2-double bond fraction.

## DISCUSSION

A previous study in guinea pigs reported that more than 47% of the administered dose of $^{14}C$-α-linolenic acid was found in the skin and fur 48 h after an oral dose (15). The aim of the present study was to examine the distribution of $^{14}C$-labeled-

**TABLE 2**
**The $^{14}C$-Label in Total Tissue Lipids (dpm/tissue) and the Specific Activity of Linoleic Acid (dpm/mg linoleic acid) Following an Oral Dose of $^{14}C$-Labeled Linoleic Acid**

| Tissue | $^{14}C$-Label in tissue lipids (dpm/total tissue weight) × $10^3$ | | Specific activity (dpm/mg linoleic acid) | |
|---|---|---|---|---|
| | High 18:3n-3 diet[a] | Low 18:3n-3 diet[a] | High 18:3n-3 diet | Low 18:3n-3 diet |
| Carcass (260)[c] | 644 ± 133[b] | 783 ± 121 | 66 ± 17 | 94 ± 15[d] |
| Adipose (26) | 373 ± 58 | 732 ± 42 | 31 ± 4 | 104 ± 17 |
| Skin + fur, carcass (99) | 255 ± 48 | 395 ± 19 | 95 ± 17 | 126 ± 43 |
| Skin + fur, head (26) | 136 ± 46 | 225 ± 24 | 188 ± 51 | 341 ± 142 |
| Liver (18) | 157 ± 13 | 274 ± 18 | 447 ± 58 | 1,058 ± 245 |
| Stomach + intestine (29) | 96 ± 3 | 115 ± 39 | 208 ± 88 | 340 ± 88 |
| Kidney + adrenal (4) | 20 ± 1 | 31 ± 5 | 236 ± 32 | 347 ± 96 |
| Lung + spleen (5) | 9 ± 0.6 | 14 ± 3 | 253 ± 69 | 544 ± 192 |
| Heart (2) | 6 ± 0.7 | 8 ± 0.5 | 205 ± 53 | 432 ± 12 |
| Brain (4) | 2 ± 0.8 | 3 ± 0.7 | 431 ± 119 | 696 ± 204 |

[a]High 18:3n-3 diet contained 1.73 g 18:3n-3 per 100 g diet, whereas the low 18:3n-3 diet had 0.03 g/100 g diet of 18:3n-3.
[b]Results are shown as mean ± SD, $n = 4$ per diet group.
[c]Mean tissue weight (g).
[d]The specific activity was significantly greater for each tissue ($P < 0.05$) on the low 18:3n-3 diet.

**TABLE 3**
**Tissue $^{14}$C Distribution (%) in Different Lipid Fractions in the High and Low 18:3n-3 Diet Groups**

| Tissue | Dietary 18:3n-3 content[a] | Cholesterol ester | Triacylglycerol | Free fatty acid | Cholesterol | Phospholipid |
|---|---|---|---|---|---|---|
| Liver | High | ND | 9 ± 1[b] | ND | ND | 91 ± 1 |
| | Low | ND | 9 ± 3 | 1 ± 0 | ND | 89 ± 3 |
| Adipose | High | ND | 99 ± 0 | ND | ND | 1 ± 0 |
| | Low | ND | 99 ± 0 | <0.2 | ND | <0.5 |
| Carcass | High | ND | 97 ± 1 | ND | ND | 3 ± 1 |
| | Low | ND | 95 ± 2 | ND | ND | 5 ± 2 |
| Skin + fur | High | 2 ± 1 | 20 ± 2 | 70 ± 1 | 3 ± 1 | 4 ± 0 |
| (carcass) | Low | 0.2 ± 0.1 | 17 ± 3 | 75 ± 4 | 4 ± 1 | 4 ± 2 |
| Skin + fur | High | 5 ± 1 | 18 ± 4 | 66 ± 2 | 7 ± 1 | 4 ± 1 |
| (head) | Low | 1 ± 0 | 19 ± 0 | 71 ± 1 | 6 ± 1 | 3 ± 0 |

[a]High 18:3n-3 diet contained 1.73 g 18:3n-3 per 100 g diet, whereas the low 18:3n-3 diet had 0.03 g/100 g of 18:3n-3.
[b]Results are shown as mean ± SD, $n$ = 4 per diet group.

linoleic acid throughout the body with a view to compare the results with the previous study using the same strain of guinea pigs maintained on semisynthetic diets for the same length of time and orally dosed with the $^{14}$C-label for the same time (48 h). As shown in Figure 1, the pattern of tissue lipid labeling with each of the EFA at this single time point was quite different. In the present linoleic acid study, the majority of the label was found in carcass and adipose lipids, with skin & fur, liver, and the intestinal lipids also being substantially labeled. The pattern of labeling of tissue lipids with linoleic acid was quite different from the former study using α-linolenic acid (15) (Fig.1), where skin and fur were the most labeled, followed by carcass, adipose, liver and intestines.

In the present experiment, the most highly labeled tissues in both the high and low α-linolenic acid diet groups (dpm/mg linoleic acid) were liver, brain, lung and spleen, stomach and intestine, and skin & fur (head). These data are quite different from our previous findings using $^{14}$C-labeled-α-linolenic acid, where the most highly labeled tissue (dpm/mg α-linolenic acid) was the skin & fur (head) by a factor of about four times over the next most highly labeled tissues (15). In the α-linolenic acid study, the order of labeling of the tissue in terms of specific activity was skin & fur of the head, liver, stomach and intestines, brain, and skin & fur of the carcass. In a study of guinea pigs given $^{14}$C-labeled linoleic acid intravenously for up to 60 min, the liver and lung were the most highly labeled tissues of 12 tissues examined (dpm/g tissue) (20). Skin and fur were not examined in that study.

Although the skin & fur were not the most highly labeled at 48 h after dosing with $^{14}$C-linoleic acid, the fur was still substantially labeled. The mass of lipid in the surface fur was insignificant, thus the specific activity of the surface fur would be much higher than the value for the combined skin & fur shown in Table 2 and perhaps the fur was one of the most highly labeled tissues.

While these comparative data suggest a substantial difference between the $^{14}$C-labeling of tissues following the oral dosing with linoleic acid (this study) and α-linolenic acid (15), it does not reveal the time course of tissue labeling since only one time point was examined. Zhou *et al.* (20) found there was a rapid loss of label from serum over 200 min, with the liver retaining up to 20% of the injected dose in the first 10 min. This report did not investigate whether skin & fur were labeled in this time nor did it provide a clue to the tissues likely to be labeled by 48 h.

Of the tissues examined (liver, carcass, adipose, and skin & fur), most of the radioactivity was found in the tissue fatty acids containing two double bonds (determined as methyl esters). This was surprising since it might be expected that in the time frame of the experiment some label would be detected in the four double bond fraction (as arachidonic acid), especially in the liver where most of the label was in the phospholipid fraction. A possible explanation is the reported low activity of the Δ-5 desaturase level in guinea pig liver compared with rat (21) and the fact that diets rich in linoleic acid inhibit the expression of the Δ-6 desaturase in liver (22). Others have noted the relatively low proportion of long-chain PUFA in guinea pig plasma, erythrocytes, liver, and heart by comparison with rat tissues (23,24) which is consistent with a low rate of desaturase activity. In contrast, in weanling rats fed an oral dose of $^{14}$C-linoleic acid, the proportion of the label associated with liver phospholipid arachidonic acid was 23% and that with linoleic acid 65%, 48 h after the dose (25). Zhou *et al.* (20) reported that at 60 min postlabeling most tissues showed less than 5% of the label as arachidonic acid, although with spleen and bone marrow the proportion was higher at 12–16% (the present study did not examine either of these two tissues separately). In most tissues, Zhou *et al.* (20) found more label in 20:3n-6 than in arachidonic acid in the short time frame of their experiment. It is unlikely that the techniques used in the two experiments could account for the different patterns of labeling of fatty acids. In the study of Zhou *et al.* (20), the fatty acids were separated by high-performance liquid chromatography, whereas in the present work the fatty acids (as methyl esters) were separated by silver nitrate TLC which clearly separated 3- and 4-double bond fatty acids from dienoic fatty acids.

It has been widely reported that EFA deficiency increases water loss through the skin and leads to the development of scaly skin, loss of hair, and a roughened hair coat in a number of different species (2,7). Although these effects are attributed to a lack of linoleic acid, some studies have indicated that α-linolenic acid might also play a role in skin and fur function

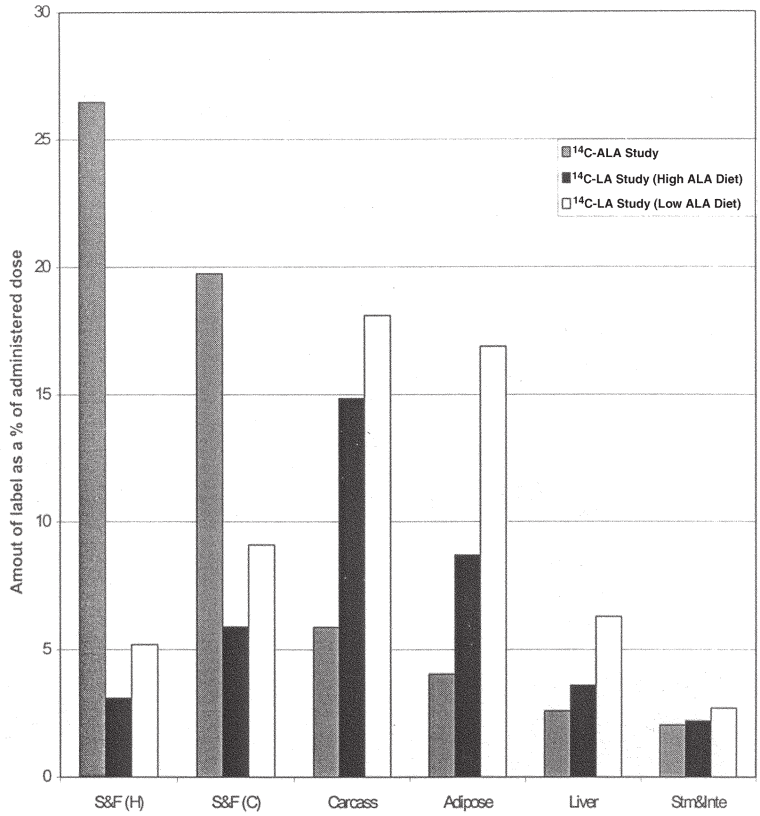


**FIG. 1.** The amount of label in tissue lipids as a percentage of administered dose. Comparison between the 1-$^{14}$C-α-linolenic acid (ALA) experiment (15) and the 1-$^{14}$C-linoleic acid experiment (high and low ALA diets). S&F (H) = skin & fur (head), S&F (C) = Skin & fur (carcass), and Stm& Inte = Stomach & Intestine.

(12–14). For example, an early study in rats showed that linseed oil contained a factor that promoted fur growth compared with a fat-deficient control group, and that α-linolenic acid was more effective than linoleic acid in restoring fur growth (12). A later study in capuchin monkeys reported skin lesions, fur loss, and abnormal behavior on a high linoleic acid, low α-linolenic acid diet, and a restoration of normal skin and fur appearance following the inclusion of linseed oil into the diet (13).

Based on the results in this experiment, it is possible to speculate that in the guinea pig, linoleic acid and α-linolenic acid may have a function in relation to fur, perhaps as a secreted lipid from sebaceous glands to protect the fur from damage by water, light, or other agents. This speculation is perhaps consistent with the use of linoleic acid in dogs to maintain their coats in good condition (9).

There was a substantially lower recovery of the $^{14}$C-label in the whole body tissue lipids in the high α-linolenic acid diet group compared with the low α-linolenic acid diet group by a factor of 0.7. In other words, approximately 40% of the label was lost as $CO_2$ or in feces in the low α-linolenic acid group compared with 60% lost in the high α-linolenic acid diet group. Pan and Storlien (26) reported that β-oxidation of α-linolenic acid increases with increasing levels of PUFA in rats. Assuming that this scenario applies in the guinea pig, could account for the increased loss (presumably by β-oxidation) in the high α-linolenic acid diet group since this group had nearly twice as much dietary PUFA compared with the low α-linolenic acid diet group (35 vs. 18% PUFA). It has been reported that in EFA-deficient rats fed 0.3% of energy as linoleic acid, less linoleic acid was oxidized to $CO_2$ compared with rats fed 10% of the energy as linoleic acid, and the retention of linoleic acid in the tissue of rats fed low EFA was greater than those fed high levels of linoleic acid (27,28).

In both diet groups, the major sites for linoleic acid, α-linolenic acid, and arachidonic acid accumulation were adipose, carcass, and skin & fur with approximately 90% of these PUFA in the whole body being found in these tissue sites. In this study, skin would include epidermis, dermis, and perhaps some subcutaneous fat. The carcass, as sampled in this study, includes muscle and bone; however, it is assumed that the majority of the lipids originated from muscle. Few other studies have examined all tissues as sites of deposition of PUFA. Cunnane and Anderson (16) examined all major tissues in young rats fed equal amounts of linoleic acid and α-linolenic acid and found that adipose, carcass, and skin were the main sites for linoleic acid and α-linolenic acid accumulation. Bowen and Clandinin (17) also report that skin is a major site of deposition of α-linolenic acid in young rats.

There were two main limitations to this study. The first was that we only took one time point (48 h) after the administration

of the oral dose of radioactively labeled fatty acid, which restricts the information regarding the turnover of the label in tissues. The aim of the study was to compare the labeling with that of the previous study using α-linolenic acid, under identical conditions. The second limitation of the study was that oral dosing of the animals may have led to some contamination of the fur from the oral dose. If contamination did occur, it would be necessary to speculate that more contamination occurred in the α-linolenic acid study than in the present study with linoleic acid, if indeed contamination was the source of the label on the fur. It should be noted that in both this and the previous study, we reported that skin contained high levels of the EFA, which may explain why this tissue becomes labeled. In order to establish if oral contamination contributes to the labeling of skin and fur, future studies will examine whether there is a similar labeling of skin and fur following either intravenous or intraperitoneal dosing of the animals.

In conclusion we report that following an oral dose of $^{14}$C-labeled-linoleic acid in guinea pigs, the skin and fur (combined) had a lower specific activity relative to other tissues, compared with previous data in guinea pigs fed with an oral dose of $^{14}$C-labeled-α-linolenic acid. Despite this, in the present experiment the fur was likely to have had a high specific activity. We also found that there was a higher recovery of the $^{14}$C-label in tissue lipids on the diet with the lowest PUFA level, suggesting a reduced β-oxidation of linoleic acid on this diet.

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# Effects of Diets Enriched in n-6 or n-3 Fatty Acids on Cholesterol Metabolism in Older Rats Chronically Fed a Cholesterol-Enriched Diet

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**ABSTRACT:** Hypocholesterolemic effects in older animals after long-term feeding are unknown. Therefore, aged rats (24 wk of age) fed a conventional diet were shifted to diets containing 10% perilla oil [PEO; oleic acid + linoleic acid + α-linolenic acid; n-6/n-3, 0.3; polyunsaturated fatty acid/saturated fatty acid (P/S), 9.6], borage oil [oleic acid + linoleic acid + α-linolenic acid; n-6/n-3, 15.1; P/S, 5.3], evening primrose oil (EPO; linoleic acid + γ-linolenic acid; P/S, 10.5), mixed oil (MIO; oleic acid + linoleic acid + γ-linolenic acid + α-linolenic acid; n-6/n-3, 1.7; P/S, 6.7), or palm oil (PLO; palmitic acid + oleic acid + linoleic acid; n-6/n-3, 25.3; P/S, 0.2) with 0.5% cholesterol for 15 wk in this experiment. There were no significant differences in the food intake and body weight gain among the groups. The liver weight in the PEO (n-6/n-3, 0.3) group was significantly higher than those of other groups in aged rats. The serum total cholesterol and very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) + low density lipoprotein (LDL)-cholesterol concentrations of the PLO (25.3) group were consistently higher than those in the other groups. The serum high density lipoprotein cholesterol concentrations of the PEO (0.3) and EPO groups were significantly lower than in the other groups at the end of the 15-wk feeding period. The liver cholesterol concentration of the PLO (25.3) group was significantly higher than those of other groups. There were no significant differences in the hepatic LDL receptor mRNA level among the groups. Hepatic apolipoprotein (apo) B mRNA levels were not affected by the experimental conditions. The fecal neutral steroid excretion of the PLO (25.3) group tended to be low compared to the other groups. The results of this study demonstrate that both n-6 fatty acid and n-3 fatty acids such as γ-linolenic acid and α-linolenic acid inhibit the increase of serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations of aged rats in the presence of excess cholesterol in the diet compared with dietary saturated fatty acid.

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Abbreviations: ALA 18:3n-3, α-linolenic acid; apo, apolipoprotein; BRO, borage oil; DG, diacylglycerol; DIG, digoxigenin; EPO, evening primrose oil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLA, γ-linolenic acid; GLC, gas–liquid chromatography; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LA 18:2n-6, linoleic acid; LDL, low density lipoprotein; MIO, mixed oil; PEO, perilla oil; PLO, palm oil; P/S, polyunsaturated fatty acid/saturated fatty acid; RT–PCR, reverse transcriptase–polymerase chain reaction; TAG, triacylglycerol; VLDL, very low density lipoprotein.

It has been reported that dietary high-linoleate safflower oil is not hypocholesterolemic in aged mice compared with perilla oil (PEO) and fish oil (n-3 fatty acids) after long-term feeding (1). However, Fukushima *et al.* (2) reported that evening primrose oil (EPO) containing about 71% linoleic acid (LA) and 9% γ-linolenic acid (GLA) has a hypocholesterolemic function compared with soybean oil containing about 53% LA and 8% α-linolenic acid (ALA) in cholesterol-fed rats after 13-wk of feeding, and that the effect of the diet could be due to the lesser amount of LA. Responses to marine lipids vary remarkably among different animal species. In the rat, dietary fish oil consistently lowers plasma cholesterol and triacylglycerol (TAG) concentrations. Although the cholesterol content of all lipoprotein fractions declines, the greatest reductions occur in lipoproteins that contain apolipoprotein (apo) B-100 or apo E (3). The fall in plasma low density lipoprotein (LDL) concentrations in rats fed fish oil is due primarily to an increase in receptor-dependent LDL uptake by the liver (3). However, there are almost no reports that ALA reduces lipoproteins containing apo B and apo E.

Numerous studies have shown that dietary polyunsaturated (P) and saturated (S) fatty acids can regulate plasma lipid and apo concentrations at the mRNA level in nonhuman primates (4–7). However, most such studies have been performed using relatively young animals. For aged rats, the data concerning the comparison between n-6 and n-3 fatty acids in this process are extremely limited, and the effects in the older animals after long-term feeding are unknown. The purpose of the present study was to investigate the effect of dietary n-6 and n-3 fatty acids in plant oils on cholesterol metabolism and the hepatic apo B and LDL receptor mRNA levels in aged rats (24 wk old) fed a cholesterol-enriched diet after long-term (15-wk) feeding.

## MATERIALS AND METHODS

*Animals and diets.* Male F344/DuCrj rats (24 wk old) were purchased from Charles River Japan Inc. (Yokohama, Japan). Twenty-five animals were housed individually in cages in a room with controlled temperature (23 ± 1°C), humidity (60 ± 5%), and light (12-h light/dark cycle). The composition of the

experimental diet was as follows (wt%): casein, 20; fat 10; cornstarch, 15; cellulose powder, 5; DL-methionine, 0.3; mineral mixture, 3.5 (AIN-76) (8); vitamin mixture, 1 (AIN-76) (8); choline bitartrate, 0.2; cholesterol, 0.5; sodium cholate, 0.125; and sucrose to 100. PEO (0.3), EPO, borage oil (BRO) (15.1), and palm oil (PLO) (25.3) were purchased from Sugiyama Yakuhin Co. (Nagoya, Japan), Summit Oil Co. (Chiba, Japan, licensed by Efamol Ltd.), Nippon Gohsei Chemical Industry Co. (Osaka, Japan), and Asahi Electric Industry Co. (Tokyo, Japan), respectively. Mixed oil (MIO) (1.7) was a mixture of PEO (0.3) and BRO (15.1) (1:2, vol/vol). The negative control group consisted of rats fed 100 g/kg PLO (25.3) containing large amounts of S.

Table 1 shows the lipid class and fatty acid compositions of the dietary fats. The rats (24 wk old) were divided into 5 groups of five animals each. They were allowed free access to experimental diets and water for 15 wk. To avoid autooxidation, each diet was stored at −20°C and freshly prepared each day. All animal procedures described conformed to recognized principles (9).

*Analytical procedures.* Blood samples (1 mL) were collected between 0800 and 1000 h from the jugular veins of fasting rats. The samples were taken into tubes without an anticoagulant. After the samples stood at room temperature for 2 h, serum was prepared by centrifugation at 1500 × *g* for 20 min. At the end of the experimental period of 15 wk, all fecal excretion during 2 d was collected. Fecal dry weights did not differ among the groups. The rats were killed by ether inhalation, and the livers quickly removed, washed with cold saline (9 g NaCl/L), blotted dry on filter paper, and weighed before freezing for storage.

*Chemical analysis.* Total cholesterol and high density lipoprotein (HDL)-cholesterol concentrations in the serum were determined enzymatically using commercially available reagent kits (assay kits for the TDX system; Abbott Laboratory Co., Irving, TX). The very low density lipoprotein (VLDL) + intermediate density lipoprotein (IDL) + LDL-cholesterol concentration was calculated as follows: VLDL + IDL + LDL-cholesterol = total cholesterol − HDL-cholesterol.

Total lipids were extracted from liver, and feces by a mixture of chloroform/methanol (2:1, vol/vol) (10). Neutral steroids in feces and liver obtained by saponification were acetylated (11) and analyzed by gas–liquid chromatography (GLC) with a DB 17 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA). Acidic steroids in feces were measured by GLC following the method of Grundy *et al.* (12).

*Reverse transcriptase–polymerase chain reaction (RT–PCR) and Southern blot analysis.* Total RNA was isolated by the acid guanidium–phenol–chloroform method, using Isogen (Nippon Gene, Tokyo, Japan) from liver (13). mRNA encoding apo B, LDL receptor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an invariant control; 14,15) were analyzed by semiquantitative RT–PCR and subsequent Southern hybridization of the PCR products with each inner oligonucleotide probe. Total RNA samples were treated with DNase RQ1 (Promega, Madison, WI) to remove genomic DNA and subjected to RT-PCR by using Moloney murine leukemia virus RT (Gibco, Gaithersburg, MD) and EX-Taq polymerase (Takara, Otsu, Japan) with apo B primers of oligonucleotides (upstream primer, 5′-GAAAGCATGCTGAAAACAACC-3′; downstream primer, 5′-AGGCCTGACTCGTGGAAGAA-3′), LDL receptor primers of oligonucleotides (upstream primer, 5′-ATTTTGGAGGATGAGAAGCAG-3′; downstream primer, 5′-CAGGGCGGGGAGGTGTGAGAA-3′), and GAPDH primers of oligonucleotides (upstream primer, 5′-GCCATCAACGACCCCTTCATT-3′; downstream primer, 5′-CGCCTGCTTCACCACCTTCTT-3′). The reaction mixtures for the PCR contained 25 pmol of each primer, 1.25 U EX-Taq polymerase, 1 × PCR buffer (Takara), and 200 μM dNTP in a 50-μL reaction volume. The expected sizes of DNA fragments amplified with these primers were 725 bp for apo B, 931 bp for the LDL receptor, and 702 bp for GAPDH. Temperature cycling was as follows: first cycle, denaturation at 95°C for 3 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Subsequent cycles were denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The thermal cycling was completed by terminal extension at 72°C for 10 min. In total, 25 cycles were performed for the apo B amplification, 35 cycles for the LDL receptor, and 20 cycles for GAPDH. Amplification products were electrophoresed on a 2% agarose gel and transferred to a nylon membrane (Biodyne B; Pall Bio-Support, East Hills, NY). Blots were hybridized with an apo B probe of a 54-base oligonucleotide (5′-TCCTTGCTTACCAAAAAGAGCTTCCAGTGTTGGCTCAAAGCCCTTTCCTTCTAA-3′), LDL receptor probe of a 54-base oligonucleotide (5′-GTGAACTTGGGTGAGTGGGCACTGATCTGAGGGGCAGGCAGGCACATGTACTGG-3′), and GAPDH probe of a 54-base oligonucleotide (5′-TGATGACCAGCTTCCCATTCTCAGCCTTGACTGTGCCGTTGAACTTGCCGTGGG-3′).

**TABLE 1**
**Lipid Class and Fatty Acid Composition (wt%) of Dietary Fats**

| | PEO | BRO | EPO | MIO[a] | PLO |
|---|---|---|---|---|---|
| Lipid class | | | | | |
| Triacylglycerol | 88 | 89 | 86 | 90 | 78 |
| Diacylglycerol | 3 | 4 | 4 | 5 | 5 |
| Sitosterol | 2 | 4 | 9 | 2 | 9 |
| Other component | 7 | 3 | 1 | 3 | 8 |
| Fatty acid | | | | | |
| 14:0 | — | 0.1 | — | 0.1 | 1.3 |
| 16:0 | 7.4 | 12.4 | 7.7 | 10.2 | 46.4 |
| 16:1n-7 | 0.6 | 0.5 | 0.4 | 0.4 | — |
| 18:0 | 1.8 | 3.5 | 2.0 | 2.9 | 3.5 |
| 18:1n-9 | 18.8 | 16.8 | 9.6 | 17.3 | 37.9 |
| 18:2n-6 | 14.3 | 38.2 | 71.1 | 28.8 | 10.1 |
| 18:3n-3 | 57.1 | 4.1 | — | 25.8 | 0.4 |
| 18:3n-6 | — | 24.2 | 9.2 | 14.5 | — |
| n-6/n-3[b] | 0.3 | 15.1 | — | 1.7 | 25.3 |
| P/S[c] | 9.6 | 5.3 | 10.5 | 6.7 | 0.2 |

[a]Mixed oil, perilla oil + borage oil (1:2, vol/vol).
[b]n-6/n-3 = (18:2n-6 + 18:3n-6)/(18:3n-3).
[c]P/S = (18:2n-6 + 18:3n-6 + 18:3n-3)/(14:0 + 16:0). PEO, perilla oil; BRO, borage oil; EPO, evening primrose oil; MIO, mixed oil; PLO, palm oil.

**TABLE 2**
**Body Weight Gain, Food Intake, and Liver Weight in Rats Fed Dietary Fats for 15 wk[a]**

| | Diet | | | | |
|---|---|---|---|---|---|
| | PEO | BRO | EPO | MIO | PLO |
| Body initial wt (g) | 340 ± 18 | 345 ± 9 | 344 ± 31 | 350 ± 14 | 344 ± 42 |
| Body wt gain (g/15 wk) | 67 ± 9 | 58 ± 8 | 74 ± 11 | 58 ± 19 | 74 ± 31 |
| Body final wt (g) | 407 ± 19 | 403 ± 16 | 418 ± 31 | 409 ± 27 | 419 ± 28 |
| Food intake (g/15 wk) | 1543 ± 65 | 1547 ± 75 | 1608 ± 121 | 1533 ± 118 | 1624 ± 14 |
| Liver wt (wet g/100 g body weight) | 4.1 ± 0.1[a] | 3.1 ± 0.2[d] | 3.4 ± 0.2[c] | 3.3 ± 0.2[c,d] | 3.8 ± 0.1[b] |

[a]Values are means ± standard deviations for five rats. Means within the same rows bearing different roman superscripts are significantly different ($P < 0.05$). See Table 1 for abbreviations.

The probe was 3′-tailing labeled with digoxigenin (DIG), using a DIG oligonucleotide tailing kit (Boehringer Mannheim, Mannheim, Germany). Prehybridization, hybridization, and detection were carried out with a DIG luminescent detection kit (Boehringer Mannheim) as recommended by the manufacturer. The relative quantity of mRNA was estimated by densitometric scanning with X-ray film.

*Statistical analysis.* Data are presented as means and standard deviations. The mean and standard deviations for serum total cholesterol, HDL-cholesterol, and VLDL + IDL + LDL-cholesterol for each time point were calculated. The significance of differences among treatment groups was determined by analysis of variance with Duncan's multiple-range test (SAS Institute, Cary, NC). Results were considered significant at $P < 0.05$.

## RESULTS

*Feed intake, rat growth, and liver weight.* The results are summarized in Table 2. There were no significant differences in the food intake and body weight gain among the groups. The liver weight in the PEO (0.3) group was significantly higher than those of the other groups.

*Tissue lipid concentration.* The serum total, VLDL + IDL + LDL-cholesterol, and HDL-cholesterol concentrations are shown in Table 3. The serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations of the PLO (25.3) group were consistently higher than those in the other groups. The serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations of all groups except for the MIO (1.7) group decreased between 8 and 15 wk. The serum HDL-cholesterol concentrations of the PEO (0.3) and EPO groups were significantly low compared with the PLO (25.3) group, at the end of the 15-wk feeding period.

Figure 1 illustrates the cholesterol concentrations in the livers of rats at the end of the 15-wk feeding period. The liver cholesterol concentration in the PLO (25.3) group was significantly higher than in the other groups, and that in the BRO (15.1) group was significantly lower than in the PEO (0.3), EPO, and PLO (25.3) groups.

**TABLE 3**
**Serum Total Cholesterol, VLDL + IDL + LDL-Cholesterol, and HDL-Cholesterol Concentrations in Rats Fed Dietary Fats for 15 wk[a]**

| Diet (mmol/L) | 0 Wk | 4 Wk | 8 Wk | 15 Wk |
|---|---|---|---|---|
| Total cholesterol | | | | |
| PEO | 2.11 ± 0.25 | 2.84 ± 0.18[c] | 5.22 ± 0.66[b] | 4.40 ± 0.34[b] |
| BRO | 2.11 ± 0.07 | 3.71 ± 0.21[b] | 4.86 ± 0.22[b] | 4.49 ± 0.67[b] |
| EPO | 1.95 ± 0.28 | 3.27 ± 0.41[b,c] | 4.92 ± 0.79[b] | 4.36 ± 0.60[b] |
| MIO | 1.97 ± 0.39 | 3.26 ± 0.52[b,c] | 4.17 ± 0.74[b] | 4.47 ± 1.27[b] |
| PLO | 2.13 ± 0.07 | 4.82 ± 0.58[a] | 8.09 ± 1.32[a] | 7.26 ± 3.61[a] |
| VLDL + IDL + LDL-cholesterol | | | | |
| PEO | 1.41 ± 0.22 | 2.18 ± 0.18[c] | 4.53 ± 0.68[b] | 3.57 ± 0.31[b] |
| BRO | 1.38 ± 0.05 | 2.91 ± 0.19[b] | 3.92 ± 0.21[b,c] | 3.54 ± 0.51[b] |
| EPO | 1.29 ± 0.26 | 2.51 ± 0.38[b,c] | 3.98 ± 0.69[b,c] | 3.45 ± 0.51[b] |
| MIO | 1.28 ± 0.28 | 2.50 ± 0.41[b,c] | 3.32 ± 0.62[c] | 3.42 ± 1.11[b] |
| PLO | 1.43 ± 0.10 | 4.08 ± 0.61[a] | 7.07 ± 1.32[a] | 6.08 ± 3.34[a] |
| HDL-cholesterol | | | | |
| PEO | 0.70 ± 0.06 | 0.65 ± 0.12[b] | 0.69 ± 0.10[c] | 0.83 ± 0.10[b] |
| BRO | 0.72 ± 0.03 | 0.80 ± 0.04[a] | 0.94 ± 0.03[a,b] | 0.95 ± 0.16[a,b] |
| EPO | 0.66 ± 0.07 | 0.77 ± 0.04[a,b] | 0.95 ± 0.11[a,b] | 0.91 ± 0.12[b] |
| MIO | 0.69 ± 0.11 | 0.77 ± 0.12[a,b] | 0.85 ± 0.12[b] | 1.05 ± 0.20[a,b] |
| PLO | 0.70 ± 0.06 | 0.74 ± 0.08[a,b] | 1.02 ± 0.10[a] | 1.19 ± 0.29[a] |

[a]Values are means ± standard deviations for five rats. Means within the same columns bearing different roman superscripts are significantly different ($P < 0.05$). VLDL, very low density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein. See Table 1 for other abbreviations.

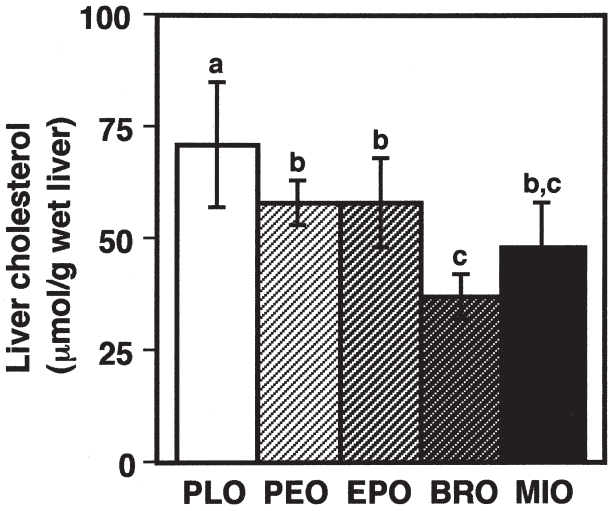


**FIG. 1.** Liver cholesterol concentration in rats fed dietary fats for 15 wk. Rats were fed a semisynthetic diet enriched with cholesterol and saturated triacylglycerol (TAG) or the same diet in which n-3 or n-6 polyunsaturated TAG replaced saturated TAG. Each value represents the means ± standard deviations for data obtained from five animals. Means values (a, b, c) were significantly different ($P < 0.05$), as determined by analysis of variance with Duncan's multiple-range test. PLO, palm oil; PEO, perilla oil; EPO, evening primrose oil; BRO, borage oil; MIO, mixed oil.

*mRNA levels of hepatic apo B and LDL receptor.* Figures 2 and 3 show representative results of Southern hybridization of PCR-amplified apo B cDNA and LDL receptor cDNA in the rat liver. Values for apo B mRNA and LDL receptor mRNA in liver are expressed relative to the average value for the PLO (25.3)-fed rat normalized to 100. There were no significant differences in the hepatic LDL receptor mRNA and apo B mRNA levels among the groups.

*Fecal lipid concentration.* Table 4 shows the effects of the dietary oils on fecal neutral steroid and bile acid concentrations in aged rats at the end of the experimental period. The excretion of cholesterol in the MIO (1.7) group was significantly higher than in the PLO (25.3) group. The excretion of coprostanol in the MIO (1.7) group was significantly higher than in the BRO (15.1) and EPO groups.

The excretion of chenodeoxycholic acid in the EPO group was highest among the groups, and the excretion of deoxycholic acid in the BRO (15.1) group was significantly higher than in the EPO, MIO (1.7), and PLO (25.3) groups. However, there were no significant differences in the excretion of cholic acid, lithocholic acid, and total bile acid among the groups.

## DISCUSSION

Ishihara *et al.* (1) suggested that n-6 fatty acid LA had no hypocholesterolemic effect in long-term feeding (120 d). In this long-term feeding (105 d) in aged rats, PEO (0.3) containing about 57% ALA was compared with EPO containing about 71% LA and 9% GLA, BRO (15.1) containing about 38% ALA and 24% GLA, MIO (1.7) containing about 26% ALA and 15% GLA, and PLO (25.3) containing about 46% palmitic acid.

This study indicated that dietary PEO (oleic acid + LA + ALA; n-6/n-3, 0.3; P/S, 9.6), BRO (oleic acid + LA + ALA; n-6/n-3, 15.1; P/S, 5.3), and MIO (oleic acid + LA + GLA + ALA; n-6/n-3, 1.7; P/S, 6.7), in the same manner as EPO (LA + GLA; P/S, 10.5), were more efficient in inhibiting increases of serum total cholesterol in long-term feeding than PLO (25.3), which contained abundant S. These results agreed with those for young rats (Fukushima, M., unpublished data). Ishihara *et al.* (1) reported that increased intake of high-linoleate vegetable oil was not useful for the prevention of hypercholesterolemia-associated diseases; however, dietary oils rich in n-3 fatty acids might be useful. However, dietary GLA is hypocholesterolemic in the same manner as PEO, which contains abundant ALA, in aged rats. Thus, our results might not agree with the results of Ishihara *et al.* (1) because our conditions were different from theirs using mice (1).

The cholesterol content in the livers of rats fed the PLO (25.3) diet was the highest among the five groups in the aged rats. On the other hand, the cholesterol content in the livers of aged rats fed abundant GLA tended to decrease more than in the EPO group. Our results using aged rats agreed with a previous report (2) that the liver cholesterol concentration in the PLO group tended to increase compared with the group

**TABLE 4**
**Fecal Steroid Concentrations in Rats Fed Dietary Fats for 15 wk[a]**

| | Diet | | | | |
|---|---|---|---|---|---|
| | PEO | BRO | EPO | MIO | PLO |
| Cholesterol | $4.39 \pm 1.11^{a,b}$ | $5.26 \pm 1.05^{a,b}$ | $6.18 \pm 1.76^{a,b}$ | $6.64 \pm 1.36^{a}$ | $3.99 \pm 2.45^{b}$ |
| Coprostanol | $0.43 \pm 0.13^{a,b}$ | $0.25 \pm 0.27^{b}$ | $0.31 \pm 0.13^{b}$ | $0.71 \pm 0.21^{a}$ | $0.55 \pm 0.28^{a,b}$ |
| CA | $0.28 \pm 0.37$ | $0.48 \pm 0.11$ | $0.37 \pm 0.18$ | $0.25 \pm 0.21$ | $0.34 \pm 0.12$ |
| CDCA | $0.24 \pm 0.14^{b}$ | $0.04 \pm 0.02^{b}$ | $0.52 \pm 0.46^{a}$ | $0.08 \pm 0.06^{b}$ | $0.02 \pm 0.01^{b}$ |
| DCA | $0.16 \pm 0.07^{a,b}$ | $0.23 \pm 0.05^{a}$ | $0.06 \pm 0.06^{c}$ | $0.11 \pm 0.08^{b,c}$ | $0.08 \pm 0.07^{b,c}$ |
| LCA | $0.78 \pm 0.72$ | $0.26 \pm 0.28$ | $0.68 \pm 0.72$ | $0.76 \pm 0.70$ | $1.14 \pm 0.54$ |
| TBA | $1.46 \pm 1.09$ | $1.01 \pm 0.20$ | $1.63 \pm 1.27$ | $1.20 \pm 0.88$ | $1.57 \pm 0.65$ |

[a]Values are means ± standard deviations of diets (μmol/g 100 g body weight/d) for five rats. Means within the same rows bearing different roman superscripts are significantly different ($P < 0.05$). CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; TBA, total bile acid. See Table 1 for other abbreviations.

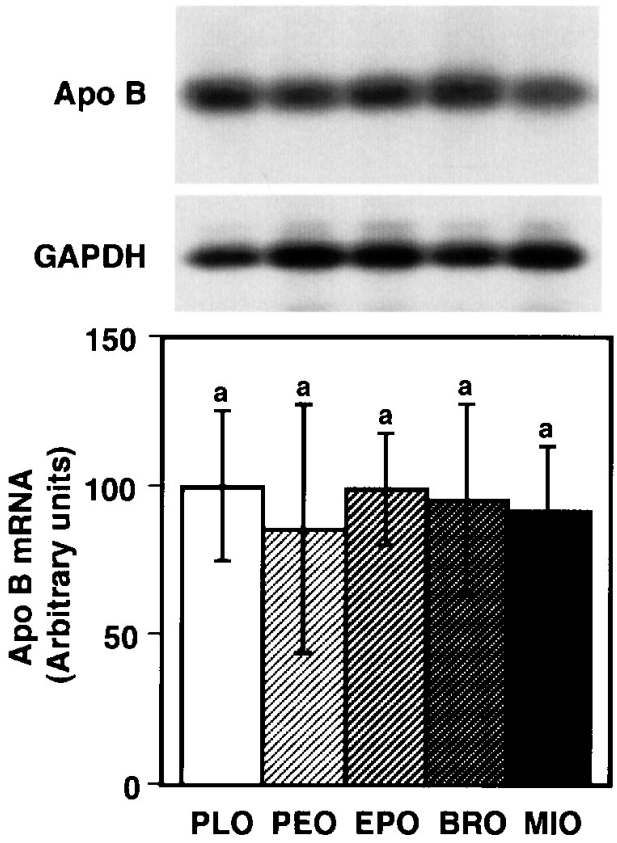


**FIG. 2.** Effect of dietary n-6 and n-3 polyunsaturated fatty acids (PUFA) on hepatic apolipoprotein (apo) B mRNA in rats fed dietary fats for 15 wk. Rats were fed a semisynthetic diet enriched with cholesterol and saturated TAG or the same diet in which n-3 or n-6 polyunsaturated TAG replaced saturated TAG. Each value represents the means ± standard deviations for data obtained from five animals. Means values were significantly different ($P < 0.05$), as determined by analysis of variance with Duncan's multiple-range test. The value of apo B mRNA was normalized to the value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and values for the rats fed the PEO, EPO, BRO, and MIO diets are expressed relative to the average values for rats fed the PLO diet, which was set to 100. Inset illustrates the representative Southern hybridization of polymerase chain reaction (PCR)-amplified apo B cDNA of hepatic RNA. See Figure 1 for other abbreviations.

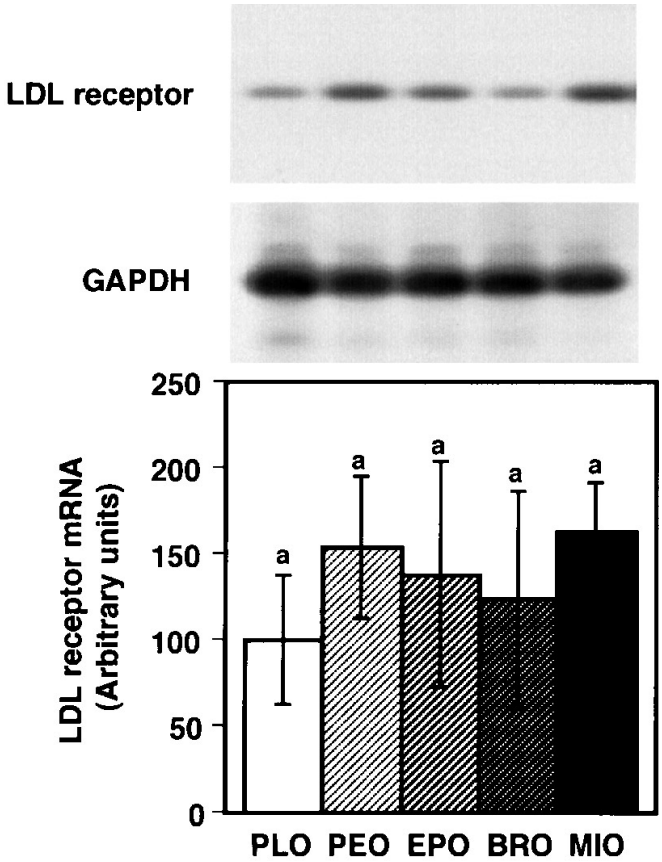


**FIG. 3.** Effect of dietary n-6 and n-3 PUFA on hepatic low density lipoprotein (LDL) receptor mRNA in aged rats fed dietary fats for 15 wk. Rats were fed a semisynthetic diet enriched with cholesterol and saturated TAG or the same diet in which n-3 or n-6 polyunsaturated TAG replaced saturated TAG. Each value represents the means ± standard deviations for data obtained from five animals. Means values were significantly different ($P < 0.05$), as determined by analysis of variance with Duncan's multiple-range test. The value of LDL receptor mRNA was normalized to the value of GAPDH mRNA, and the rats fed the PEO, EPO, BRO and MIO diets are expressed relative to the average values for rats fed the PLO diet, which was set to 100. Inset illustrates the representative Southern hybridization of PCR-amplified LDL receptor cDNA of hepatic RNA. See Figures 1 and 2 for other abbreviations.

containing GLA in young rats. Ventura *et al.* (3) reported that dietary n-6 or n-3 fatty acid increased hepatic LDL receptor activity compared with dietary S in the rat. However, there were no significant differences in the hepatic LDL receptor mRNA levels among the groups. Dietary cholesterol presumably suppresses hepatic LDL receptor activity *via* this mechanism (16) and, indeed, dietary cholesterol has been shown to reduce hepatic LDL receptor mRNA levels in nonhuman primates (17). In this study, it is possible that dietary cholesterol suppressed hepatic LDL receptor mRNA levels in all groups. Ihara-Watanabe *et al.* (18) have reported that the activity and mRNA expression of HMG-CoA reductase were lower in rats on an ALA-diet than in those on a GLA-diet without cholesterol, though there were no significant differences in the activity and mRNA expression of HMG-CoA reductase between the ALA-diet and GLA-diet during cholesterol feeding. There were no significant differences in the excretion of neutral and acidic sterol concentrations between n-6 and n-3 fatty acid groups in this experiment. In this respect, the rat is highly resistant to the effects of dietary cholesterol. The major factors appear to be a much higher basal rate of hepatic bile acid synthesis in the rat, allowing a much greater inflow of dietary cholesterol to be balanced by suppression of hepatic sterol synthesis (19). It may be that the excretion of neutral and acidic steroid concentrations did not affect the response of serum LDL cholesterol and liver cholesterol in n-6 and n-3 fatty acid groups in this experiment.

In conclusion, the results of this study demonstrate that both n-6 and n-3 fatty acids inhibit increases of serum total cholesterol and VLDL + IDL + LDL-cholesterol concentrations as compared with S in the presence of excess cholesterol in the diet after long-term feeding.

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# Acute Effect of Ethanol on 7-Hydroperoxycholesterol in Muscle and Liver

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**ABSTRACT:** We tested the hypotheses that ethanol sensitivities of muscle and liver can be discerned in the initial periods of ethanol exposure, especially when acetaldehyde levels are markedly raised with cyanamide, an aldehyde dehydrogenase inhibitor. To test this, we measured cholesterol hydroperoxides in soleus (Type I) and plantaris (Type II) muscle in four groups of rats acutely (i.e., 2.5 h) exposed to: [S] saline (control), [Cy] cyanamide, [EtOH] ethanol, or [Cy + EtOH] cyanamide + ethanol. Comparative reference was also made to the response of the liver. After 2.5 h, ethanol alone significantly increased 7α-hydroperoxycholest-5-en-3β-ol (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH) levels in plantaris muscle. Identical qualitative effects were seen in rats treated with cyanamide + ethanol, but there was no discernible difference between groups [EtOH] and [Cy + EtOH]. In both the soleus muscle and liver, none of the treatments with either ethanol or cyanamide + ethanol had any effect on any of the measured parameters. This is the first report of a differential response of 7α-OOH and 7β-OOH in Type II, compared to Type I predominant muscles, and the first time that muscle has been shown to be more sensitive than the liver in terms of its lipid marker response to oxidative stress. Perturbations in the muscle membrane lipid domain may contribute to impairment of muscle in alcoholism.

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Skeletal muscle abnormalities affect up to 60% of alcohol abusers compared with an estimated 15–20% prevalence for cirrhosis (1). Type II (anaerobic, fast-twitch) fibers are particularly affected, whereas Type I (aerobic, slow-twitch) are relatively resistant to alcohol-induced muscle disease, although they are both affected in extreme circumstances (1). Despite the frequent occurrence of alcohol-induced muscle disease, the pathogenic mechanisms are unknown, although both perturbed membrane function and increased generation of reactive oxygen species have been proposed (2–4). Previous studies have identified 7α- (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH) in skeletal muscles formed *via* reactive oxygen species. They are therefore putative markers of oxidative stress, and are increased in both Types I (represented by the soleus) and II (represented by the plantaris) fiber-predominant skeletal muscles of rats 1 d after an acute ethanol load (5). These changes are attributable to perturbations in the membrane lipid domain by reactive oxygen species, a hypothesis supported by the observation that the membrane phospholipid profiles are also altered. Altered membrane function may initiate the deleterious processes that precipitate the ethanol-induced reductions in protein synthesis (6–10).

We have shown that an acute bolus of ethanol reduces the synthesis of muscle proteins (6,8,11). When acetaldehyde levels are raised with the aldehyde dehydrogenase inhibitor cyanamide, greater reductions in protein synthesis are obtained, implicating acetaldehyde as a potent protein synthetic perturbant (7).

Paradoxically, the changes in cholesterol hydroperoxides and fatty acid profile in 24-h dosed rats are equally observed in both Types I and II fibers. This is at variance with the known fiber sensitivities to alcohol (reviewed in Refs. 2–4). Certainly, Type I muscles have a higher antioxidant status (such as catalase, glutathione peroxidase, and superoxide dismutase activities and α-tocopherol concentrations) than the susceptible Type II muscles (12). It is quite possible that marked differential effects of ethanol-induced changes in membrane lipids will be observed at earlier time points such as at 2.5 h, as originally demonstrated for protein synthesis alterations (8,11). Furthermore, there is no information on how these metabolites change when endogenous acetaldehyde levels are raised in the presence of ethanol. Finally, previous data for muscle cholesterol hydroperoxides have been taken in isolation (5), without reference to the effects on the liver which is a major target organ for alcohol's deleterious effects, particularly with respect to oxidative stress (2).

To resolve the issues raised above we measured cholesterol hydroperoxides in Types I and II muscles and in liver in alcohol-dosed rats with or without cyanamide pretreatment.

## MATERIALS AND METHODS

*Materials.* 3,5-Di-*tert*-butyl-4-hydroxytoluene, luminol (3-aminophthaloylhydrazine), and cytochrome c (from horse heart, type VI) were purchased from Wako Pure Chemical Co.

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Abbreviations: 5α-OOH, 5α-hydroperoxycholest-6-en-3β-ol; 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol; CL, chemiluminescence; DIH, 2,4-dinitrophenyl hydrazine-1,3-indandione-1-azine; HPLC, high-performance liquid chromatography.

(Osaka, Japan). Acetaldehyde, cyanamide, ethanol, and 2,4-dinitrophenyl hydrazine-1,3-indandione-1-azine (DIH) were purchased from Sigma-Aldrich Chemical Co. (Poole, Dorset, United Kingdom). Acetonitrile, methanol, and hydrochloric acid for the high-performance liquid chromatography (HPLC) determination of acetaldehyde were obtained from BDH Chemicals (Poole, Dorset, United Kingdom). Cholesterol hydroperoxides, 5α-hydroperoxycholest-6-en-3β-ol (5α-OOH), 7α-OOH, and 7β-OOH were synthesized as described previously (13,14). β-Sitosterol-5α-hydroperoxide (as internal standard) was prepared by irradiating a solution of β-sitosterol and hematoporphyrin in pyridine (13).

*Animal treatments.* Animals were housed in a Home Office-approved Comparative Biology Unit. All experimental animal work was conducted at King's College under approval from the appropriate local and national authorities. Male Wistar rats were obtained from accredited commercial suppliers at about 60 g body weight. They were maintained in a temperature- and humidity-controlled animal house for 1 wk until they weighed approximately 100–150 g. They were then ranked and divided into four groups of equal mean body weight. All rats had access to food throughout the study period up to and including the postinjection period. The experimental treatment of the rats involved two steps, i.e., pretreatment (30 min) followed by treatment (150 min) stages as follows: [S], saline + saline; [Cy], cyanamide + saline; [EtOH], saline + ethanol; [Cy + EtOH], cyanamide + ethanol. Rats were pretreated with an intraperitoneal injection (5 mL/kg body weight) of solution containing either saline (0.15 mol NaCl/L) or cyanamide (0.5 mmol/kg body weight). At 30 min after the pretreatment injection, rats were treated with an intraperitoneal injection (10 mL/kg body weight) of solution containing either saline (0.15 mol NaCl/L) or ethanol (75 mmol/kg body weight). At 150 min after commencement of the treatment step, rats were killed by decapitation. A separate group of identically treated rats was used for acetaldehyde (to facilitate rapid trapping of this volatile agent) and lipid determinations. For cholesterol hydroperoxides, soleus (Type I fiber-predominant) muscle, plantaris (Type II fiber-predominant) muscle, and liver were dissected.

*HPLC-chemiluminescence (CL) analysis of cholesterol hydroperoxides.* Cholesterol hydroperoxides were determined by reversed-phase HPLC-CL, comprising two LC-10AD vp pumps (Shimadzu, Kyoto, Japan), a SPD-6A spectrophotometric detector (Shimadzu), a CLD-10A CL detector (Shimadzu), and a Chromatopac C-R4A integrator (Shimadzu). A TSK gel Octyl-80Ts column (Tosoh, Tokyo, Japan) was used. One pump delivered the mobile phase of methanol/water/acetonitrile (89:9:2, by vol). The second pump delivered the chemiluminescent reagent, which consisted of cytochrome c and luminol in alkaline borate buffer as described previously (15). After the column eluant had passed through an ultraviolet detector set at 210 nm, it was mixed with the luminescent reagent in the postcolumn mixing joint of the CL detector. Standard curves were prepared from the analyses of 1–20 ng of 5α-OOH and 7β-OOH and from 0.5–10 ng of 7α-OOH using 5 ng of the internal standard. Regression lines of the ratios of hydroperoxides to the internal standard vs. the standard concentrations were linear.

*Tissue extraction procedures for lipids.* For peroxidation products, total lipid was extracted by adding 5 mL of ice-cold chloroform/methanol (2:1, vol/vol), containing 0.005% (vol/vol) butylated hydroxytoluene (as antioxidant) and 500 pmol β-sitosterol 5α-hydroperoxide as the internal standard, to approximately 0.1 g of muscle or liver, and homogenizing under ice-cold conditions. The homogenate was mixed with 5 mL of chloroform/methanol (2:1, vol/vol) and 1 mL of distilled water, vortexed vigorously, and centrifuged ($800 \times g$). The chloroform layer was aspirated, concentrated in a rotary evaporator, and dried under nitrogen. A cholesterol-rich fraction was isolated from the total lipid by solid phase extraction. A silica column (Sep-Pak; Waters Co., Milford, MA) packed with aminopropyl-derivatized silica ($-NH_2$) was initially conditioned by washing with 5 mL of acetone and 10 mL of *n*-hexane. The total lipid sample, dissolved in a small amount of chloroform, was added to the column, then flushed with a mixture of 2 mL chloroform and 1 mL isopropanol, giving an eluate that mainly consisted of cholesterol. This was concentrated in a rotary evaporator and dried under a nitrogen stream. The cholesterol-rich fraction was dissolved in methanol and stored until analysis.

*Acetaldehyde determinations.* The conditions for the acetaldehyde determinations were essentially those described in detail previously (16). Briefly, the trapping agent was prepared by dissolving 30 mg DIH in 100 mL of methanol and acetonitrile (80:20, vol/vol), and then storing at 4°C. The blood was immediately collected *via* heparinized funnels into tubes containing the aldehyde trapping agent DIH (16). All tubes used for blood collection were pre-cooled on ice and all samples were maintained at 0–4°C during the experiments. For determination of muscle acetaldehyde, the combined gastrocnemius and plantaris muscles were rapidly dissected and immediately homogenized in DIH trapping agent. The combined analysis of both the gastrocnemius and plantaris was necessary to minimize the period between death and suspension of tissue homogenate in DIH. In addition, the amount of plantaris and soleus muscle was too small (less than 0.1 g for each leg) to analyze acetaldehyde levels in each muscle. Samples of liver tissue were similarly treated. The blood (1 g) or tissue (about 1 g) was added to 4 mL of ice-cold DIH reagent in a glass vial, capped, and the contents were either immediately mixed thoroughly (i.e., blood) or homogenized (i.e., muscle or liver) with a Polytron Highspeed Homogenizer (Philip Harris, United Kingdom). After centrifugation, the supernatants were mixed with hydrochloric acid and incubated in a water bath at 37°C for 10 min. The samples were then stored at −70°C until analysis. Samples were then subjected to quantitative analysis by HPLC with a Shimadzu system controller with a LC-6A pressure pump and SIL-6A autoinjector (Dysons Instruments Ltd., Tyne and Wear, United Kingdom) and Hypersil MOS 2 column (Phenomonex, Macclesfield, United Kingdom), as described previously (16,17). The azine derivatives of acetaldehyde were detected

by fluorescence spectroscopy (excitation 430 nm, emission 525 nm; Merck Hitachi, Luterworth, United Kingdom). Assays employed standard solutions of acetaldehyde (0.1–10 mmol/L).

*Statistics.* All data are expressed as mean ± SD of 5 or 6 observations in each group. Differences between groups were assessed by nonparametric Kruskal-Wallis test.

## RESULTS

*Cholesterol hydroperoxides.* There was clear separation of cholesterol hydroperoxides (5α-OOH, 7α-OOH, and 7β-OOH) together with the internal standard β-sitosterol-5α-hydroperoxide in standard solutions as well as in muscle samples. Lipid extracts from muscle contained 7α-OOH and 7β-OOH, but not 5α-OOH, as previously described (5).

The mean concentrations of 7α-OOH and 7β-OOH in muscle of control rat are shown in Table 1. In plantaris muscle from Group [S], 7α-OOH (0.81 nmol/g) was significantly lower than 7β-OOH (1.98 nmol/g). In the soleus muscle from Group [S], 7α-OOH (0.43 nmol/g) was lower than 7β-OOH (0.58 nmol/g), but it did not reach statistical significance. In both muscles, values of 7α-OOH were lower compared to 7β-OOH which may reflect the fact that 7α-OOH is easily epimerized to 7β-OOH (5). Compared to soleus muscle, significantly higher values of 7α-OOH and 7β-OOH were found in plantaris muscles confirming previous observations (5). High levels of 7α-OOH and 7β-OOH in plantaris compared to the soleus muscle may reflect the lower antioxidant capacity of Type II fiber-rich muscles (12,18–21). Moreover, in liver the concentrations of 7α-OOH and 7β-OOH were approximately 10-fold higher than muscle (Table 1).

Following acute treatment with cyanamide, concentrations of 7α-OOH and 7β-OOH were unaffected in all three tissues.

In response to ethanol, there were no significant changes in 7α-OOH and 7β-OOH in soleus muscle (Table 1). However, there were significant increases in 7α-OOH and 7β-OOH in plantaris muscle at 2.5 h following acute ethanol administration (Table 1). Evidence for the greater relative sensitivity of the Type II fibers compared to the Type I fibers was obtained by examining the soleus/plantaris ratio of individual analytes in each rat. Thus, the soleus/plantaris ratio for 7α-OOH decreased from 0.56 to 0.26 ($P < 0.01$), and a similar change was observed for 7β-OOH (from 0.37 to 0.16, $P < 0.025$). There was no change in the liver hydroperoxides in response to ethanol (Table 1).

The effects of raising acetaldehyde with cyanamide (i.e., Group [S] vs. [Cy + EtOH]) were similar to treatments with ethanol alone (i.e., Groups [S] vs. [EtOH]). Thus, compared to Group [S], significant elevations in 7α-OOH and 7β-OOH were observed in plantaris muscle following treatment with cyanamide + ethanol (in both instances, increases were approximately 70%, $P < 0.01$). Neither 7α-OOH nor 7β-OOH in soleus muscle or liver, respectively, were affected following treatment with cyanamide + ethanol, i.e., Groups [S] vs. [Cy + EtOH], $P > 0.05$ not significant (Table 1). Furthermore, compared to Group [Cy], significant elevation in 7β-OOH and elevation in 7α-OOH (not significant) were observed in plantaris muscle following treatment with cyanamide + ethanol (increase of 7β-OOH was 64%, $P < 0.01$).

*Ethanol levels.* Blood ethanol levels were not measured in the present experiment, but under identical experimental conditions using rats of the same size and age, plasma values of ethanol were as follows (all data as mean; mmol/L) [S], <1; [Cy] <1; [EtOH], 33; [Cy + EtOH] 46 (7) . The statistics are as follows; [S] vs. [EtOH], $P < 0.001$; [S] vs. [Cy + EtOH], $P < 0.001$; [EtOH] vs. [Cy + EtOH], $P < 0.01$ (7).

*Acetaldehyde levels*. Table 2 shows the acetaldehyde levels in the blood of rats in the four groups. There were no differences in blood acetaldehyde levels between the Groups [S] and [Cy]. Blood acetaldehyde levels increased sixfold in rats treated with ethanol (Group [EtOH]

**TABLE 1**
**7-Hydroperoxycholesterol Levels[a] in Skeletal Muscle and Liver of Rats 2.5 h After Acute Ethanol Administration**

| | S | Cy | EtOH | Cy + EtOH |
|---|---|---|---|---|
| 7α-Hydroperoxycholest-5-en-3β-ol (nmol/g) | | | | |
| Soleus | 0.43 ± 0.22 | 0.41 ± 0.15 | 0.34 ± 0.12 | 0.34 ± 0.07 |
| Plantaris | 0.81 ± 0.22 | 0.88 ± 0.28 | 1.27 ± 0.16[b] | 1.38 ± 0.51[b] |
| Soleus/plantaris ratio | 0.56 ± 0.11 | 0.49 ± 0.09 | 0.26 ± 0.08[c] | 0.25 ± 0.08[c] |
| Liver | 7.9 ± 1.2 | 8.3 ± 1.5 | 8.9 ± 2.0 | 9.1 ± 1.6 |
| 7β-Hydroperoxycholest-5-en-3β-ol (nmol/g) | | | | |
| Soleus | 0.58 ± 0.32 | 0.68 ± 0.37 | 0.51 ± 0.19 | 0.52 ± 0.13 |
| Plantaris | 1.98 ± 0.57 | 2.13 ± 0.68 | 3.16 ± 0.18[b] | 3.50 ± 0.62[b,d] |
| Soleus/plantaris ratio | 0.37 ± 0.07 | 0.27 ± 0.14 | 0.16 ± 0.06[c] | 0.14 ± 0.03[c] |
| Liver | 20.8 ± 4.0 | 21.7 ± 3.8 | 21.0 ± 4.3 | 22.9 ± 2.1 |

[a]Values are mean ± SD. S, saline + saline; Cy, cyanamide + saline; EtOH, saline + ethanol; Cy + EtOH, cyanamide + ethanol. Saline, 0.15 mol NaCl/L; cyanamide, 0.5 mmol/kg body weight; ethanol, 75 mmol/kg body weight. The study design included a pretreatment and treatment stage to ensure all rats were treated identically. Other details are contained in the the Materials and Methods section.
[b]Significantly greater than S ($P < 0.05$).
[c]Significantly smaller than S ($P < 0.05$).
[d]Significantly greater than Cy ($P < 0.05$).

**TABLE 2**
**Acetaldehyde Levels[a] in Blood and Muscle After Acute Ethanol Administration With or Without Cyanamide**

| | S | Cy | EtOH | Cy + EtOH |
|---|---|---|---|---|
| Blood (nmol/mL) | 5 ± 2 | 5 ± 2 | 31 ± 14[b] | 2495 ± 119[b,c] |
| Muscle (nmol/g) | 51 ± 10 | 52 ± 10 | 62 ± 28 | 827 ± 123[b,c] |
| Liver (nmol/g) | 31 ± 17 | 33 ± 14 | 75 ± 29[b] | 1202 ± 180[b,c] |

[a]Values are mean ± SD.
[b]Significantly greater than S ($P < 0.001$).
[c]Significantly greater than Cy ($P < 0.001$).

compared to Group [S]). In Group [Cy + EtOH], blood acetaldehyde was 500-fold higher compared to either Group [S] or Group [Cy] and significantly higher than levels in Group [EtOH].

Acetaldehyde concentrations in muscle of cyanamide + ethanol rats (Group [Cy+EtOH]) increased 16-fold compared to Group [S] (Table 2). Acetaldehyde concentrations in liver of cyanamide + ethanol rats increased 38-fold compared to Group [S] (Table 2).

## DISCUSSION

We examined hydroperoxide as an index of free radical activity (5,22,23). 7α-OOH and 7β-OOH are formed as a consequence of oxidative stress *per se* rather than other routes of metabolism (22). The HPLC-CL quantification of 7α-OOH and 7β-OOH have been utilized as a marker of lipid peroxidation in human erythrocyte membranes (22). More direct assessment of conjugated diene levels, or reaction of lipid peroxidation products with thiobarbituric acid or protein carbonyl, are limited as they are nonspecific and possibly insensitive (5). In the present investigation, we report increased levels of 7α-OOH and 7β-OOH cholesterol hydroperoxides in the plantaris muscle of acutely ethanol-dosed rats, an effect not seen in the soleus muscle or liver. We therefore describe for the first time evidence for the differential effects of ethanol-induced increases in 7α-OOH and 7β-OOH cholesterol hydroperoxides in accordance with the known antioxidant properties of the different muscle types.

In these studies we analyzed the soleus and plantaris, which are Type I and II fiber-predominant muscles, respectively. We showed that there were significant biochemical differences between these two muscles in control rats in accordance with previous observations. This included a higher concentration in plantaris muscle of 7α-OOH and 7β-OOH. There were some differences between cholesterol hydroperoxides in control rats of the present experiments (Table 1) and previously published studies (5). These differences possibly reflect the markedly divergent nutritional states of rats in the two studies, which were fed (having access to food throughout the study period up to and including the postinjection period) in the present study (see the Materials and Methods section), compared to starved in the previous analysis (5).

There are particular difficulties in examining biochemical mechanisms in individual Type I and II fibers, mainly because insufficient material can be analyzed. To resolve this, the soleus and plantaris muscle (the latter being equivalent to the extensor digitorum longus muscle used by some groups) muscles were taken to represent Type I and Type II fibers, respectively. Previous comparisons of these two muscles have included diverse subject areas such as signal transduction, the examination of muscle with respect to the fasting-feeding transition, the metabolism of muscle triacylglycerol and glycogen in response to dietary modification, and the effect of exercise (see for example Ref. 24). Their suitability is further supported by the observation that in response to alcohol (albeit with chronic alcohol feeding models) individual Type II fibers within both the soleus and plantaris display atrophy and that the individual Type I fibers in the soleus and plantaris muscles are resistant (25). Furthermore, the suppression of protein synthesis in acutely dosed rats is much less in Type I muscles (8). We believe that the resilience of the Type I fiber is related to the antioxidant status of these muscles compared to the Type II fibers.

Previous studies have also shown that muscle and liver respond differently to alcohol. For example, after 2.5 h, acute ethanol reduces the synthesis rates of skeletal muscle but not liver (11,26). Further evidence for the differential responses of tissues are obtained from studies showing that ethanol administration reduces the antioxidants catalase and glutathione in liver, but not in muscle (27). The reduction of hepatic catalase and glutathione may reflect the utilization of these protective agents (27).

Moreover, in the present studies we used cyanamide, a potent inhibitor of aldehyde dehydrogenase. The increase in acetaldehyde in response to cyanamide + ethanol was 500-fold in blood, 16-fold in muscle, and 38-fold in liver compared to Group [S]. Although levels of ethanol and acetaldehyde were extremely high, we failed to observe changes of cholesterol hydroperoxides in liver due to the high antioxidant capacity of this tissue. For example, α-tocopherol concentrations are considerably higher in the liver than in skeletal muscle (26). Previous studies have shown that ethanol reduces skeletal muscle protein synthesis by approximately 30%, and cyanamide + ethanol treatment reduces protein synthesis in skeletal muscle by approximately 65% (7). As far as we are aware, the magnitude of this reduction in protein synthesis in such acute conditions is the highest ever reported. Cyanamide pre-dosing therefore offers the opportunity of measuring metabolic perturbations when there is a marked suppression of protein synthesis. Acetaldehyde-mediated muscle damage probably results from inhibition of protein synthesis rates, but free radical-mediated muscle damage may not result from high acetaldehyde levels, because acetaldehyde can react with protein to form protein adduct but cannot react with lipids themselves. Alternatively, it is possible that only trace amounts of acetaldehyde can cause increases in cholesterol hydroperoxides as was observed in plantaris muscle of rats only dosed with ethanol. Nevertheless, we can conclude that the failure to observe changes in soleus muscle and liver is not due to limitations in the dosing regimen but rather

a reflection of the antioxidant defences or other cytoprotective mechanisms within these tissues.

In conclusion, this is the first report of a differential response of 7α-OOH and 7β-OOH in Type II, compared to Type I predominant muscles and the first time that muscle has been shown to be more sensitive than the liver in terms of its response with respect to lipid markers of oxidative stress. These results signify perturbations in the membrane, which may have important consequences for the pathogenesis of alcohol-induced muscle disorders.

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# Incorporation and Distribution of Saturated and Unsaturated Fatty Acids into Nuclear Lipids of Hepatic Cells

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**ABSTRACT:** Liver nuclear incorporation of stearic (18:0), linoleic (18:2n-6), and arachidonic (20:4n-6) acids was studied by incubation *in vitro* of the [1-$^{14}$C] fatty acids with nuclei, with or without the cytosol fraction at different times. The [1-$^{14}$C] fatty acids were incorporated into the nuclei as free fatty acids in the following order: 18:0 > 20:4n-6 ≫ 18:2n-6, and esterified into nuclear lipids by an acyl-CoA pathway. All [1-$^{14}$C] fatty acids were esterified mainly to phospholipids and triacylglycerols and in a minor proportion to diacylglycerols. Only [1-$^{14}$C]18:2n-6-CoA was incorporated into cholesterol esters. The incorporation was not modified by cytosol addition. The incorporation of 20:4n-6 into nuclear phosphatidylcholine (PC) pools was also studied by incubation of liver nuclei *in vitro* with [1-$^{14}$C]20:4n-6-CoA, and nuclear labeled PC molecular species were determined. From the 15 PC nuclear molecular species determined, five were labeled with [1-$^{14}$C]20:4n-6-CoA: 18:0-20:4, 16:0-20:4, 18:1-20:4, 18:2-20:4, and 20:4-20:4. The highest specific radioactivity was found in 20:4-20:4 PC, which is a minor species. In conclusion, liver cell nuclei possess the necessary enzymes to incorporate exogenous saturated and unsaturated fatty acids into lipids by an acyl-CoA pathway, showing specificity for each fatty acid. Liver cell nuclei also utilize exogenous 20:4n-6-CoA to synthesize the major molecular species of PC with 20:4n-6 at the *sn*-2 position. However, the most actively synthesized is 20:4-20:4 PC, which is a quantitatively minor component. The labeling pattern of 20:4-20:4 PC would indicate that this molecular species is synthesized mainly by the *de novo* pathway.

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Saturated and unsaturated fatty acids are important cellular components involved in different biological processes. In cells, fatty acids are essential structural and functional components of all membranes, and they are principally esterified to glycerolipids. Besides the traditional roles of fatty acids as nutrients and as a source of metabolic energy and structural components of complex lipids, fatty acids *per se* have physiological and pathological effects. Fatty acids are the source of signaling molecules involved in cellular transduction (1). A correlation between fatty acid alteration in phospholipid (PL) composition and initiation of apoptotic cell death has also been described (2,3). In particular, arachidonic and linoleic acid metabolites were reported to have both metastatic and mitotic potential (4,5).

However, in addition to the biological functions described above, fatty acids are also important determinants and mediators in gene expression (6–9). Dietary polyunsaturated fatty acids (PUFA) have been shown to decrease mRNA levels and transcription of several rat hepatic lipogenic genes such as S14 protein, glucose-6-phosphate dehydrogenase, fatty acid synthase (10–12), and stearoyl-CoA desaturase in different tissues and species (7–9,13). It has been proposed that the genes of stearoyl-CoA desaturase are co-regulated by fatty acids and cholesterol through a common class of transcription factor, the sterol regulatory element binding proteins (9). Also, arachidonic acid has been found to have a rapid inhibitory effect on gene transcription of stearoyl-CoA desaturase, and this repression is probably due to a noneicosanoid role of 20:4n-6 (8,14,15). If this is the case, it is very important to know how nuclear lipid metabolism operates in the cell.

Nuclear lipid metabolism is very active. However, its precise topology and the relationship between nuclear lipids and cell functions have not been fully elucidated. The most abundant PUFA from total lipids in hepatic cell nuclei are those of the n-6 series which represent 46% of the total fatty acid composition, arachidonic acid being the main one (23%) (16). Moreover, recent studies have proposed that the nucleus is a key subcellular site for enzymes involved in the release and metabolism of arachidonic acid (17). Thus, arachidonic acid is not only an important nuclear structural membrane component (16) but also the primary substrate for the synthesis of eicosanoids. In nuclei, arachidonic acid may come from: (i) cell cytoplasm where it can be synthesized in the endoplasmic reticulum from 18:2n-6 acid which is desaturated by Δ6 desaturase to 18:3n-6 acid, elongated to 20:3n-6, and finally desaturated by Δ5 desaturase to 20:4n-6 (18); (ii) hydrolyzed from other lipids; (iii) synthesized in nuclei by Δ5 desaturase from 20:3n-6 previously activated as 20:3n-6-CoA by means of nuclear long-chain fatty acyl-CoA synthetase (16,19). We have also investigated the distribution of 20:3n-6 and its desaturation product, 20:4n-6, into nuclear lipids during *in vitro* Δ5 desaturation (20). As previously demonstrated by others

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Abbreviations: CE, cholesterol ester; DG, diacylglycerol; ELSD, evaporative light scattering detector; FABP, fatty acid binding protein; FFA, free fatty acid; GC, gas chromatography; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; IM, incubation mixture; N, nuclear pellet; PC, phosphatidylcholine; PL, phospholipid; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acid; RHPLC, reversed-phase HPLC; TG, triacylglycerol; TLC, thin-layer chromatography.

in fibroblasts, the nuclear membrane is an important compartment for the uptake and release of arachidonate from PL, which in this way, become available for eicosanoid production (21). It is very interesting to note that another desaturase has recently been described in rat liver nuclei, the Δ9 desaturase (22).

Taking into acount that fatty acids regulate gene expression (9) and that information on nuclear fatty acid metabolism is scarce, the aim of the present study has been to investigate the incorporation and distribution of saturated and unsaturated fatty acids into nuclear lipids and to determine nuclear phosphatidylcholine (PC) pools. For this reason, cell liver nuclei were incubated *in vitro* with [1-$^{14}$C] stearic (18:0), linoleic (18:2n-6), or arachidonic (20:4n-6) acids with and without a cytosol fraction. To study nuclear PC pools, molecular species of PC were determined and the incorporation of [1-$^{14}$C]20:4n-6-CoA into them was also studied.

## MATERIAL AND METHODS.

*Materials.* [1-$^{14}$C]Stearic acid (18:0) (56.0 mCi/mmol, 99% radiochemically pure), [1-$^{14}$C]linoleic acid (18:2n-6) (54.7 mCi/mmol, 98.5% radiochemically pure, 1% *trans* isomer), [1-$^{14}$C]arachidonic acid (20:4n-6) (54.9 mCi/mmol, 99% radiochemically pure), and [1-$^{14}$C]arachidonyl-CoA (20:4n-6-CoA) (51.6 mCi/mmol, 97% radiochemically pure) were purchased from New England Nuclear Corp. (Boston, MA). Cofactors used were provided by Sigma Chemical Co. (St. Louis, MO), thin-layer chromatography (TLC) precoated silica gel G 20 × 20 cm plates were from Merck (Buenos Aires, Argentina) and all unlabeled fatty acids were from Nu-Chek-Prep Inc. (Elysian, MN). 1,2-Diarachidonoyl-*sn*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All chemicals and solvents were of analytical and high-performance liquid chromatography (HPLC) grade.

*Animals*. In this study, international regulations for animal care were observed. Male Wistar rats of 60–70 d of age, weighing 180–200 g were maintained on a commercial standard pellet diet (Nutrimento rat chow 3; Escobar, Argentina) and tap water *ad libitum*. The diet contained 4.0% of total lipid; its fatty acid composition was as follows 16.7% 16:0 (palmitic acid), 0.8% 16:1 (palmitoleic acid), 4.9% 18:0 (stearic acid), 21.8% 18:1 (oleic acid), 52.4% 18:2n-6 (linoleic acid), and 4.3% 18:3n-3 (linolenic acid). All animals were subjected to a daily photoperiod of 12-h light and 12-h darkness (midnight being the midpoint of the dark period).

*Preparation of homogenate and subcellular fractions*. Rats were killed by decapitation at 8 A.M. to equalize circadian effect (23). Livers from 3–5 animals were pooled and homogenized in sucrose 0.25 M in TKM buffer (0.05 M Tris-HCl, pH 7.5, 0.0025 M KCl, 0.005 M $MgCl_2$) 1:2 (wt/vol). All steps were carried out at 4°C. Highly purified nuclei were isolated from liver homogenate by sucrose-density ultracentrifugation using the method of Blobel and Potter (24), modified by Kasper (25) as described in a previous work (16). Concentration of nuclei in terms of protein was determined by the method of Lowry *et al.* (26) using crystalline bovine serum albumin as standard. The postmicrosomal supernatant, prepared as described previously (16), was centrifuged three times at 100,000 × *g* for 60 min to obtain the cytosolic fraction, then stored at −80°C (18 mg cytosol protein/mL of homogenizing solution). Cytosol fraction was checked for purity by enzymatic analysis as described previously (16). The nuclear fraction was resuspended in 25% glycerol (10 mM Tris-HCl, pH 7.9) and stored at −80°C until used.

*Criteria for nuclear purity.* Nuclear preparations were checked for purity by electron microscopy and enzymatic analysis as described previously (16). To assess the levels of possible contamination produced by endoplasmic reticulum, cytosol, lysosomes, and mitochondria, the nuclear fraction was assayed for the respective marker enzymes, arylesterase (27), lactate dehydrogenase (28), acid phosphatase (29), and succinate dehydrogenase (30). Each assay was performed in duplicate. Negligible amounts of microsomal, cytosolic, lysosomal, and mitochondrial marker enzymes were found in the isolated nuclei as described previously (16). Based upon the above morphological and biochemical assays, the isolated nuclei were judged highly homogeneous and pure.

*Incorporation of radioactive fatty acids.* The incorporation reactions were initiated by the addition of the nuclei (6 mg of nuclear protein) to flasks containing, unless otherwise indicated, 68 µg cytosolic protein, 2.0 µM of [1-$^{14}$C]-labeled fatty acid (18:0, 18:2n-6, or 20:4n-6), 41.7 mM NaF, 41.7 mM $K^+$ phosphate buffer (pH 7.4), 0.15 M KCl, 0.25 M sucrose, 5.0 mM $MgCl_2$, and 1.6 mM *N*-acetylcysteine, final pH 7.4, 60 µM CoA (sodium salt), 1.3 mM ATP, in a final volume of 1.6 mL according to the procedure described previously (16). Samples that were 2, 4, and 8 µM in [1-$^{14}$C]20:4n-6-CoA were incubated with nuclei with the same incubation mixture but CoA and ATP were omitted. Reaction mixtures were incubated in open tubes under constant shaking at 36°C. Blank incubations without the protein source were done concurrently. After 2.5, 5, 10, and 20 min, nuclei, as a nuclear pellet (N), were separated from the incubation mixture (IM) by centrifugation at 5,000 × *g* at 4ºC for 10 min.

*Lipid analysis.* Lipids from each fraction (N and IM) were extracted by the procedure of Folch *et al.* (31), and the radioactivity of the chloroform (*c*) and water/methanol (*w*) phases was assessed by liquid scintillation counting. Lipids were recovered from the original chloroform extract (IM*c* and N*c*) and separated into different classes by TLC on precoated silica gel G plates, using hexane/diethylether/acetic acid (80:20:1, by vol) as mobile phase. The plates were then subjected to radiochromatographic scanning on a Berthold Ld-2723, Dunnschicht Scanner (Wildbad, Germany) and peaks corresponding to lipid location were compared to those of known lipid standards that were visualized by exposure to iodine vapor. Individual lipids were scraped off from the plate into glass centrifuge tubes, and neutral lipids (DG: diacylglycerols, TG: triacylglycerols, CE: cholesterol esters, and FFA: free fatty acids) and polar lipids were extracted from the silica gel with chloroform/methanol/hexane (2:1:3, by vol)

and assayed for $^{14}C$ by liquid scintillation counting. Fatty acids from individual lipids were analyzed by gas–liquid chromatography (GLC).

*Fatty acid analysis.* Fatty acid methyl esters from nuclear lipids were prepared with $BF_3$/MeOH according to the method of Morrison and Smith (32) and analyzed by gas chromatography (GC), using a Shimadzu 9A GC (Tokyo, Japan) fitted with an Omegawax 250 fused-silica column, 30 m × 0.25 mm, with 0.25 µm phase (Supelco, Bellefonte, PA). Peaks were identified by comparing the retention times with those from a mixture of standard methyl esters.

*Molecular species.* PC fractions for molecular species separation were isolated from the lipid extracts by HPLC using a Merck-Hitachi L-6200 pump (Darmstadt, Germany). Detection was performed using an Evaporative Light-Scattering Detector (5000 ELSD) purchased from Alltech Associates (Deerfield, IL). To separate the PL classes, we used as stationary phase an Econosil Silica normal-phase, 250 × 4.6 mm analytical column purchased from Alltech, packed with 10 µm spherical particles. A guard column packed with the same material was also used. Elution was performed at a flow of 1 mL/min at ambient temperature throughout the separation by a gradient of hexane/isopropanol/dichloromethane 40:48:12 to hexane/isopropanol/dichloromethane/water 40:42:8:8 for 15 min followed by additional elution with the latter solvent for 15 min (33). PC and other components were collected manually from the column effluent using a flow splitter (Alltech) located between the column and the detector. The column effluent was monitored by an ELSD operating at a $N_2$ gas flow rate of 2.2 mL/min and a drift tube temperature of 90°C. The eluate was evaporated to dryness under a stream of nitrogen and redissolved in chloroform/methanol 1:1.

Resolution of molecular species was performed on two 5 µm end-capped Lichrosphere 100, 250 × 4 mm, RP18 columns in series, obtained from Merck (Darmstadt, Germany). Isocratic elution was applied with a mobile phase composed of acetonitrile/methanol/triethylamine 40:58:2 at a rate of 1 mL/min at 10°C. The column effluent was monitored by an ELSD operating at a gas flow rate of 1.8 mL/min and a drift tube temperature at 100°C (34).

The individual PC molecular species were identified by determining the fatty acid composition of each peak as follows and by comparison with the literature (34). Ninety percent of the column eluate was collected using the flow splitter with a fraction collector LKB 2212 Helirac (Bromma, Sweden) every 30 s; the other 10% of eluate from the splitter was analyzed in the ELSD. According to the HPLC–ELSD mass chromatograms, tubes with eluates from the same peak were gathered manually and interesterified for capillary GLC.

PC-radiolabeled samples were injected onto the RP18 column with unlabeled PC of liver homogenate used as internal standard in order to reach the appropriate amount of mass for the ELSD detection. Column eluate (90%) was collected every 30 s by using a flow splitter with a fraction collector LKB 2212 Helirac for subsequent determination of radioactivity by liquid scintillation counting of each tube collected. The remaining 10% eluate from the splitter was quantified in the ELSD, and the HPLC mass chromatograms of each sample were used to identify the radiolabeled peaks of PC molecular species. Radioactivity in each peak was calculated by the addition of the disintegrations per minute from the corresponding eluates, and then as a percentage of the counts recovered in all peaks for that run.

*Data presentation*. Biochemical analyses were run in duplicate. All experiments were carried out five times on nuclear fractions isolated from pooled livers from 3–5 animals.

## RESULTS

*Time course of [1-$^{14}$C]18:0, [1-$^{14}$C]18:2n-6, and [1-$^{14}$C]-20:4n-6 fatty acid incorporation into nuclear lipids*. In order to study the incorporation of the most abundant saturated and unsaturated nuclear fatty acids into nuclear lipids, liver cell nuclei were incubated *in vitro* with [1-$^{14}$C]18:0, [1-$^{14}$C]-18:2n-6, or [1-$^{14}$C]20:4n-6 with or without cytosol. After incubation, the mixture was centrifuged and N was separated from the IM. Lipids from each fraction were extracted by the procedure of Folch *et al.* (31), and the incorporation of radioactivity was measured by liquid-scintillation counting (Fig. 1). The incorporation patterns are expressed as a percentage of the amounts of starting radioactivity (time zero) that contained 100% of the respective [1-$^{14}$C]fatty acid (as analyzed by HPLC).

By using this procedure, on average, 95% of the total radioactivity was recovered in N and IM. In N, for the three fatty acids tested, more than 95% of the radioactivity was incorporated into lipids (fraction N*c*) and less than 5% into nuclear water-soluble components (fraction N*w*) (Fig. 1A). The incorporation profiles of fatty acids into N and IM at all times tested were not affected by the presence of cytosol in the incubation mixture (data not shown).

The incorporation of the three labeled fatty acids into total nuclear lipids (N*c*) showed an early and rapid uptake followed by a later saturation;18:0 was almost fully incorporated (90%) into nuclei, whereas, 18:2n-6 and 20:4n-6 were incorporated 67 and 50%, respectively, at the latest time tested (Fig. 1A, N*c*).

The time-dependent profiles of the fatty acids that had not been incorporated into nuclear lipids and that had remained in the IM are shown in Figure 1B. When nuclei were incubated with 20:4n-6, 90% remained as FFA, whereas with 18:2n-6, 50% remained as FFA and the rest were found to be water-soluble components. In the incubation of nuclei with 18:0, the amount of radioactivity found in the IM was small (less than 10%) and as FFA, since the rest was incorporated in the nuclear lipids (Fig. 1B).

*Incorporation and distribution of [1-$^{14}$C]18:0, [1-$^{14}$C]-18:2n-6, and [1-$^{14}$C]20:4n-6 into nuclear lipid classes.* Figure 2 shows the time course of the incorporation of [1-$^{14}$C]18:0, [1-$^{14}$C]18:2n-6, and [1-$^{14}$C]20:4n-6 into nuclear lipid classes. The radioactivity was found in PL, TG, DG, CE, and FFA. First, [1-$^{14}$C]18:0 was highly incorporated into

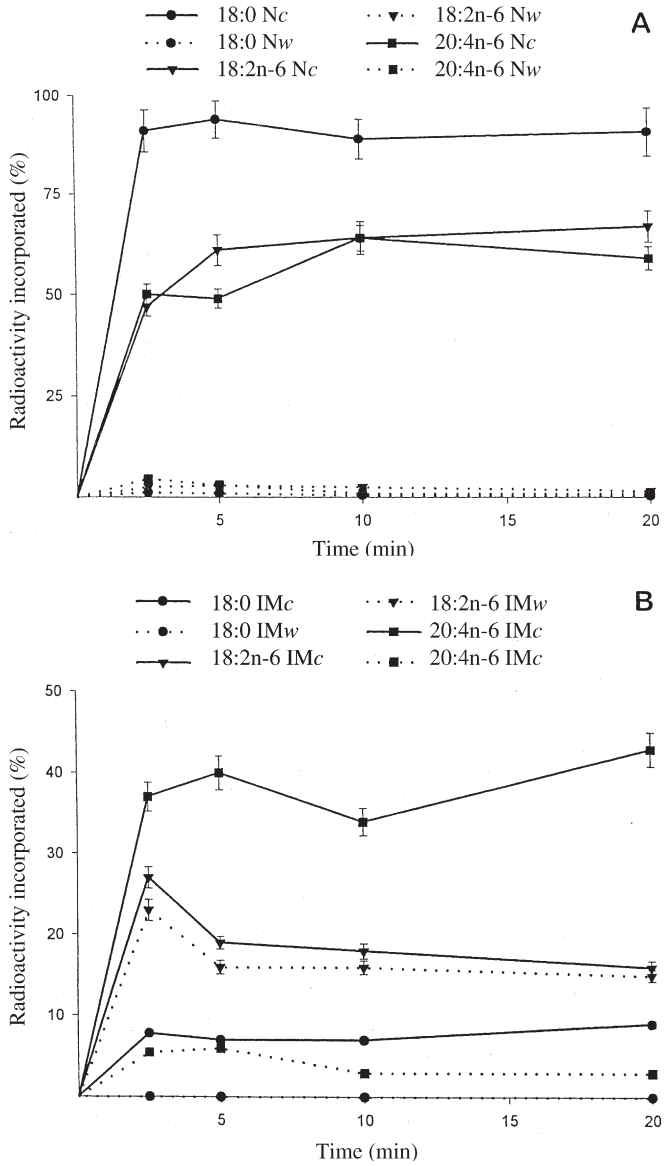


**FIG. 1.** Time-course incorporation of radioactivity into lipids. (A) Nuclear fraction (N) and (B) incubation mixture (IM). Liver nuclei were incubated with [1-$^{14}$C]18:0, [1-$^{14}$C]18:2n-6, or [1-$^{14}$C]20:4n-6 fatty acids. At different time points the incubation mixture was centrifuged to separate the nuclei from the incubation mixture. Lipids from each fraction were extracted by the method of Folch *et al.* (31), and the radioactivity at the chloroform (*c*) and water/methanol (*w*) phases was assessed by liquid scintillation counting. Results are the mean of five experiments ± SE.

nuclear lipids as FFA (78%) whereas [1-$^{14}$C]20:4n-6 was initially incorporated as FFA and esterified to PL (37 and 46%, respectively). Then the incorporated 18:0 and 20:4n-6 FFA decreased after esterification into nuclear lipids. On the other hand, the early incorporation of [1-$^{14}$C]18:2n-6 into cell nuclei as FFA was less and almost saturated from the beginning.

The three fatty acids studied were rapidly esterified mainly into nuclear PL, and this incorporation increased from [1-$^{14}$C]18:2n-6 to [1-$^{14}$C]18:0 and [1-$^{14}$C]20:4n-6 after 20 min of incubation. The incorporation of [1-$^{14}$C]18:2n-6 and [1-$^{14}$C]20:4n-6 into nuclear TG was not as rapid as into PL, increasing throughout the time tested. The incorporation of [1-$^{14}$C]18:0 into nuclear TG reached a low plateau very early, 5 min after incubation, indicating a low selectivity for this acid. The esterification of 18:0 into TG was saturated. This saturation cannot be due to a lack of substrate in the system since the amounts of 18:2 and 20:4 incorporated into TG were higher and increased throughout the test period. The incorporation of fatty acids into nuclear DG was only quantitatively important for [1-$^{14}$C]18:0. From the three fatty acids tested, [1-$^{14}$C]18:2n-6 was the only one esterified to CE under the experimental conditions used. This incorporation, after an initial delay of 2.5 min, increased in the next incubation times tested.

Three controls were done to determine if the esterification of 18:0, 18:2n-6, and 20:4n-6 acid into nuclear lipids followed an acyl-CoA-dependent pathway. Regarding these controls, [1-$^{14}$C]18:0, [1-$^{14}$C]18:2n-6, and [1-$^{14}$C]20:4n-6 acids were incubated, omitting the necessary cofactors for long-chain acyl-CoA synthetase (CoA and ATP) in the IM (Table 1). In these controls, [1-$^{14}$C]18:0, [1-$^{14}$C]18:2n-6, and [1-$^{14}$C]20:4n-6 acids were either incorporated into the nuclei or remained in the IM, exclusively as FFA. These results indicate that the esterification of 18:0, 18:2n-6, and 20:4n-6 into liver nuclear TG, DG, CE (only for 18:2n-6), and PL follows an acyl-CoA-dependent pathway. The incorporation profiles of fatty acids into N were not affected by the presence of cytosol in the incubation mixture as shown in Figure 2.

*PC molecular species of liver cell nuclei.* Typical reversed-phase HPLC (RHPLC) with an ELSD separation of PC molecular species of liver nuclei is illustrated in Figure 3A. In using this procedure, 15 molecular species of PC were identified and quantitated. They are shown in Table 2. The most abundant molecular species are 18:0-20:4 (36.7%), 16:0-20:4 (19.6%), 16:0-18:2n-6 (13.4%), and 18:0-18:2n-6 (11.1%). Arachidonic acid is mainly esterified in four molecular species at the *sn*-2 position of PC: 18:0-20:4, 16:0-20:4, 18:1-20:4, and 18:2-20:4, and in a minor species (0.25%) at both positions *sn*-1 and *sn*-2 in a 20:4-20:4 molecular species. Diarachidonyl PC molecular species coeluted with 18:2-22:6 species in approximately equal amounts, as revealed by the fatty acid analysis of peak 1 (Fig. 3A) which was identified

**TABLE 1**
**Effect of ATP and CoA on [1-$^{14}$C]Fatty Acid Incorporation[a]**

| Incubation conditions | Incorporation of radioactivity as FFA (%) | | | | | |
|---|---|---|---|---|---|---|
| | [1-$^{14}$C]18:0 | | [1-$^{14}$C]18:2n-6 | | [1-$^{14}$C]20:4n-6 | |
| | N*c* | IM*c* | N*c* | IM*c* | N*c* | IM*c* |
| With ATP and CoA | 40 | 100 | 12 | 53 | 10 | 93 |
| Without ATP and CoA | 100 | 100 | 100 | 100 | 100 | 100 |

[a]Liver nuclei were incubated with [1-$^{14}$C]18:0, [1-$^{14}$C]18:2n-6, or [1-$^{14}$C]20:4n-6 fatty acids with or without ATP and CoA for 20 min. After the incubation the samples were assayed as described in Figure 2. Results are presented as a percentage of the total $^{14}$C radioactivity incorporated as free fatty acids into nuclear lipids (N*c*: chloroform phase) and into the incubation mixture (IM*c*: chloroform phase).

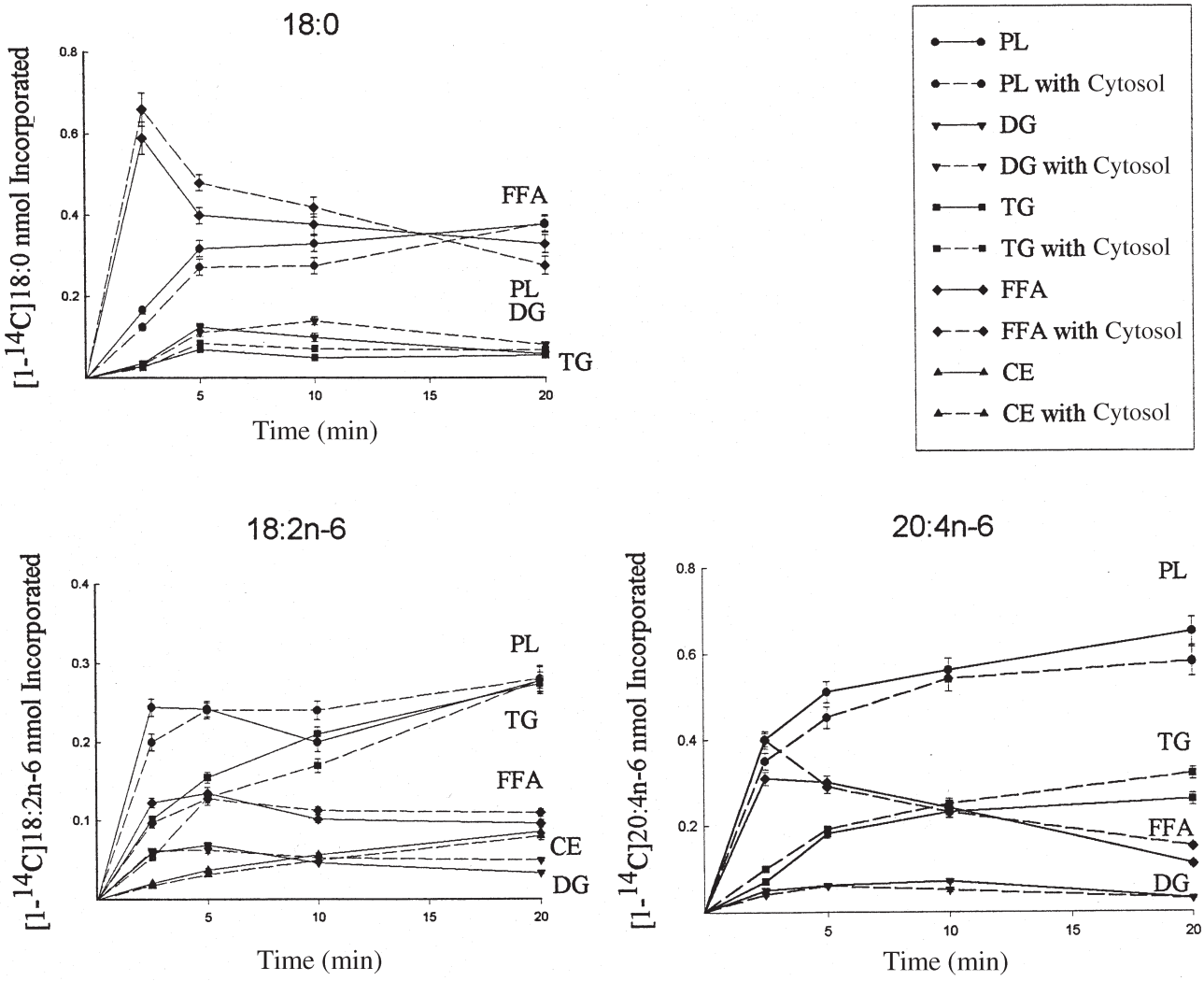


**FIG. 2.** Time-course incorporation of [1-$^{14}$C]18:0, [1-$^{14}$C]18:2n-6, or [1-$^{14}$C]20:4n-6 fatty acids into nuclear lipid classes. Liver nuclei were incubated with [1-$^{14}$C]18:0, [1-$^{14}$C]18:2n-6, or [1-$^{14}$C]20:4n-6 fatty acids with or without cytosol. After the incubation the fraction containing nuclei was separated as described in Figure 1; lipids were extracted and separated by thin-layer chromatography. Scanning of the thin-layer plates was performed as described in the Materials and Methods section. The $^{14}$C was recovered in phospholipids (PL) and in neutral lipids as triacylglycerols (TG), diacylglycerols (DG), cholesterol esters (CE), and free fatty acids (FFA). Results are the mean of five experiments ± SE.

by comparison with a commercial standard of 20:4-20:4 PC and by comparison with literature data (35).

*Incorporation of [1-$^{14}$C]20:4n-6-CoA into PC molecular species.* Liver cell nuclei were incubated *in vitro* with 2, 4, and 8 µM [1-$^{14}$C]20:4n-6-CoA, and the typical RHPLC-ELSD separation of labeled PC molecular species is shown in Figure 3B. From the 15 molecular species of liver nuclear PC, five peaks were labeled and identified by means of HPLC–mass chromatogram of each sample as described in the Materials and Methods section. Labeled peak 1 was identified with a commercial standard of 20:4-20:4 PC as described above.

Specific radioactivity of the molecular species of liver nuclear PC is presented in Table 2. The distribution of radioactivity among the five peaks was the same for the three concentrations of [1-$^{14}$C]20:4n-6-CoA tested. Therefore, data from these samples are presented together. These results could be due to the fact that [1-$^{14}$C]20:4n-6-CoA esterified into PL was saturated at all concentrations analyzed and that the excess of acyl-CoA added to the incubation and not esterified into PL, TG, and CE was hydrolyzed to FFA by nuclear acyl-CoA hydrolase (36) as shown in Figure 4.

The molecular species of PC showing the highest specific radioactivity after the incubation of liver nuclei with [1-$^{14}$C]-20:4n-6-CoA were 20:4-20:4 and, in a minor proportion, 18:2-20:4. In liver nuclei, 20:4-20:4 PC is quantitatively the minor molecular species, since it only represents 0.25 µmol% of total PC. On the other hand, the most abundant molecular species of liver nuclei PC, 18:0-20:4 and 16:0-20:4, showed the lowest specific radioactivity (Table 2).

These results demonstrated that liver cell nuclei can utilize exogenous 20:4n-6-CoA for the synthesis of molecular species with 20:4n-6 at the *sn*-2 position, although the most actively and newly synthesized PC contained 20:4n-6 at both

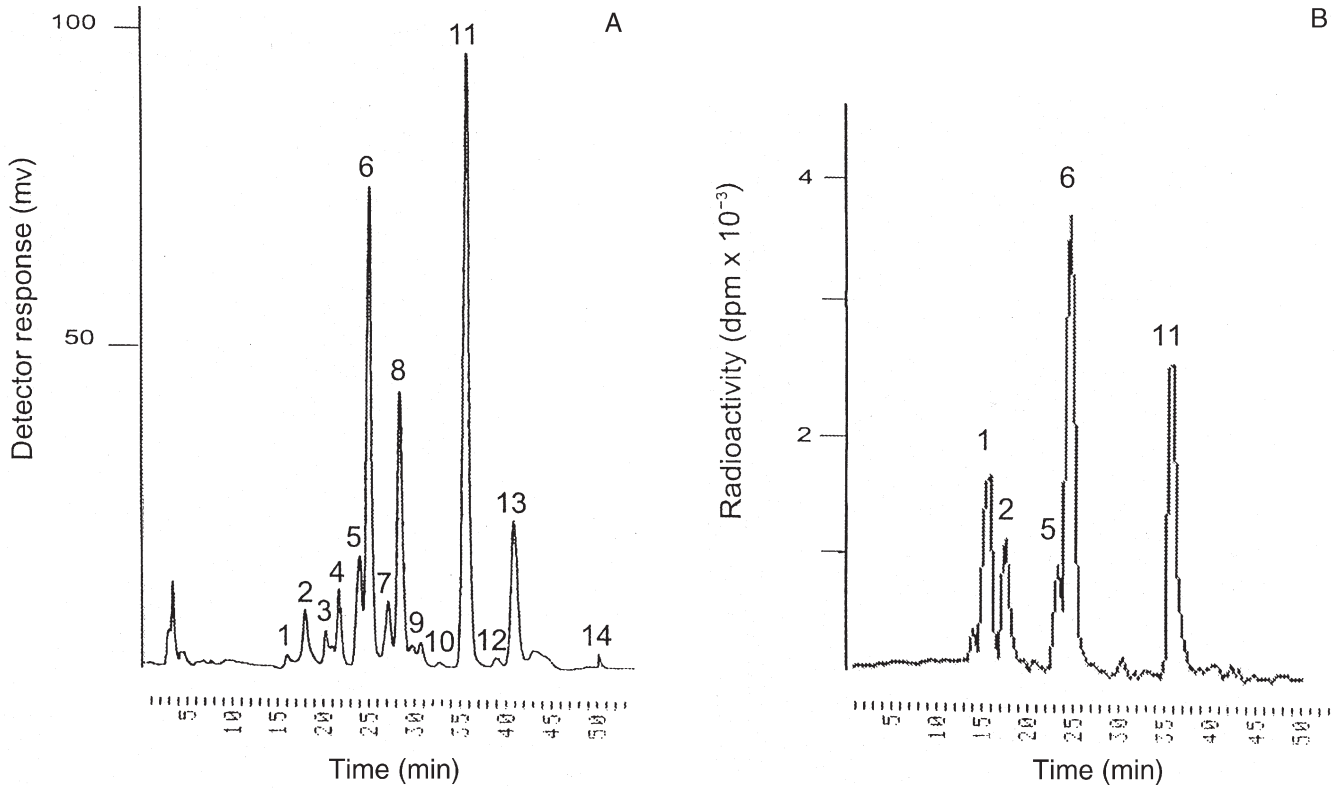


**FIG. 3.** Molecular species of phosphatidylcholine (PC) from liver cell nuclei. Molecular species of PC were analyzed by reversed-phase high-performance liquid chromatography as described in the Materials and Methods section. The identification and quantitation of each peak is presented in Table 2. (A) Typical chromatogram of mass distribution using evaporative light-scattering detector. (B) Labeled molecular species of PC after incubation of liver nuclei *in vitro* with [1-$^{14}$C]20:4n-6-CoA.

**TABLE 2**
**Incorporation of [1-$^{14}$C]20:4n-6-CoA in Phosphatidylcholine Molecular Species of Liver Cell Nuclei**

| Peak[a] | Molecular species | Incorporation[b] µmol% ($n$ = 10) | dpm % ($n$ = 5) | dpm·µmol$^{-1}$ ($n$ = 5) |
|---|---|---|---|---|
| **1** | **20:4-20:4** | **0.25 ± 0.03** | **15.5 ± 0.7** | **17.3 ± 1.1** |
| 1 | 18:2-22:6 | 0.25 ± 0.02 | — | — |
| **2** | **18:2-20:4** | **2.1 ± 0.2** | **11.2 ± 0.9** | **1.5 ± 0.1** |
| 3 | 18:2-18:2 | 1.9 ± 0.1 | — | — |
| 4 | 16:0-22:6 | 3.6 ± 1.7 | — | — |
| **5** | **18:1-20:4** | **4.7 ± 0.6** | **8.9 ± 0.4** | **0.54 ± 0.03** |
| **6** | **16:0-20:4** | **19.6 ± 0.6** | **38.5 ± 2.1** | **0.56 ± 0.04** |
| 7 | 18:1-18:2 | 2.8 ± 0.3 | — | — |
| 8 | 16:0-18:2 | 13.4 ± 0.6 | — | — |
| 9 | 16:0-20:3 | 0.2 ± 0.03 | — | — |
| 10 | 18:0-22:6 | 1.7 ± 0.4 | — | — |
| **11** | **18:0-20:4** | **36.7 ± 1.9** | **30.4 ± 2.3** | **0.22 ± 0.01** |
| 12 | 16:0-18:1 | 1.1 ± 0.2 | — | — |
| 13 | 18:0-18:2 | 11.1 ± 0.7 | — | — |
| 14 | 18:0-18:1 | 0.15 ± 0.01 | — | — |

[a]Peak numbers correspond to those shown on the HPLC chromatogram in Figure 3. In peak 1, two different molecular species coeluted from the HPLC (18:2-22:6 and 20:4-20:4).
[b]Data were recalculated from peak areas (µmol%), dpm collected per peak (dpm %), and specific radioactivities calculated from total dpm and total µmol (dpm·µmol$^{-1}$). Values are means of $n$ incubations analyzed in duplicate. Boldface corresponds to molecular species with arachidonic acid. Disintegrations per minute, dpm.

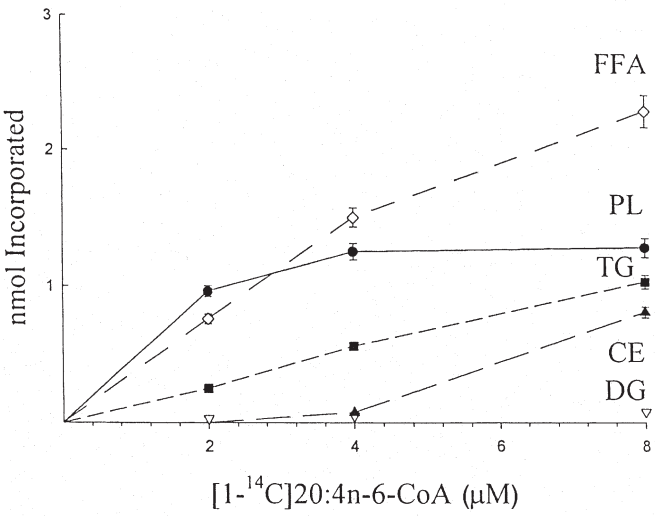


**FIG. 4.** Incorporation of [1-$^{14}$C]20:4n-6-CoA into nuclear lipid classes. Liver nuclei were incubated with different concentrations of [1-$^{14}$C]20:4n-6-CoA for 10 min. Nuclear lipids were extracted and separated as described for Figure 2. Results are the mean of five experiments ± SE. ●, PL; ▽, DG; ■, TG; ◇, FFA; ▲, CE. For abbreviations see Figure 2.

the *sn*-1 and *sn*-2 positions of the molecule. As Schmid *et al.* reported (35), the high specific radioactivity in this minor species would indicate that 20:4-20:4 PC was synthesized *de novo via* phosphatidic acid.

## DISCUSSION

In addition to the traditional roles of fatty acids as nutrients and sources of metabolic energy and structural components of complex lipids, fatty acids *per se* have physiological and pathological effects; they also regulate gene expression.

Regulation of gene expression by fatty acids is an event that takes place inside the nuclei of eukaryotic cells. It may be produced by a direct effect of fatty acids or fatty acyl-CoA (9,37) over gene expression, or through the activation of factors translocated into the nucleus (37). For instance, peroxisome proliferator-activated receptor-α can be activated by fatty acids (37), and PUFA-receptor element may regulate gene expression of stearoyl-CoA desaturase (9). On the other hand, hepatocyte nuclear factor-4α (HNF-4α) may bind acyl-CoA and be activated (38). Fatty acids in the nuclei may come from cytosol, be hydrolyzed from other nuclear lipids, or be synthesized in the same nuclei. Since information is scarce on nuclear fatty acid metabolism, on the precise topology of nuclear lipid metabolism, and on the relationship between nuclear lipids and cell functions, the aim of the present study was to investigate the incorporation and distribution of stearic (18:0), linoleic (18:2n-6), and arachidonic (20:4n-6) acids with or without the presence of the cytosol fraction in the presence of ATP and CoA.

The results showed that fatty acids tested were taken up by the nuclei and esterified into nuclear lipids by an acyl-CoA pathway. Fatty acids would be activated to the corresponding fatty acyl-CoA esters by the nuclear fatty acyl-CoA synthetase (19). We found different patterns of incorporation and esterification for 18:0, 18:2n-6, and 20:4n-6 fatty acids, showing a nuclear selectivity for them (Figs. 1 and 2). From all fatty acids tested, 20:4n-6 was the most esterified into nuclear lipids with respect to 18:2n-6 and 18:0.

In this study, the profiles of fatty acid incorporation and distribution into nuclear lipids were not affected by the presence of cytosol in the incubation mixture (Fig. 2). In consequence cytosolic proteins are apparently not necessary in this process. One of the reasons for which a cytosol fraction was added to the incubation mixture was to test the effect of some cytoplasmic proteins, since under physiological conditions, and as a result of their low solubility in water, fatty acids are transported through extracellular and intracellular aqueous spaces bound to albumin (39) and cytoplasmic fatty acid-binding proteins (FABP) (40), respectively. Recently, a nuclear FABP (41) and an acyl-CoA binding protein (37) have also been described.

In agreement with our results, Baker and Chang (42,43) found that neuronal nuclei isolated from cerebral cortices of rabbits esterified *in vitro* [$^{14}$C]oleate and [$^{3}$H]arachidonate into complex lipids. The incorporation was dependent upon ATP, CoA and acyl-CoA synthetase. A nuclear acyl-CoA synthetase was also observed by Stadler and Franke in chicken erythrocytes (44). In contrast, Surette and Chilton (45) found that isolated nuclei of human monocyte-like THP-1 were unable initially to incorporate arachidonic acid into their PL in the absence of cellular cytosol. These authors reported that this inability was due to the lack of fatty acyl-CoA synthetase and/or acyltransferase activity in their nuclear preparation of these cells, and that these enzymes would be present in their cytosol preparation. These results would indicate a different mechanism for the lipid nuclear metabolism between liver and cerebral cortex and inflammatory cells. Nuclei from liver (19) and cerebral cortex (42,43) have enzymatic activity of acyl-CoA synthetase whereas inflammatory cell nuclei would lack this enzymatic activity (45).

In our experiments, the 18:0 fatty acid was directly incorporated from the IM into the nuclei as FFA, whereas 20:4n-6 was initially incorporated as FFA and esterified to PL. The acids 18:0 and 20:4n-6 were mainly esterified into PL, and in a minor proportion into TG and DG by an acyl-CoA pathway. This incorporation increased throughout the incubation time (Fig. 2).Therefore, these results are in agreement with a previous report (20), showing that 20:4n-6 synthesized *in vitro* from 20:3n-6 by liver nuclear Δ5 desaturase is also esterified mainly into PL, and in a lesser proportion into TG and DG. On the other hand, unlike liver nuclei, neuronic nuclei from the cerebral cortex esterify 18:1 and 20:4n-6 into TG in a greater proportion than into PL, whereas in these cell microsomes, 20:4n-6 is incorporated mainly into PL with respect to TG (43).

The main difference found between 18:2n-6 and 18:0 and 20:4n-6 incorporation into nuclear lipids was that 18:2n-6 was largely esterified into both PL and TG (Fig. 2). Besides, 18:2n-6 was also esterified to CE. These results indicate a specific selectivity in nuclear fatty acid esterification.

It is known that all cells readily take up FFA from the culture medium and incorporate them into cellular PL and TG (46). In particular, when arachidonic acid is taken up by mammalian cells, it is initially esterified into nuclear glycerolipids, then it will regulate gene expression (47) and/or move into other cellular compartments as suggested by Schievella *et al.* (17).

In nuclei, most lipids are PL and structural components of nuclear membranes, although there are also PL in a minor proportion that are associated with chromatin and nuclear matrix (48–50). The main nuclear PL is PC, which constitutes over 56% of liver nuclear glycerophospholipids (data not shown). Taking into account that arachidonic acid was mainly esterified into PL (Fig. 2), we studied arachidonic acid nuclear pools analyzing PC molecular species. In using RHPLC-ELSD, 15 molecular species of PC were identified and quantified as shown in Table 2. The most abundant nuclear molecular species of PC contained arachidonic acid at the *sn*-2 position, and they were: 18:0-20:4 (36.7%) and 16:0-20:4 (19.6%). They were followed by less abundant molecular species containing linoleic acid also at the *sn-2* position:

16:0-18:2n-6 (13.4%) and 18:0-18:2n-6 (11.1%). The main PC nuclear pools of arachidonic acid are the following molecular species: 18:0-20:4, 16:0-20:4, 18:1-20:4, 18:2-20:4, these being arachidonic acid esterified at the *sn*-2 position of glycerol. A PC molecular species was also found, though in a minor proportion (0.25 %), in which both *sn*-1 and *sn*-2 positions were esterified to arachidonic acid. The pattern of molecular species of nuclear PC is the same as that in the whole organ (data not shown).

These results led us to consider that an active transesterification of 20:4n-6 exists in liver nuclei. Therefore, to get further information about the incorporation of arachidonic acid into nuclear cell pools, we incubated liver nuclei *in vitro* with [1-$^{14}$ C]20:4n-6-CoA and determined the labeled PC molecular species (Fig. 3B). From the 15 molecular species of liver nuclear PC, five peaks were labeled and identified. The specific radioactivity of these are presented in Table 2.

As expected, the most abundant molecular species containing arachondic acid:18:0-20:4 and 16:0-20:4 PC incorporated the highest label, but by far the highest specific radioactivity was found in the double 20:4-20:4 PC molecular species, which is a minor molecular species. Schmid *et al*. (35) reported that when the percentage of radioactivity exceeds the mol percentage, it is assumed that the species are primarily formed through *de novo* synthesis. In this regard, the same mechanism would be active in liver nuclei.

Generally, double 20:4-20:4 PC molecular species are present in very low levels in different animal tissues (51–53). However, Schmidt *et al*. (35,54) have shown that when arachidonic acid is offered to cells, the first step is the *de novo* synthesis of a molecular species of PC and phosphatidylethanolamine having arachidonic acid bound to both the *sn*-1 and *sn*-2 positions. But once 20:4-20:4 PC is formed, a remodeling occurs at the *sn*-1 position, with the result that the predominant species are 18:0-20:4n-6 and 16:0-20:4 PC. An analogy can be made with cell nuclei; then we can infer that a remodeling process is also active in the nuclei. That is, the 20:4-20:4 PC molecular species in liver cell nuclei is a minor component because it is synthesized and apparently remodeled by translocation. The *sn*-1 position would be mainly replaced by 16:0 and 18:0, the fatty acids that are generally found at this position.

It is not yet clear whether these diunsaturated PC molecular species that are in minor proportion in the cell and nuclei have physiological functions *per se*, or if they are only metabolic intermediates in *de novo* synthesis and lipid remodeling. By the way, PL containing arachidonic acid at the *sn*-1 position of glycerol are particularly important since these PL are precursors of anandamide (*N*-arachidonylethanolamine), which is a ligand of the cannabinoid receptor in mammalian tissues (55).

It is also important to remark that the enzymes responsible for metabolizing arachidonic acid to prostaglandins and leukotrienes have also been localized in the nuclear envelope (56–58); and phospholipase $A_2$, which hydrolyzes 20:4n-6 from PL and provides this substrate for these processes, translocates to the nuclear membrane (17). This fact implies that the nucleus is an important site for the control of some arachidonic acid cellular effects.

In conclusion, these findings indicate that liver cell nuclei possess the necessary enzymes to incorporate exogenous saturated and unsaturated fatty acids into nuclear lipids by an acyl-CoA-dependent pathway, showing a nuclear specificity for each fatty acid in these processes. Liver cell nuclei can also utilize exogenous 20:4n-6-CoA for the synthesis of the main molecular species of PC, the main species with 20:4n-6 at the *sn*-2 position, and the minor species with 20:4n-6 at both the *sn*-1 and *sn-2* positions of the molecule. The labeling pattern of 20:4-20:4 PC would indicate that this molecular species is synthesized mainly by the *de novo* pathway.

## ACKNOWLEDGMENTS

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# Purification and Characterization of an Extracellular Lipase from *Penicillium candidum*

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**ABSTRACT:** *Penicillium candidum* produces and secretes a single extracellular lipase with a monomer molecular weight of 29 kDa. However, this enzyme forms dimers and higher molecular weight aggregates under nondenaturing conditions. The lipase from *P. candidum* was purified 37-fold using Octyl-Sepharose CL-4B and DEAE-Sephadex columns. The optimal assay conditions for lipase activity were 35°C and pH 9. The lipase was stable in the pH range of 5–6 with a pI of 5.5, but rapid loss of the enzyme activity was observed above 25°C. Tributyrin was found to be the best substrate for the *P. candidum* lipase, among those tested. Metal ions such as $Fe^{2+}$ and $Cu^{2+}$ inhibited enzymatic activity and only $Ca^{2+}$ was able to slightly enhance lipase activity. Ionic detergents inhibited the activity of the enzyme, whereas nonionic detergents stimulated lipase activity.

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Lipases [EC 3.1.1.3] are enzymes that hydrolyze ester bonds at oil–water interfaces producing mono- and diacylglycerols, glycerol, and free fatty acids (1). These enzymes have received much attention during the last 10 yr because of their potential industrial applications. Examples of their uses are resolution of racemic mixtures, production of optically active compounds, and ester synthesis. Currently they are mainly used in the dairy industry. In this field lipases have applications in flavor development of certain types of cheese, flavor enhancement, cheese ripening acceleration, and production of cheese-like products (2). Filamentous fungi are the preferred sources for industrial uses of lipases because their enzymes are extracellular. The most utilized species belong to the genera *Rhizopus*, *Rhizomucor*, *Geotrichum*, *Penicillium,* and *Aspergillus* (3).

*Penicillium candidum* is a deuteromycete that produces an extracellular, inducible lipase suitable for dairy flavor development. Previously, we reported some studies on enzyme formation in this fungus (3). In this paper we describe the purification of the lipase from *P. candidum* and the general characteristics of the purified enzyme.

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Abbreviations: DEAE, diethylaminoethyl; IEF, isoelectric focusing; MOPS, 3-[*N*-morpholino]propanesulfonic acid; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; U, units of lipase activity.

## EXPERIMENTAL PROCEDURES

*Strain. Penicillium candidum* was acquired from the Instituto de Investigaciones Biomédicas Culture Collection, U.N.A.M., Mexico. This strain was preserved as reported by Celerin and Fergus (4).

*Culture conditions.* All fermentations were performed at 27°C and 160 rpm in 500-mL Erlenmeyer flasks with 100 mL of "D" medium (1% glucose, 1% casamino acids, 0.2% $KNO_3$, 0.1% $KH_2PO_4$, 0.05% $MgSO_4$), using 0.2% olive oil as inducer.

*Enzyme assays. (i) Spectrophotometric assay*. The method previously described by Isobe *et al.* (5) was used to determine lipolytic activity using the spectrophotometric measurement of *p*-nitrophenol release. The assay was performed using 2.5 mM *p*-nitrophenyl laurate as substrate in 20 mM Tris-HCl, pH 7.2, and 2% Triton X-100. One milliliter of the reaction mixture was incubated for 10 min at 37°C. Optical density at 410 nm was read before and immediately after incubation. Activity against different *p*-nitrophenyl derivatives was analyzed using this same method with 2.5 mM of each substrate. One unit of lipase activity is defined as the amount of enzyme which liberates 1 μmol *p*-nitrophenol/min under these assay conditions.

*(ii) Emulsion method*. Lipase activity was measured by determination of butyric acid released during hydrolysis as determined by measuring change in pH (6). Tributyrin (80 μmol) was emulsified in 3 mL of Tris-HCl 20 mM, pH 8. This mixture was incubated for 30 min at 37°C under constant agitation. Lipase-catalyzed hydrolysis of tributyrin was followed potentiometrically with a Beckman (Fullerton, CA) pH-meter model 3500. Activity against mono-, di-, and triglycerides was tested using the same reaction mixture and incubation conditions but changing the substrate (80 μmol each). One unit is defined as the amount of enzyme which liberates 1 μmol free fatty acid/min under the assay conditions.

*Protein measurement.* Protein in the enzyme preparation was determined by the method of Lowry *et al*. (7) using bovine serum albumin as standard.

*Electrophoresis*. Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions was performed as described by Laemmli (8), in 12% running and 4% stacking gels. Protein was stained using the silver stain method reported by Rosenberg (9). Low molecular weight markers were used with the following reference proteins: phosphorylase B (97.4 kDa),

bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) (Bio-Rad, Richmond, CA).

*Zymography. In situ* lipase activity was detected by zymography following sodium dodecyl sulfate (SDS)-PAGE. The gel was placed onto a solid lipase detection medium containing tributyrin and incubated at 37°C for 60 min (10).

*Enzyme purification. Penicillium candidum* was grown for 72 h, and culture medium was filtered through Whatman #1 paper (Whatman, Maidstone, England). The solution was concentrated by inverse dialysis against powdered sugar overnight at 4°C. Ammonium sulfate was added until the solution reached 20% saturation at 0°C overnight with agitation. The saturated solution was centrifuged for 20 min at 20,000 × *g* and 4°C. Ammonium sulfate was then gradually added to the supernatant until it reached 75% saturation under the same conditions. The solution was centrifuged for 20 min and the activity-containing precipitate was resuspended in 20 mM citrate buffer, pH 6.5.

The fraction containing enzymatic activity was adjusted to 1 M $(NH_4)_2SO_4$ and was applied to an Octyl Sepharose CL-4B column (Pharmacia, Uppsala, Sweden) (11 × 215 mm) previously equilibrated with 20 mM citrate buffer pH 6.5 containing 1 M $(NH_4)_2SO_4$. The column was washed with the same buffer and the enzyme was eluted by a decreasing step gradient of $(NH_4)_2SO_4$ in the same buffer, from 1 to 0 M, at a flow rate of 0.75 mL/min. Activity in each fraction was measured by the spectrophotometric method as described previously. The active fractions were combined and concentrated to 2 mL using an Amicon centriflo membrane cone (Amicon, Beverly, MA). The concentrated enzyme solution was loaded onto a DEAE-Sephadex A-50 column (Sigma, St. Louis, MO) pre-equilibrated with 50 mM citrate buffer, pH 6.5 containing 1% Nonidet P-40 (USB, Cleveland, OH). The column was washed with the same buffer to remove the unadsorbed material. The enzyme was eluted by an increasing step gradient of NaCl (0–500 mM) in the same buffer at a flow rate of 0.5 mL/min. Lipase fractions were pooled and concentrated by ultrafiltration with an Amicon centriflo membrane cone. As recovery of enzyme activity after the DEAE-column was low, lipase characterization was performed using the enzyme obtained after the octyl-sepharose column.

*Isoelectric focusing*. Electrofocusing was performed on a Phast System according to the manufacturer's instructions using IEF 3-9 Phast Gels (Pharmacia). Standards used were trypsinogen (pH 9.3), basic lentin lectin (pH 8.65), lentin lectin middle (pH 8.45), lentin lectin acidic (pH 8.15), basic myoglobin (pH 7.35), acidic myoglobin (pH 6.85), human carbonic anhydrase B (pH 6.55), bovine carbonic anhydrase B (pH 5.85), β-lactoglobulin A (pH 5.2), and amylglucosidase (pH 3.5) (Bio-Rad). Proteins were detected by Coomassie staining and zymography.

*Effect of pH and temperature on lipase activity*. Enzyme aliquots obtained after the octyl-sepharose column were incubated without substrate for 10 min at different temperatures from 4 to 75°C. Remaining activity was measured by the spectrophotometric assay. The optimal temperature for activity was determined by increasing the temperature in steps of 5°C, from 20 to 65°C.

The effect of pH on enzyme stability was tested using buffered solutions adjusted to different pH values (acetate buffer pH 4–6 and phosphate buffer pH 6–9) and incubating without substrate for 24 h at 37°C. After incubation the pH values remained constant. The remaining activity was determined by the spectrophotometric method.

Optimal pH was analyzed by the emulsion method using different pH values from 5 to 11 (citrate buffer from 5 to 6, 3-[*N*-morpholino]propanesulfonic acid (MOPS) buffer from 7 to 9 and bicarbonate buffer from 9 to 11). In order to avoid buffer effects on the enzyme assays, the pH optimum was determined using several sets of buffers obtaining similar results: Tris-HCl pH 7–8, glycine pH 9–10, MOPS, 7–9, bicarbonate pH 9–11 and borate pH 9–10.

*Effect of metal ions.* The effect of metal ions on lipase activity was measured using the spectrophotometric assay. The reaction mixture was supplemented with various salts at a final concentration of 1 or 10 mM. The salts tested were monovalent, such as KCl and NaCl, and divalent, such as $CaCl_2$, $MgCl_2$, $MnCl_2$, $SrCl_2$, $CoCl_2$, $CuCl_2$, and $FeCl_2$.

*Effect of detergents.* The enzyme solution was incubated for 1.5 h at 37°C in 20 mM citrate buffer, pH 5.5 containing 0.1 or 1% (wt/vol) of each detergent. Lipase activity was tested at 0 and 1.5 h by the spectrophotometric method. The detergents assayed were Nonidet P-40 (USB), Triton X-100, Tween 20, Tween 80 (Sigma), sodium cholate, sodium deoxycholate (Research Organics, Cleveland, OH), and Zwittergent (Fluka, Buchs, Switzerland).

*N-terminal sequence analysis*. The N-terminal sequence of *P. candidum* lipase was analyzed using an automated Protein Sequencer 491, Procise (Applied Biosystems, Foster City, CA), consisting of a gas-phase sequencer and liquid sequencer.

## RESULTS

*Lipase purification*. In order to obtain lipase from *P. candidum,* the fungus was grown for 72 h until late log phase. The extracellular medium was concentrated and then precipitated with ammonium sulfate. The sample obtained after precipitation with ammonium sulfate was applied to an octyl-sepharose chromatography column. The elution pattern showed a single peak with lipase activity (data not shown). This peak was eluted only after using 1% detergent, which indicates the hydrophobic character of this enzyme. The active samples were pooled and applied to a DEAE-Sephadex column. The enzyme was purified to homogeneity (about 36.7-fold) with a low recovery of activity (0.8%). A summary of the purification is shown in Table 1. Purified preparations had specific activities of approximately 14,000 units of lipase activity (U/mg).

*Enzyme characterization*. In order to determine the size of the lipase, we analyzed the protein using denaturing and non-

**TABLE 1**
**Summary of the Purification of the *Penicillium candidum* Lipase[a]**

| Purification step | Total protein (mg) | Total activity (U) × $10^3$ | Specific activity (U/mg) | Yield (%) | Purification factor (%) |
|---|---|---|---|---|---|
| Crude sample | 991.89 | 375.81 | 378 | 100.0 | — |
| $(NH_4)_2SO_4$ precipitation | 9.74 | 78.21 | 8,030 | 20.8 | 21.2 |
| Octyl-Sepharose CL-4B[b] | 0.74 | 11.56 | 15,715 | 3.8 | 41.5 |
| DEAE-Sephadex A-50[c] | 0.23 | 3.25 | 13,898 | 0.8 | 36.7 |

[a]Enzyme activity was determined by a spectrophotometric method, using 2.5 mM *p*-nitrophenyl laurate in 20 mM Tris-HCl, pH 7.2, and 2% Triton X-100 at 37°C.
[b]Pharmacia, Uppsala, Sweden.
[c]Sigma Chemical, St. Louis, MO.

denaturing PAGE. The enzyme was detected by silver staining and the activity by zymography after electrophoresis. The molecular weight of the enzyme was 29 kDa as determined by SDS-PAGE (Fig. 1). However, under nondenaturing conditions, the purified lipase migrated as a dimer and tetramer with molecular weights of approximately 64 and 130 kDa, respectively (Fig. 2). This behavior has been observed in other lipases from the genus *Penicillium* (11–14). Attempts to disaggregate the lipase using organic solvents such as ethanol and isopropanol failed due to denaturation of the enzyme causing loss of activity. Di- and tetra-aggregates showed lower lipase activity than the monomer on zymography assays (data not shown). Electrofocusing assays followed by zymography showed the isoelectric point to be 5.5.

*Effect of pH and temperature on lipase activity and stability.* The pH optimum for *P. candidum* lipase was studied in a range from pH 5 to 11 using the emulsion method. The enzyme was found to be most active at pH 9.0 and was most stable in the pH range from 4 to 6 when incubated at 37°C for 30 min (Fig. 3).

The effects of temperature on enzyme activity were examined through a range of 4 to 75°C using the spectrophotometric method. The optimal temperature was about 35°C, as shown in Figure 4. This temperature optimum is similar to that of other fungal lipases such as those from *Fusarium* sp. YM-30 (15), *P. caseicolum* (16), and *P. simplicissimum* (13).

Stability was tested after incubation at different temperatures for 10 min. The enzyme was stable at temperatures below 25°C. At 35°C the lipase retained 70% of its full activity while at 75°C, the enzyme completely lost activity. Remaining activity was determined using spectrophotometric assay.

*Effect of metal ions*. Table 2 indicates the effects of various metal ions on enzyme activity as determined by the spectrophotometric method. Calcium chloride at 1 mM was the only salt with a significant positive effect on lipase activity, although at 10 mM the effect became slightly negative. When $FeCl_2$ and $CuCl_2$ were added, the effect on enzyme activity was strongly negative at both concentrations tested. A negative effect on the lipase was observed upon adding $CoCl_2$, $MnCl_2$, and KCl only at a concentration of 10 mM. With NaCl, $MgCl_2$, and $SrCl_2$ the lipolytic activity was not affected.

*Substrate specificity*. To study the substrate specificity of *P. candidum* lipase, we tested esterase and lipase activities by using *p*-nitrophenyl derivatives and triglycerides, respectively. Hydrolysis of mono-, di-, and triglycerides with different fatty acid chain lengths was tested by the emulsion method. The *P. candidum* lipase preferred triglycerides to

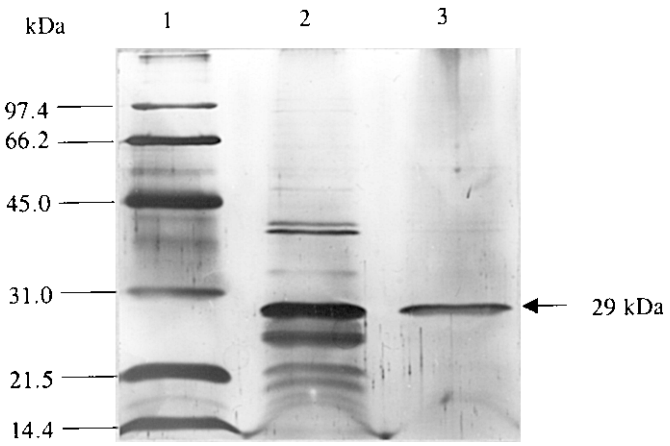


**FIG. 1.** Sodium dodecylsulfate-polyacrylamide gel electrophoresis (12%) of *Penicillium candidum* lipase. Lane 1: low molecular weight marker proteins; lane 2: octyl-sepharose eluate fraction; lane 3: DEAE-Sephadex eluate fraction. The arrow indicates the lipase.

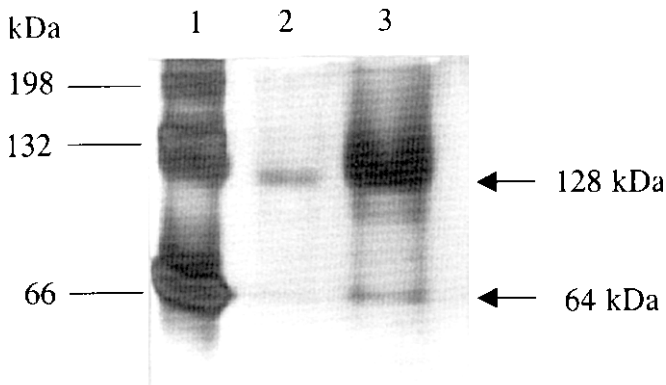


**FIG. 2.** Nondenaturing gel electrophoresis (12% acrylamide) conditions of the purified lipase from *Penicillium candidum* detected by silver staining. Lane 1: molecular weight marker for native electrophoresis (bovine serum albumin); lanes 2 and 3: purified lipase (0.18 and 2.9 μg of protein, respectively). Arrows indicate bands of di- and tetramers of the purified lipase.

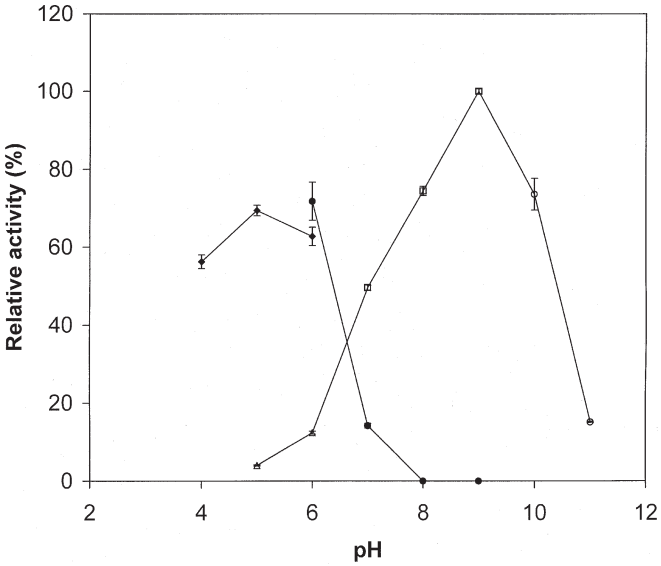


**FIG. 3.** pH effect on *Penicillium candidum* lipase activity. Optimal pH was determined using 20 mM citrate buffer pH 5–6 (△), 20 mM 3-[*N*-morpholino]propanesulfonic acid pH 7–9 (□), and 20 mM bicarbonate buffer pH 9–11 (○). Stability at different pH values: (◆) at pH 4–6 in acetate buffer, (●) at pH 6–9 in phosphate buffer. Optimal pH was determined by the emulsion method and pH stability by the spectrophotometric method. In both cases the reaction mixture was incubated at 35°C.

mono- or diglycerides such as monocaprin, monolaurin, monoolein, dicaprin and diolein. These substrates were hydrolyzed at 2–18% compared to tributyrin, its best substrate. For example, tricaprin was 41.5% hydrolyzed and its mono- and diglyceride were 11 and 13.5% hydrolyzed, respectively. Among various triglycerides, *P. candidum* enzyme showed preference for tributyrin and to a lesser degree for tricaprin, trilaurin, and triolein (Table 3).

When measuring its activity on *p*-nitrophenyl derivatives, we found the enzyme showed higher activity against *p*-nitrophenyl palmitate compared to the other substrates. Although palmitate and stearate are insoluble, the enzyme displayed higher activity on a suspension of these monoesters (Table 3).

*Effect of detergents.* Because the purified *P. candidum* lipase aggregates, causing a decrease in enzyme activity, we decided to test the effect of several detergents on its activity. *Penicillium candidum* lipase activity was assayed at concentrations of 0.1 and 1% (wt/vol) of several detergents

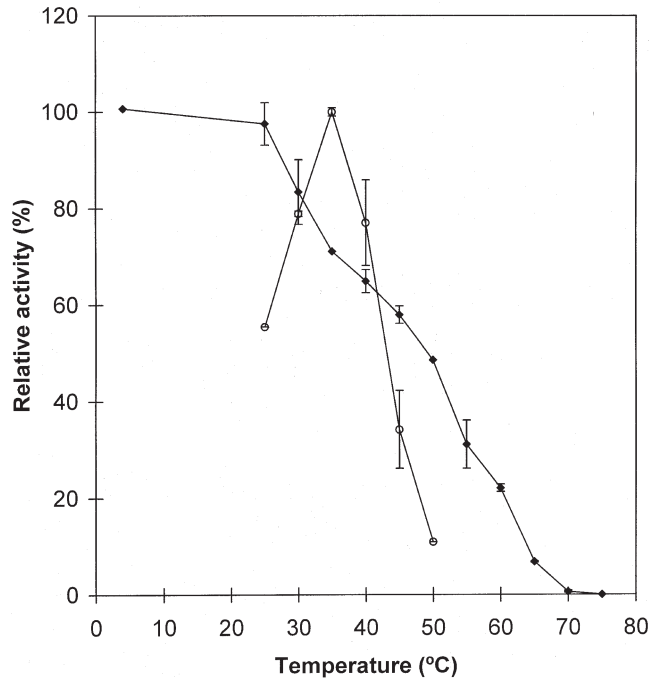


**FIG. 4.** Temperature effect on *Penicillium candidum* lipase activity. Optimal temperature (○), temperature stability (◆). Effects were determined by the spectrophotometric method using 20 mM citrate buffer pH 5.5.

**TABLE 2**
**Effect of Metal Ions on *Penicillium candidum* Lipase Activity**

| Substance | Relative activity during the assay (% ± SD)[a] | |
|---|---|---|
| | 1 mM[b] | 10 mM[b] |
| Control | 100.00 ± 3.07 | 100.00 ± 3.78 |
| $CaCl_2$ | 118.83 ± 3.07 | 94.68 ± 6.25 |
| $MgCl_2$ | 101.22 ± 3.23 | 97.08 ± 2.02 |
| $MnCl_2$ | 96.34 ± 0.70 | 74.76 ± 1.68 |
| $SrCl_2$ | 104.88 ± 3.92 | 95.14 ± 2.57 |
| $CoCl_2$ | 102.47 ± 2.23 | 57.07 ± 7.29 |
| $CuCl_2$ | 24.39 ± 1.22 | 4.85 ± 2.57 |
| $FeCl_2$ | 0.00 ± 0.00 | 0.00 ± 0.00 |
| KCl | 102.86 ± 5.08 | 79.87 ± 3.06 |
| NaCl | 101.22 ± 0.70 | 97.42 ± 5.64 |
| SDS | 97.56 ± 4.62 | 78.81 ± 3.21 |
| EDTA | 98.49 ± 0.29 | 54.05 ± 1.82 |

[a]The activities are expressed relative to the activity obtained without agent, and represent the arithmetic mean ± SD of at least three determinations.
[b]Final concentration in the reaction mixture. Enzyme activity was determined by the spectrophotometric method, using 2.5 mM *p*-nitrophenyl laurate in 20 mM Tris-HCl pH 7.2 and 2% Triton X-100 at 37°C. SDS, sodium dodecylsulfate.

**TABLE 3**
**Specificity of *Penicillium candidum* Lipase Using *p*-Nitrophenyl Derivatives and Triglycerides**

| Substrate | % Relative activity (% ± SD)[a] | |
|---|---|---|
| | *p*-Nitrophenyl derivatives[b] | Triglycerides[c] |
| 4:0 | 11.23 ± 0.29 | 100.00 |
| 6:0 | 2.86 ± 0.99 | 5.80 ± 0.57 |
| 8:0 | 6.99 ± 0.43 | ND |
| 10:0 | 27.55 ± 0.85 | 41.55 ± 1.93 |
| 12:0 | 47.53 ± 2.89 | 15.56 ± 0.94 |
| 14:0 | ND | 5.04 ± 0.41 |
| 16:0 | 100.00 ± 6.96 | 2.6 ± 0.31 |
| 18:0 | 72.31 ± 1.86 | 4.83 ± 3.86 |
| 18:1 | ND | 13.04 ± 2.89 |

[a]Activities are expressed relative to the activity obtained without agent, and represent the arithmetic mean ± SD of at least three determinations.
[b]Enzyme activity was measured by the spectrophotometric method using 2.5 mM of each substrate in 20 mM Tris-HCl, pH 7.2 with 2% Triton X-100 at 37°C.
[c]Lipase activity was determined by the emulsion method using 80 µmol of each triglyceride in 20 mM Tris-HCl, pH 8 at 37°C. The specific activity at 100% was 1,206.1 U/mg. ND, not determined.

**TABLE 4**
**Effect of Detergents (0.1 and 1.0% wt/vol) on Purified Lipase Activity**

| Detergent | Relative activity[a] (% ± SD)[b] | | | |
|---|---|---|---|---|
| | 0.1% | | 1% | |
| | 0 h | 1.5 h | 0 h | 1.5 h |
| None | 100.0 ± 15 | 100.0 ± 10 | 100.0 ± 13 | 100.0 ± 8 |
| Triton X-100[c] | 120.9 ± 7 | 134.0 ± 20 | 124.4 ± 6 | 104.7 ± 2 |
| Tween 20[c] | 117.5 ± 14 | 117.8 ± 6 | 104.3 ± 7 | 69.3 ± 9 |
| Tween 80[c] | 97.3 ± 12 | 71.4 ± 19 | 120.6 ± 5 | 65.9 ± 13 |
| Nonidet P-40[c] | 108.1 ± 14 | 127.3 ± 16 | 130.1 ± 7 | 106.2 ± 2 |
| Zwittergent[c] | 150.6 ± 10 | 162.9 ± 17 | 467.5 ± 19 | 17.5 ± 2 |
| Sodium deoxycholate | 110.8 ± 16 | 42.4 ± 26 | 554.8 ± 18 | 105.6 ± 24 |
| Sodium cholate | 87.1 ± 12 | 99.6 ± 7 | 168.7 ± 1 | 125.3 ± 9 |

[a]Effect of detergents was analyzed by incubation of lipase for 1.5 h at 37°C, in 20 mM citrate buffer (pH 5.5) with each detergent, and remaining activity was determined using the spectrophotometric method with *p*-nitrophenyl laurate as substrate.
[b]Activities are expressed relative to the activity obtained without detergent and represent the arithmetic mean ± SD of at least three determinations.
[c]Nonidet P-40 (USB, Cleveland, OH; Triton X-100, Tween 20, and Tween 80 (Sigma, St. Louis, MO); sodium cholate and sodium deoxycholate (Research Organics, Cleveland, OH); and Zwittergent (Fluka, Buchs, Switzerland).

(Table 4). At 0 h and concentrations of 0.1%, all detergents had a positive effect except Tween 80 and sodium cholate, which showed a negative influence on lipase activity. The highest activity was obtained with Zwittergent at this concentration. At 1%, the initial activity (0 h) was high with all detergents, with sodium deoxycholate having the highest positive effect. However, after 1.5 h all activities decreased compared to the activity at the beginning of incubation. The most drastic reduction was caused by Zwittergent; it decreased lipase activity by approximately 83% compared to the control.

*N-terminal sequence analysis*. The N-terminal amino acid sequence of the lipase from *P. candidum* was found to be: STAAGAAFPDLHHAAALS. This sequence has been compared to the sequences of N-terminal fungal lipases as deposited in the TrEMBL and Swissprot databases: (http://www.ebi.ac.uk/). The 18 N-terminal amino acids of the *P. candidum* lipase showed high identity to the sequence reported for the *P. expansum* lipase (11). However, *P. candidum* lipase showed low identities to the N-terminal amino acid sequences of other fungal lipases (*Fusarium, Humicola, Rhizopus, Rhizomucor,* and *P. camembertii*). *Penicillium candidum* lipase shared only the Phe and Ala residues in positions 8 and 16, respectively, with other filamentous fungi sequences (Table 5).

## DISCUSSION

The fungus *P. candidum* produced a single extracellular lipase with a molecular weight of 29 kDa as determined by SDS-PAGE. However, when the sample was electrophoresed under native conditions, the enzyme showed dimeric and tetrameric forms. This behavior was also observed during the purification of enzymes from *P. expansum* (11), *P. simplicissimum* (13), *P. citrinum* (12), and *P. chrysogenum* (14). As with these lipases, the use of detergents with the *P. candidum* lipase is necessary to avoid aggregation which causes loss of activity. Lipases from some species of *Bacillus*, *Pseudomonas,* and *Staphylococcus* (17,18), as well as many from the genus *Penicillium,* are susceptible to this problem (11,13, 14,19,20). In our case, *P. candidum* lipase formed di-, tetra-, and higher aggregates, which requires the addition of detergents for optimal activity. This suggests that disaggregation of the enzyme would allow the lipase to have more contact with its substrate. This could also be the reason for the low purification factor after anionic chromatography. Studies using other lipases reported that aggregation caused difficulties in the estimation of enzymatic specific activity (13).

The finding that the enzyme has a pH optimum of 9 for activity is uncommon. Most lipases, both intracellular and extracellular, appear to be optimally active between pH 6 and 7 (21). However, an alkaline pH optimum has been reported for some fungal lipases such as those from *P. caseicolum,* pH 9 (15); *Fusarium* sp. YM-30, pH 7–8 (14); *P. expansum,* pH 9 (11); and *A. flavipes,* pH 8.8 (21). This suggest that *P. candidum* lipase could be useful as an ingredient in household detergents. Although it is unstable at alkaline pH values (optimal stability pH 4–6), this could be avoided using microen-

**TABLE 5**
**Comparison of Sequence of the 20 N-Terminal Amino Acids of Lipase from *Penicillium candidum* and Other Fungi**

| Fungi[a] | | Number of amino acids |
|---|---|---|
| Fus | AVTVTTQDLSNFRFYLQHADAAYC | 1–24 |
| Hum | EVSQDLFNQFNLFAQYSAAAYC | 1–22 |
| Pcm | DVSTSELDQFEFWVQYAAASYY | 1–22 |
| Rhm | SIDGGIRAATSQEINELTYYTTLSANSYC | 1–30 |
| Rhd | SDHHKVVAATTAQIQEFTKYAGIAATAYC | 1–30 |
| Pex | AVAASAAFPDLXRAAKLSSA | 1–20 |
| **Pca** | **STAAGAAFPDLHHAAALS** | **1–18** |

[a]Fus: *Fusarium heterosporum,* Hum: *Humicola lanuginosa,* Pcm: *Penicillium camembertii,* Rhm: *Rhizomucor miehei,* Rhd: *Rhizopus delemar,* Pex: *Penicillium expansum,* Pca: *Penicillium candidum.*

capsulation techniques. There are many reports of fungal lipases with a pH stability between 5 and 8. However, incubation times were shorter than those tested in this work (24 h).

The lipase from *P. candidum* preferred *p*-nitrophenyl derivatives with acyl lengths of 16:0 and 18:0 as substrates, whereas *p*-nitrophenyl esters with short chains (10:0 or less) were poorly hydrolyzed. A similar specificity has been reported only for the *P. simplicissimum* lipase (13). However, when the *P. candidum* enzyme was tested in emulsified substrates, it showed a preference for triglycerides with fatty acid chains of different lengths such as tributyrin, tricaprin, trilaurin, and triolein. More importantly, of triolein, stearate, palmitate, and butyrate, only triolein was capable of inducing lipase formation (3). Therefore, we consider this enzyme is a true lipase. Furthermore, when this fungus was grown on solid medium containing triolein as sole carbon source, it produced clear zones of hydrolysis. However, the rate of hydrolysis was slower compared to that using tributyrin on the same solid medium. For this reason, although tributyrin is now considered a questionable substrate for lipase activity, it was important to include it in the activity determination by the emulsion method. Helistö and Korpela (22) analyzed the activity of lipases from different sources on *p*-nitrophenyl substrates and triolein. They also observed low correlation between hydrolysis of different *p*-nitrophenyl derivatives and triolein. These authors suggested that the differences in specificity between *p*-nitrophenyl substrates and triolein might be due to the fact that these kinds of enzymes often have both lipase and esterase activities (22). The *P. candidum* lipase specificity is very similar to that of lipases used for food applications such as *P. caseicolum* (15). In the food industry, it is important to generate delicate flavors in dairy products. These are obtained with the release of short fatty acids during hydrolysis. Therefore, the enzyme from *P. candidum* could be used for the production of cheese-like flavors and related dairy products.

The positive effect of $CaCl_2$ has been observed in other fungal enzymes like *P. expansum* (11). In contrast, calcium had a strong negative effect on the lipase from *P. citrinum* (12). In fungi, it has been suggested that calcium ion is necessary for enzyme folding but is not essential to its catalytic activity (19).

EDTA had a negative effect on *P. candidum* lipase activity, confirming that this enzyme could be a metalloenzyme. Both $Fe^{2+}$ and $Cu^{2+}$ strongly inhibit lipolytic activity. Their effects on the lipase were similar to those found for several microbial lipases such as the *Fusarium* sp. YM-30 (14), *A. oryzae* (23), *H. lanuginosa* (24), *P. citrinum* (17), and *Beauveria bassiana* (25) lipases. For *B. bassiana*, it was demonstrated that iron ions inhibit the lipase by direct binding, and this effect can be reverted by the use of chelating agents such as EDTA.

Many lipase genes have been cloned from mammalian tissues, fungi, and bacteria, and their primary structures have been deduced from the nucleotide sequences. These lipases are classified into several families on the basis of their homologies. Most fungal lipases can be classified into *R. miehei* and *G. candidum* families (26). However, *P. expansum* lipase cannot be classified into either of these two families, as was previously reported (11). The N-terminal amino acid sequence similarity of *P. candidum* lipase to *P. expansum* lipase reveals that they can be defined within a separate family of *Penicillium* lipases with similar biochemical characteristics and substrate specificity. The most interesting behavior of these two enzymes is their tendency for aggregation, a characteristic that differentiates them from other fungal triacylglycerol lipases and from the *P. camembertii* mono- and diacylglycerol lipase.

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# Bovine Milk Gangliosides: Changes in Ceramide Moiety with Stage of Lactation

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**ABSTRACT:** The stage of lactation is one of the most important factors that influence milk composition. Changes in fatty acids from triacylglycerols and phospholipids have already been reported. In this study, we looked for a lactational change in the ganglioside lipid moiety since ganglioside contents and patterns vary strongly with stage of lactation. Individual gangliosides from four stages were isolated, methanolyzed to cleave the bonds between individual constituents, and derivatized for gas–liquid chromatography and gas chromatography/mass spectrometry analyses. Ceramide components, both fatty acids (as methyl esters derivatives) and long-chain bases, were identified and quantified. The results pointed to a marked change in ceramide from colostrum to milk that was characterized by a dramatic decrease in saturated and the longest-chain fatty acids as well as an increase in 18:1 and 18:2. The major long-chain base along lactation was a recently described structure, 3-ethoxy-15:0 sphinganine. Other new long-chain base structures appeared in these gangliosides. All these changes suggest differences in the fluidity of the fat globule membrane, reflecting physiological variations in cows with respect to milk production.

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Gangliosides are important constituents of neural membranes, and they also appear in extraneural tissues and body fluids. In milk, they are localized in the milk fat globule membrane (MFGM), where they are assumed to play a role in the defense of the newborn against infection (1). Ganglioside GM1 (GM ganglioside species have one sialic acid, GD have two sialic acids; see Ref. 2) binds cholera toxin although the ability to bind other ligands such as viruses, bacteria and hormones has also been reported (2). These would act as false ligands in the gut, blocking the pathogenic agents and their enterotoxins and preventing newborns from contracting possible infections.

The composition of milk fat is strongly influenced by physiological, seasonal, and nutritional factors (3). The ganglioside content varies with stage of lactation, being very high in colostrum and decreasing in ensuing stages. Nevertheless, the main gangliosides in bovine milk are always GD3 (>60%) and GM3. Several changes in their concentrations with stage of lactation have been reported (4), but until now no reports about changes in the ceramide moiety have been made.

The stage of lactation mainly affects the content of short-chain fatty acids (SCFA) of milk, since their proportions are very low early in lactation and increase over the following weeks. At the same time, the uptake of long-chain fatty acids (LCFA) from adipose stores decreases. A negative correlation between the proportions of several SCFA ($C_6$–$C_{14}$) and unsaturated $C_{18}$ compounds has been reported (5).

A theoretical dual origin for milk fatty acids (FA) has been proposed (3): SCFA would be synthesized in the mammary gland from plasma acetate or β-hydroxybutyrate while LCFA would derive directly from circulating lipoproteins. $C_{16}$ FA could have both origins.

Plasma FA have two sources: dietary lipids and those stored in adipose tissue. In nonruminant animals, circulating FA reflect the composition of the food ingested, but in the rumen of cattle, metabolism must be considered. Rumen microorganisms degrade food polysaccharides to produce some volatile FA and metabolic intermediates, such as propionate, butyrate or isobutyrate, which are used to synthesize their own odd-numbered or branched-chain FA. Moreover, rumen bacteria carry out extensive hydrogenation of $C_{18}$ polyunsaturated fatty acids (PUFA), yielding high amounts of 18:0 (6,7). Thus, the true uptake by cows consists mainly of saturated LCFA and small amounts of PUFA, although in general ruminants do not seem to suffer from any lack of essential FA (8). An extensive study of changes in triacylglycerol FA from bovine milk throughout the course of lactation (40 wk) has been carried out (9).

In the present work we determined the possible influence of lactation stage on the ceramide composition of gangliosides. Four different and particularly important stages in lactation were chosen: the two-day colostrum, milk from the midlactation stage (mature milk), and an earlier stage with intermediate features between colostrum and mature milk, known as transitional milk. Finally, we considered late lacta-

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Abbreviations: BSA, bovine serum albumin; C, chloroform; FA, fatty acids; FAME, fatty acid methyl esters; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; GSL, glycosphingolipids; HFBAA, heptafluorobutyric anhydride; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; IgM, immunoglobulin M; LCB, long-chain bases; LCFA, long-chain fatty acids; M, methanol; mAb, monoclonal antibody; MFGM, milk fat globule membrane; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; PBS, phosphate-buffered saline; PIBM, poly(isobutyl methacrylate); PUFA, polyunsaturated fatty acids; RT, retention time; SCFA, short-chain fatty acids; TLC, thin-layer chromatography; VLCFA, very long chain fatty acids.

tion milk, just before the dry period, when the cow is pregnant again. We obtained gangliosides from all these samples and studied the ceramide components following a recently proposed method (10).

## EXPERIMENTAL PROCEDURES

*Samples.* Four Spanish-Brown cows were used in this study. Calving took place in December–January. A 300-mL milk sample from each animal was obtained on postpartum day 2 (colostrum), day 15 (transitional milk), day 90 (mature milk), and in the tenth month (late lactation milk). Samples were always obtained from the morning milking, frozen at −20ºC, lyophilized, and homogenized to ensure accurate distribution of the components.

*Chemicals*. Heptafluorobutyric anhydride (HFBAA) was from Fluka (Buchs, Switzerland) and high-performance liquid chromatography (HPLC)-grade acetonitrile from SDS (Peypin, France). Anhydrous acetonitrile was obtained by addition of calcinated calcium chloride (Prolabo, Paris, France), followed by storage in a closed vessel. The methanolysis reagent was obtained by dissolving anhydrous gaseous HCl (up to 0.5 M) at −50ºC in anhydrous methanol (M) previously redistilled on magnesium turnings. The 25QC3/BP1 column was from SGE trance SARL (Villeneuve St. Georges, France) and the CP-Sil5 CB capillary column was from Chrompak France (Les Ullis, France).

Monoclonal antibodies P3, 14F7, R24, and G1 were a kind gift from Dr. Ana María Vázquez (Centro de Inmunología Molecular, Havana, Cuba). Anti-A2B5 [mouse immunoglobulin M (IgM)] was provided by Roche Molecular Biochemicals (Mannheim, Germany). Anti-9-*O*-acetyl-GD3 (clone JONES, mouse IgG), mouse polyvalent Ig G-, Ig M-, Ig A-biotin conjugated, ExtrAvidin®-alkaline phosphatase, and FAST BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) were from Sigma (St. Louis, MO). Poly(isobutyl methacrylate) (PIBM) was from Aldrich Chemical Company (Milwaukee, WI), and *n*-hexane was from Merck (Darmstadt, Germany). Albumin from bovine serum (BSA) was provided by Fluka.

*Extraction of gangliosides.* Gangliosides were isolated as previously described (4). Briefly, lyophilized milk was homogenized twice with 10 vol of cold acetone (−20ºC) to remove neutral lipids and filtered. The solid residue was successively extracted with 10 vol of chloroform/methanol (C/M) (2:1, 1:2, and 1:1, vol/vol). The combined extracts were evaporated to dryness, taken up in 10 vol of C/M (2:1), and subjected to a Folch partition (4). The upper phases, containing crude gangliosides, were combined and dialyzed against distilled water (volume ratio of sample to dialysate, 1:1000) at 4ºC for 2 d. Water was changed every 6–8 h. After dialysis, the material was lyophilized and dissolved in C/M (2:1).

The ganglioside content was quantified as lipid-bound sialic acids by the resorcinol assay (11). Gangliosides were separated by high-performance thin-layer chromatography (HPTLC) using solvent system A: C/M/0.2% $CaCl_2$ (5:4.5:1, by vol). Individual gangliosides (ganglioside pattern) were analyzed with a dual-wavelength thin-layer chromatography (TLC) densitometer (Shimadzu CS 9000; Kyoto, Japan) after separation by HPTLC. Gangliosides were visualized by spraying the plates with the resorcinol and orcinol reagents.

For the gas chromatography (GC) and GC–mass spectrometry (GC–MS) assays, individual gangliosides were purified by preparative TLC using solvent system B: methyl acetate/C/M/*n*-propanol/0.25% KCl, (25:20:20:20:17, by vol) (12).

*Methanolysis and acylation.* Ganglioside samples were dried under a nitrogen stream. Methanolysis reagent (300 µL) was added, and closed tubes were incubated for 20 h at 80ºC. Samples were evaporated to dryness under a nitrogen stream and derivatized with 25 µL of HFBAA and 200 µL of acetonitrile for 30 min at 100ºC. After cooling to room temperature, samples were dried and dissolved in an appropriate volume of anhydrous acetonitrile.

*GC*. Each HFBAA derivative was injected into a Shimadzu GC-14A gas chromatograph equipped with a Ross injector and a 25-m capillary column (25QC3/BP1, 0.5-mm film phase). The injector and flame-ionization detector temperatures were 260ºC. The temperature program was 1.2ºC/min from 100 to 140ºC, and then 4ºC/min up to 240ºC. This temperature was maintained for 10 min. The carrier gas (helium) pressure was 0.8 bar. This program allowed a good separation of monosaccharides into their different isomers; it has been used to identify each ganglioside before the GC–MS analyses (10).

*GC–MS analyses.*The GC separation was performed on a Carlo Erba GC 8000 gas chromatograph (Milan, Italy) equipped with a 60 m × 0.32 mm CP-Sil5 CB low-bleed/MS capillary column, 0.25 mm film phase. The temperature of the Ross injector was 280ºC, and the temperature program was as follows: 90ºC for 3 min, then 5ºC/min up to 260ºC. The temperature was held at 260ºC for purposes of cleaning. This column was coupled to a Finnigan Automass II mass spectrometer (mass limit 1000). Analyses were performed in the electron impact mode (ionization energy 70eV; source temperature 150ºC) (10).

*Immunostaining assay.* Immunostaining on HPTLC plates was performed as previously reported (13). Gangliosides from each stage of lactation were chromatographed with solvent system A. Once dried, the plates were soaked with 0.1% PIBM in *n*-hexane for 75 s and kept overnight at room temperature for drying. The plates were blocked with 1% BSA in Tris-0.1 N HCl for 30 min. They were then incubated with each monoclonal antibody (mAb) at room temperature for 2 h. After washing with phosphate-buffered saline (PBS), plates were incubated with conjugated biotin (1:2000 in 1% BSA in PBS) for 1 h 30 min, and then with streptavidin-alkaline phosphatase (1:1000 in the same buffer) for 1 h 30 min before developing with the substrate.

*Statistical assays.* In order to find statistically significant differences among the four stages of lactation, an analysis of variance test was applied in each case.

## RESULTS

*Ganglioside content.* The ganglioside contents at each stage of lactation, expressed as lipid-bounded sialic acids [mg/kg of milk, expressed as means ± SD ($n$ = 4 cows); three replicates were made per cow and sample time], were as follows: colostrum, 3.5 ± 1.7; transitional milk, 1.2 ± 0.5*; mature milk, 0.9 ± 0.4*; and late lactation milk, 1.8 ± 1.4. The asterisks indicate statistically significant differences ($P$ < 0.05) that were found between colostrum and transitional and mature milk but not between colostrum and late lactation milk nor among the other stages. No statistically significant differences among cows were found. The ganglioside content was high in colostrum but then slowly decreased until day 90, after which it increased again in late lactation milk.

Several gangliosides were separated on HPTLC (solvent system A) as resorcinol- and orcinol-positive spots and named G1 to G6 according to their mobility (increasing polarity). They were identified by co-migration with authentic standards and the ganglioside pattern previously found in cow's milk (4). Gangliosides were also analyzed by the HPTLC-overlay method, using specific mAb.

Ganglioside G1 co-migrated with standard GM3. G1 reacts with mAb 14F7 [specific for *N*-glycolylneuraminic acid (NeuGc)-containing GM3]. After mild hydrolysis (formic acid, pH 2) of the ganglioside, both *N*-acetylneuraminic acid (NeuAc) and NeuGc were found as sialic acid moieties by TLC. These data suggest that G1 is a mixture of NeuGc- and NeuAc-containing GM3. With TLC, G2 was found in the monosialoganglioside region, and it reacted with the JONES anti-*O*-acetyl GD3 mAb. This mAb only detected one band in colostrum and transitional milk but two bands in mature and late lactation milk. G2 was identified as *O*-acetyl GD3. Only traces of G2 were found in the first stages of lactation, but a clear increase was detected as lactation progressed.

G3 showed a mobility pattern identical to that of GD3. R24, a mAb with a high degree of specificity against GD3, reacted strongly with G3. The structure of G3 was therefore assumed to be GD3. Additionally, NeuAc and NeuGc were detected in the TLC analyses after hydrolysis, suggesting the presence of NeuGc- and NeuAc-containing GD3. G1 and G3 also showed a positive reaction with mAb P3, which is specific for NeuGc-containing gangliosides. The staining was very prominent in colostrum and weaker in the other stages, suggesting that the content of NeuGc in gangliosides decreases during the course of lactation.

G5 was located in the trisialoganglioside region of the chromatogram. G5 also reacted with the anti-A2B5 mAb (specific for GT3 as well as for *O*-acetyl GT3). According to this, G5 was identified as GT3. G4 and G6 were tentatively designated on the basis of their mobility on TLC plates and previous data (14). G4 could be a monosialoganglioside with a branched oligosaccharide chain, and G6 might be a trisialoganglioside with the same branched oligosaccharide chain. A specific mAb against GM1, called G1, was also used. No reaction was found in any stage of lactation, pointing to the notion that cow's milk does not contain GM1.

*Ceramide content.* Individual gangliosides were separated by TLC (solvent system B) and analyzed by GC–MS. FA and long-chain bases (LCB) of the different individual gangliosides were determined (data not shown). Table 1 shows the FA average content of gangliosides from the different stages. Values are means of the FA content of the different individual

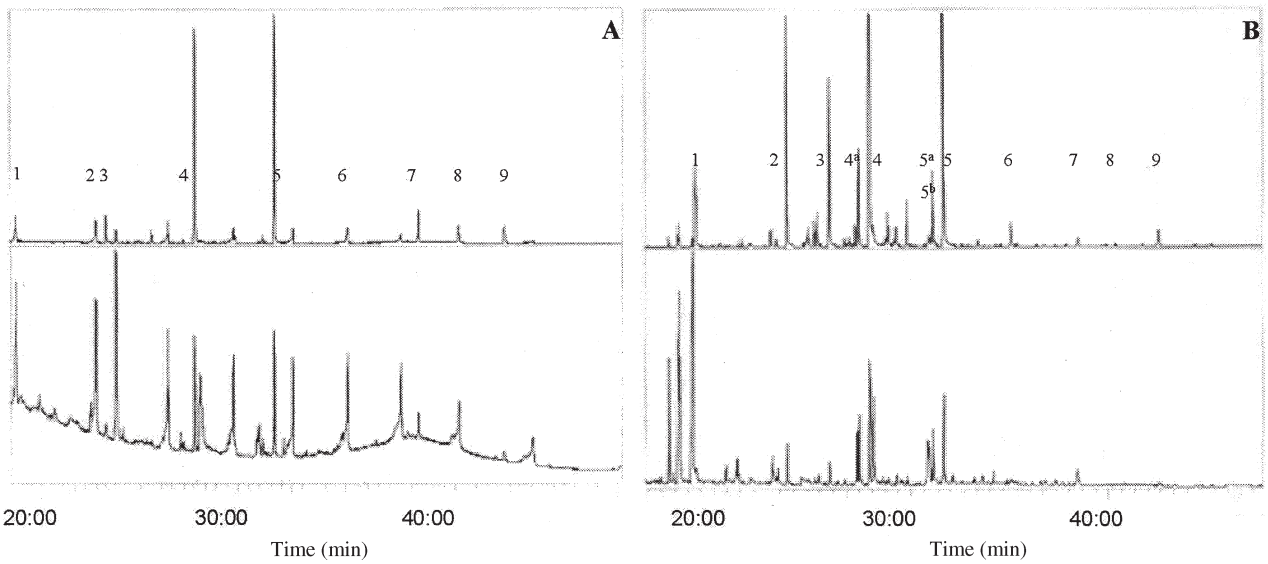


**FIG. 1.** Gas chromatogram of fatty acid methyl ester content of colostral (A) and transitional (B) gangliosides. Samples were methanolyzed (20 h at 80°C) and derivitized with heptafluorobutyric anhydride (30 min at 100°C). A Carlo Erba GC 8000 gas chromatograph coupled to a Finnigan Automass II mass spectrometer was used. The gas chromatograph was equipped with a 60 m × 0.32 mm CP-Sil5CB low-bleed/MS capillary column. Peak 1, 12:0; 2, 14:0; 3, 15:0; 4, 16:0; 4a, 16:1; 5, 18:0; 5a, 18:1; 5b, 18:2; 6, 20:0; 7, 22:0; 8, 23:0; 9, 24:0.

**TABLE 1**
**Content[a] (%) of Fatty Acids and Long-Chain Bases (LCB) of Gangliosides from the Different Stages of Lactation**

| Fatty acids | Colostrum | Transitional milk | Mature milk | Late lactation milk |
|---|---|---|---|---|
| 12:0 | 2.46 ± 4.3 | 1.08 ± 1.4 | 0.75 ± 0.7 | 1.03 ± 1.6 |
| 13:1 | — | 0.01 ± 0.02 | — | — |
| 13:0 | — | 0.43 ± 0.7 | 0.06 ± 0.1 | — |
| 14:1 | — | 0.35 ± 0.3 | 0.07 ± 0.1 | — |
| 14:0 | 6.86 ± 2.4 | 6.88 ± 1.5 | 6.21 ± 1.7 | 4.37 ± 2.5 |
| 15:1 | — | 0.09 ± 0.2 | — | — |
| 15:0[b] | 3.36 ± 2.2 | 5.54 ± 2.3 | 3.63 ± 1.3 | 2.43 ± 1.4 |
| 16:1 | 0.73 ± 1.4 | 9.01 ± 2.1 | 4.08 ± 3.2 | 2.82 ± 2.6 |
| 16:0 | 37.94 ± 4.3 | 26.61 ± 3.5 | 35.59 ± 6.4 | 45.26 ± 17.7 |
| 17:1 | — | 0.60 ± 1.05 | — | — |
| 17:0[b] | 1.98 ± 2.5 | 3.49 ± 1.9 | 2.45 ± 0.6 | 2.74 ± 2.1 |
| 18:2 | — | 10.00 ± 4.6 | 3.54 ± 2.6 | 6.17 ± 4.4 |
| 18:1 | 1.32 ± 2.8 | 11.56 ± 1.2 | 14.12 ± 3.6 | 8.04 ± 8.9 |
| 18:0 | 27.92 ± 6.8 | 18.46 ± 3.1 | 22.59 ± 2.8 | 21.96 ± 5.2 |
| 19:0 | 0.09 ± 0.2 | 0.17 ± 0.2 | 0.03 ± 0.05 | 0.07 ± 0.1 |
| 20:0 | — | 1.12 ± 1.1 | 1.13 ± 1.3 | 0.41 ± 0.4 |
| 21:0 | — | 0.48 ± 0.6 | — | — |
| 22:1 | 0.91 ± 2.2 | — | — | — |
| 22:0 | 15.03 ± 7.1 | 2.65 ± 1.9 | 3.81 ± 1.6 | 2.88 ± 3.7 |
| 23:0 | — | 0.11 ± 0.2 | 0.34 ± 0.4 | 0.04 ± 0.06 |
| 24:1 | — | — | 0.57 ± 1.1 | — |
| 24:0 | 1.39 ± 2.1 | 0.93 ± 0.2 | 0.91 ± 0.6 | 1.59 ± 1.8 |
| 25:0 | — | 0.36 ± 0.3 | 0.14 ± 0.2 | 0.10 ± 0.1 |
| 26:0 | — | 0.07 ± 0.1 | — | 0.09 ± 0.2 |
| Long-chain bases | | | | |
| 8-Me-3-*O*-ethoxy-$C_{14}$ spha | 3.36 ± 3.9 | 5.40 ± 1.9 | 4.36 ± 2.4 | 2.96 ± 0.6 |
| 3-*O*-Ethoxy-$C_{15}$ spha | 62.82 ± 10.3 | 53.20 ± 12.0 | 43.57 ± 2.59 | 65.25 ± 9.6 |
| 3-*O*-Ethoxy-$C_{17}$ spha | 19.07 ± 9.5 | 19.45 ± 11.2 | 19.75 ± 7.7 | 13.47 ± 6.6 |
| $C_{18}$ sphingosine | 9.53 ± 4.4 | 19.22 ± 4.3 | 31.35 ± 5.3 | 16.33 ± 6.9 |
| $C_{20}$ phytosphingosine | 5.22 ± 1.1 | 2.73 ± 1.8 | 0.98 ± 0.25 | 1.99 ± 2.5 |

[a]Values, expressed as mean ± standard deviation, are means of the fatty acid and LCB contents of the different gangliosides from each stage of lactation.
[b]Including the branched isomers. Spha, sphinganine.

gangliosides from each stage of lactation. FA were identified as fatty acid methyl esters (FAME) by the typical ions at *m/z* 74 and *m/z* 87 (Fig. 1). Each FAME could be identified by its retention time (RT) as well as by its molecular ion.

Several FA ($C_{12}$–$C_{26}$) were identified by MS analysis, including odd-numbered chains. No FA below $C_{12}$ was detected, although these are very common in milk fat (triacylglycerols). The most abundant were $C_{16}$ and $C_{18}$, whose proportions were over 65% of total saturated FA in gangliosides. The FA 22:0 was well represented in colostrum, but its percentage was below 4% in the other stages.

Monounsaturated FA—from 13:1 to 24:1—were also detected. Palmitoleic (16:1) and oleic (18:1) acids represented 69.9, 64.47, 81.32, and 63.7% of the total unsaturated FA from colostral, transitional, mature, and late lactation gangliosides, respectively. The only PUFA found was 18:2.

The saturated FA content in gangliosides changed over the course of lactation. Significant differences were found between colostrum and transitional ($P < 0.01$), mature ($P < 0.01$), and late lactation ($P < 0.05$) milk. Statistical assays revealed no differences among the last three stages, although a tendency to increase was observed, as seen in Figure 2. $C_{16}$

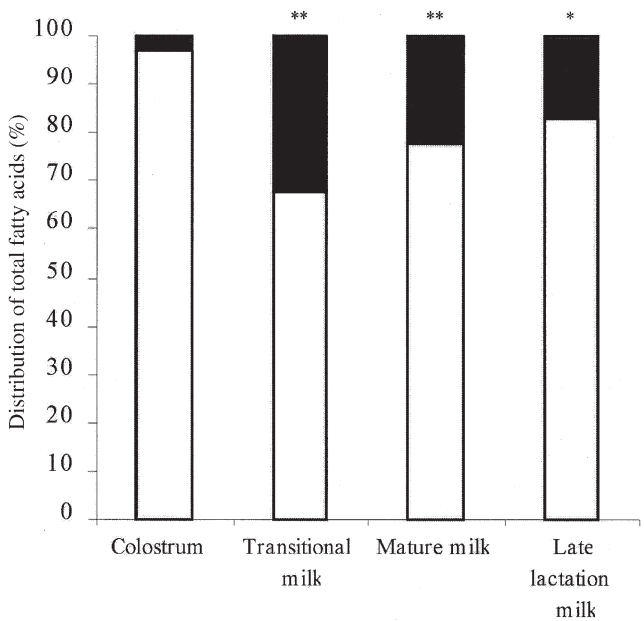


**FIG. 2.** Distribution of saturated (□) and unsaturated (■) fatty acids. Statistically significant differences from colostrum are shown: *$P < 0.05$, **$P < 0.01$.

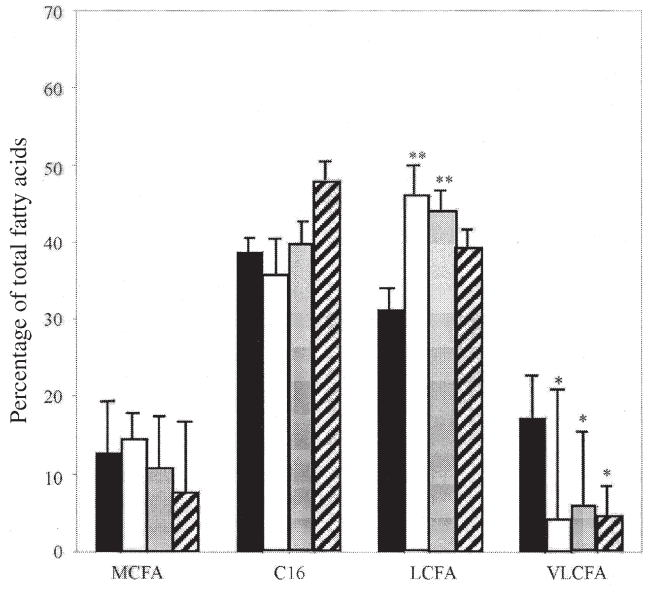


**FIG. 3.** Lactational variation of fatty acids: medium-chain fatty acids (MCFA, $C_{12}$–$C_{15}$), $C_{16}$ (includes 16:0 and 16:1), long-chain fatty acids (LCFA, $C_{17}$–$C_{21}$), and very long chain fatty acids (VLCFA, $C_{22}$–$C_{26}$). Statistically significant differences from colostrum are shown: $^*P < 0.05$, $^{**}P < 0.01$). Closed bar, colostrum; open bar, transitional milk; shaded bar, mature milk; striped bar, late lactational milk.

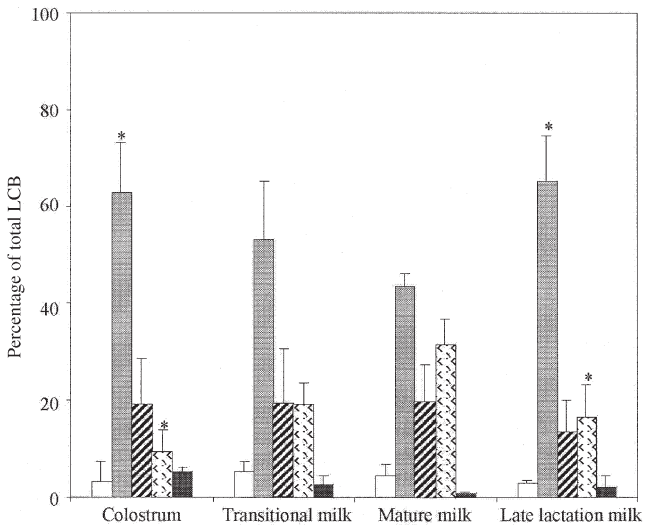


**FIG. 4.** Lactational variation of long-chain bases (LCB). Statistically significant differences from mature milk ($^*P < 0.01$) are shown. Open bar, 9-Me-3-*O*-ethoxy-$C_{14}$ sphinganine; shaded bar, 3-ethoxy-$C_{15}$ sphinganine; striped bar, 3-ethoxy-$C_{17}$ sphinganine; patterned bar, 18:1 sphingosine; closed bar, 20:0 phytosphingosine.

was the most abundant FA in all gangliosides for each stage, followed by 18:0.

Very long chain fatty acids (longer than $C_{20}$, VLCFA) decreased over the course of lactation, since in colostrum the proportion of $C_{18}$ FA was twice that of VLCFA but 10-fold higher in the rest of the stages (Fig. 3). A similar trend was found for $C_{16}$. In fact, the sum of $C_{16}$ and $C_{18}$ increased during the course of lactation from 67.9% in colostrum to 84.2% in the tenth month postpartum. The most radical changes occurred in the first 2 wk after calving: 22:0 decreased dramatically from 15% (on day 2) to 2.6% (on day 15) while unsaturated $C_{16}$ and $C_{18}$ increased to the same extent (palmitoleic, 0.73 to 9.01%; oleic, 1.32 to 11.56%).

The acids 15:0 and 17:0 were the only branched-chain FA detected in our analyses. They never represented more than 1.5% of the total, and they did not appear in all gangliosides. The branched $C_{15}$ was characterized by the molecular ion at *m/z* 256 (corresponding to the $C_{15}$ FAME) and by an ion at M – 29, indicating that it belonged to the anteiso series. The branched 17:0, whose molecular ion appeared at *m/z* 284, was also an anteiso branched FA.

Taking into consideration the three most abundant gangliosides (GM3, GD3, and GT3), we observed a within-group variability of certain FA: in colostrum, 18:0 varied from 38.1 (in GM3) to 18.8% (in GD3), and 22:0 represented 8.6% of the total in GT3 but represented 27.9% of FA in GD3. Transitional gangliosides were more homogeneous, like those of mature milk, although linoleic acid represented 4.68 and 16.13% of FA from transitional GD3 and GT3, respectively, and palmitic acid varied from 29.9 (in GD3) to 42.2% (in GT3) in mature milk. The most extreme values were found in 16:0 from late lactation gangliosides: from 25.2% in GD3 to 69.4% in GT3, and oleic acid represented 21.6% of total FA from GD3 although it was not detected in the most polar gangliosides.

By GC–MS, LCB were separated as heptafluorobutyric derivatives. In the electron impact mode, sphingosines (monounsaturated and dihydroxylated bases) gave a typical fragment ion at *m/z* 290, allowing unambiguous identification of two isomers at RT of 1.001 and 1.010 (relative to 18:0). $C_{20}$ phytosphingosine was found at RT 1.100. Three new LCB, recently reported (15), were predominant in milk gangliosides (Fig. 4). They gave a typical ion at *m/z* 510 corresponding to the substituted first three carbons. The most abundant one is 3-ethoxy-$C_{15}$ sphinganine, whose spectrum is shown in Fig-

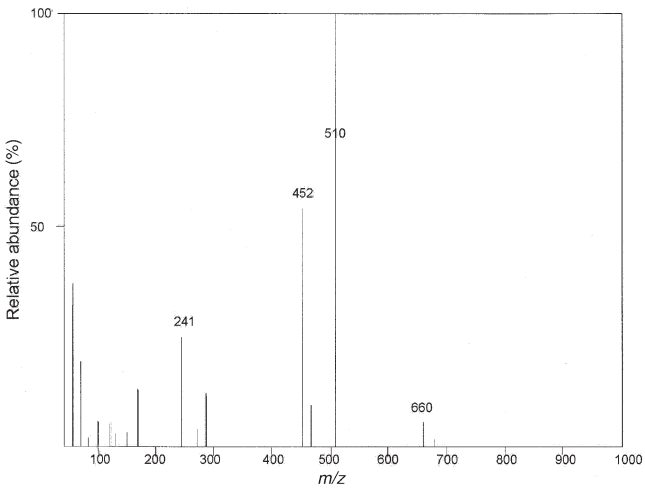


**FIG. 5.** Electron impact spectrum showing the characteristic ion sequence of 3-ethoxy-$C_{15}$ sphinganine.

ure 5. Table 1 also shows the LCB average content of gangliosides from the different stages of lactation. Values are means of the LCB content of the different individual gangliosides from each stage of lactation.

## DISCUSSION

Lipids are the most variable component in milk. The most abundant lipids are triacylglycerols, representing 97–98% of the total, followed by phospholipids and cholesterol. Glycosphingolipids (GSL) represent only 0.6% and are localized in the MFGM, although a small fraction of them is dispersed in the aqueous phase. Glucosyl- and lactosyl-ceramide are the main GSL in bovine milk (16), and GD3 is the most abundant ganglioside.

Our results are in agreement with a previous report demonstrating a decrease in the ganglioside content of the milk as lactation progresses (4). Nonstatistically significant variations among individual cows were observed. The individual ganglioside content (ganglioside pattern) revealed six different gangliosides. G1, G3 and G5 were identified as GM3, GD3, and GT3, respectively, and they account for 63–83% of total gangliosides depending on the stage of lactation. Several authors have also reported that about 80–90% of the gangliosides in bovine milk (expressed as lipid-bound sialic acid) consist of hemato-series gangliosides, such as GM3, GD3, and GT3. G4 and G6 were designated as a monosialo- and a trisialoganglioside, respectively, with a branched oligosaccharide chain, as previously reported (14). Since no specific monoclonal antibodies are available against these two gangliosides and since the amount of the latter was insufficient to determine their structure, their identification remains unclear. G2 was identified as *O*-acetyl GD3. We detected one band in colostrum and transitional milk but two bands in mature and late lactation milk. By using TLC, two bands in the region between GM3 and GM1 corresponding to alkali-labile gangliosides and reacting with JONES were observed in buttermilk (17). These were identified as 7,9-*O*-diacetyl GD3 (higher mobility) and a mixture of 7- and 9-*O*-acetyl GD3 (lower mobility). These data suggest that our G2 ganglioside could be a mixture of *O*-acetylated derivatives of ganglioside GD3.

We also detected several NeuGc-containing gangliosides (G1 and G3) by the HPTLC-overlay method. The staining was very strong in colostrum but weaker in the other stages, suggesting that the content of NeuGc in milk gangliosides decreases as lactation progresses. These data are consistent with other observations previously reported by our group (18). We did not detected ganglioside GM1 in bovine milk. This result is in contrast with a previous study reporting the presence of GM1 in bovine milk (19). However, our results are in agreement with the individual ganglioside content of milk and the biosynthetic pathways of these gangliosides. Milk gangliosides mainly consist of hemato-series gangliosides (GM3, GD3, and GT3). Other minor gangliosides, such as *O*-acetyl GD3 and *O*-acetyl GT3, share the same biosynthetic pathway, and there are also monosialo- and trisialogangliosides with a branched chain. Most researchers have failed to detect any ganglioside of the classical a-pathway of ganglioside synthesis in milk. Thus, the presence of GM1 in cow's milk would be unlikely. If it were present, this would in trace amounts, below the limit of detection by TLC immunostaining.

Regarding the ceramide content, it was found that MFGM gangliosides were richer in VLCFA (47.4%) than in 16:0 (25.6%) (20). Nevertheless, Palmquist and Schanbacher (21) found lower amounts of VLCFA (3.7%) than 16:0 (22.6%) in MFGM lipids. Except for the colostrum, our results are in agreement with the latter authors. According to several investigators (3), the proportion of the longest FA ($>C_{16}$) decreases as the mammary gland assumes responsibility for the major part of FA synthesis and as uptake from plasma ceases. Since MFGM comes from mammary gland cells, changes in the FA content of MFGM should reflect changes in mammary gland metabolism. Although we found no differences in the global content of LCFA, which persisted at around 45–50% throughout lactation, a marked decrease in the amounts of VLCFA was detected in the postcolostrum stages. Studying lactational changes in several phospholipids, some authors (22) have reported that 40–50% of sphingomyelin moieties have FA chains longer than $C_{20}$ (VLCFA) whereas other workers (23) have described the same characteristic in MFGM cerebrosides. However, these latter authors also showed that skim milk cerebrosides are richer in $C_{16}$ than in VLCFA. All these differences could be due to the influence of dietary fat intake (21).

The presence of branched-chain FA in milk lipids has been reported (24). These branched-chain FA would be of ruminal origin, since this kind of FA constitutes more than 20% of the total in rumen bacteria and plays an important role in membrane fluidity (25). The same source has been reported for odd-numbered FA.

Although there was an increase in the $C_{18}$ sphingosine percentage in mature milk, the 3-ethoxy-$C_{15}$ sphinganine predominated throughout lactation. This LCB has never been reported in milk gangliosides, nor has 3-ethoxy-$C_{17}$ sphinganine and 9-methyl-3-ethoxy-$C_{14}$ sphinganine. Nevertheless, $C_{18}$ sphingosine had been reported as the most abundant LCB in previous studies (14,26). Since those results had been obtained by GC, differences are probably due to the methodology employed.

To determine the biological significance of these changes in ceramide, membrane fluidity must be addressed. Changing both FA and LCB could control the fluidity of the membrane. In colostrum, the presence of high amounts of VLCFA and saturated FA would suggest a thick structure. In other stages of lactation the increase of unsaturated FA content points to a more fluid membrane. The increase of the length of LCB leads to a larger hydrophobic volume and affects the membrane organization (27). Gangliosides with longer LCB contribute to membrane thickening. Thus, the physiological changes in FA synthesis would be balanced by the proportions of the longest LCB.

The role of milk gangliosides in the protection of newborns against infection has been discussed for a long time (2). Milk

gangliosides could act as alternative receptor analogs for bacteria and/or toxins in the gut, preventing bacterial infection. The orientation of the carbohydrate moiety of membrane gangliosides may be influenced by the composition of ceramide. Furthermore, the ceramide moiety is essential in the lateral phase separation of gangliosides leading to the formation of ganglioside-enriched microdomains. This feature could be important in the presentation and/or accessibility of the ganglioside binding site (epitope) (28). Specific ceramide moieties involved in *Escherichia coli* binding have been reported. NeuGc-GM3 with highly hydroxylated ceramide was found to bind *E. coli* K99 more strongly than nonhydroxylated forms (29). Adhesion is mediated by electrostatic interactions between the bacterial adhesin and the carboxyl group of sialic acid (30). Changes in the position of this group could lead to variations in the adhesion properties, modulating the inhibitory activity of gangliosides.

A detailed knowledge of the ceramide composition of milk gangliosides and their interactions with bacteria could be useful to develop synthetic analogs, possibly easier to obtain than natural compounds.

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# Comparative Investigation of Human Stratum Corneum Ceramides

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**ABSTRACT:** The stratum corneum (SC) requires ceramides, cholesterol, and fatty acids to provide the cutaneous permeability barrier. SC lipids can be analyzed by normal-phase high-performance thin-layer chromatography (HPTLC). However, without further analysis, some uncertainty remains about the molecular composition of lipids represented by every TLC band of an unknown sample. We therefore analyzed each ceramide band further by subjecting the isolated lipids to a direct coupling of reversed-phase high-performance liquid chromatography and electrospray ionization–mass spectrometry (HPLC/ESI-MS, or LC/MS). LC/MS analysis and ESI-MS/MS negative ion and collision-induced dissociation experiments revealed that ceramide band 4 contained not only *N*-(ω-OH-acyl)acyl-6-OH-sphingosine, Cer(EOH), but also *N*-(α-OH-acyl)-sphingosine. Band 5 exclusively contained *N*-acyl-6-OH-sphingosine. Our results demonstrate the benefit of LC/MS analysis for selective identification of human SC ceramides. Moreover, the combination of HPTLC for pre-separation and LC/MS for identification of lipids is an even more powerful tool for detailed ceramide analysis.

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The skin barrier protects the skin against excessive loss of water (1,2) as well as environmental influences (3,4). This feature is attributed mainly to the amount and composition of the intercellular lipid matrix in the stratum corneum (SC), which consists mainly of ceramides, free fatty acids, and sterols (5–7). Since alterations of barrier lipid composition are known to result in a functional impairment of the skin barrier or to delay barrier repair (8–10), different types of healthy human skin and skin diseases have been previously investigated for possible changes in overall barrier lipid composition (10–13).

Analysis of ceramides is usually performed by thin-layer chromatography (TLC) (13–15). Further analysis of ceramide fractions separated by TLC has previously been carried out by Wertz *et al.* (14). After hydrolysis and separation by gas chromatography Wertz *et al.* demonstrated that every ceramide species comprises a range of subspecies with different chain lengths in the sphingoid base as well as in the amide-linked fatty acid. More information about the molecular structure of the different ceramide species can be obtained by nuclear magnetic resonance (NMR) spectroscopy (15,16). Ceramides can also be separated by high-performance liquid chromatography (HPLC) on silica gel as well as on diol- and reversed phases with evaporative light-scattering detection (ELSD) (17–20). The separation of benzoyl derivatives of ceramides on reversed-phase (RP) material, as detected by ultraviolet (UV) absorbance or by atmospheric pressure chemical ionization mass spectrometry (MS), has also been described (21,22). Furthermore, direct coupling of high-performance liquid chromatography (HPLC) with an electrospray ionization (ESI) mass spectrometer has been reported (23).

In this study we compared two methods for the analysis of human SC ceramides; high-performance thin-layer chromatography (HPTLC) and direct coupling of nonaqueous RP HPLC/ESI ion trap mass spectrometry (LC-MS). To verify the HPTLC assignment of ceramide bands and to ensure the comparability of both methods, isolated SC lipids were first analyzed by HPTLC. The ceramide bands were investigated further by means of LC/MS and mass spectrometric fragmentation of specific ions (MS/MS) and collision-induced dissociation (CID) fragmentation experiments.

## MATERIALS AND METHODS

*Chemicals and reagents.* Ceramide reference standards [*N*-(α-OH-acyl]-sphingosine, Cer(AS); *N*-acyl-sphingosine, Cer(NS)] were obtained from Sigma (Deisenhofen, Germany). *N*-Acyl-4-OH-sphinganine [Cer(NP)] and *N*-(α-OH-acyl)-4-OH-sphinganine [Cer(AP)] were from Cosmoferm (Delft, The Netherlands). Ammonium acetate (analytical grade), cholesterol, palmitoleic acid, and all solvents (HPLC or analytical grade) were purchased from Merck (Darmstadt, Germany). Primulin was obtained from Sigma (Deisenhofen).

*SC lipid sampling.* SC lipids were collected from healthy Caucasian males and females in March 1999. Informed consent

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Abbreviations: Cer(AP), *N*-(α-OH-acyl)-4-OH-sphinganine; Cer(AS), *N*-(α-OH-acyl)-sphingosine; Cer(EOH), *N*-(ω-OH-acyl)acyl-6-OH-sphingosine; Cer(EOP), an ω-esterified ceramide containing phytosphingosine as a sphingosine backbone and an ω-esterified linoleic acid; Cer(EOS), *N*-(ω-OH-acyl)acyl-sphingosine; Cer(NH), *N*-acyl-6-OH-sphingosine; Cer(NP), *N*-acyl-4-OH-sphinganine; Cer(NS), *N*-acyl-sphingosine; CID, collision-induced dissociation; ELSD, evaporative light-scattering detection; ESI–MS, electrospray ionization—mass spectrometry; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; LC–MS: liquid chromatography–mass spectrometry; MS, mass spectrometry; MS/MS, mass spectrometric fragmentation of specific ions; NMR, nuclear magnetic resonance; RP, reversed-phase; SC, stratum corneum; TLC, thin-layer chromatography; UV, ultraviolet.

was obtained from all volunteers participating in the study. Lipids were collected from skin sites located on the volar aspect of the forearms by a single stripping with cyanoacrylate resin. The stripped SC sheets were extracted twice with hexane/ethanol (95:5, vol/vol) according to Imokawa *et al.* (12). The combined solvents were evaporated to dryness (SpeedVac, Jouan Unterhaching, Germany) and the lipid-residue was stored at –20°C until chromatographic separation.

*HPLC.* RP-HPLC analyses were performed with an HPLC system (1100 series; Agilent, Palo Alto, CA). The ceramides were separated on a Grom Sil 120 ODS 3-CP, 125 × 2 mm, 3 µm column (Grom Analytik + HPLC GmbH, Herrenberg-Kayh, Germany) at ambient temperature. Methanol containing 0.2 mM ammonium acetate (A) and *t*-butyl methyl ether (B) were used as the mobile phase in a binary gradient (200 µL $min^{-1}$). The program steps were: 0 min, 95% A, 5% B; 0.5 min, 95% A, 5%, B; 11 min, 70% A, 30% B; 13 min, 60% A, 40% B; 20 min, 60% A, 40% B; 20.5 min, 95% A, 5% B; and 30.0 min, 95% A, 5% B.

*MS.* MS was carried out with an Esquire ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an atmospheric pressure ion-source and an ESI interface (Agilent). The HPLC effluent was delivered in total into the ion source. Nitrogen was used as nebulizing (18 psi) and drying gas (7 L $min^{-1}$, 300°C). Negative ions were acquired from 200–1200 *m/z*. The capillary voltage was +3800 V, and the capillary exit was –180 V. The ion trap was set to a sampling value of 3000, with a maximum accumulation time of 300 ms. CID fragmentation spectra were performed with a capillary exit voltage of –195 V. ESI MS/ MS-experiments were performed by isolating single ions in the ion trap (width: 3 *m/z*) and fragmentating them with an amplitude of 1.8 V.

All pseudomolecular and fragment ion masses shown in the figures are nominal masses with the mass defect omitted.

*HP TLC of SC lipids.* Lipid samples were dissolved in 200 µL chloroform/methanol (2:1, vol/vol) and applied to HPTLC Silica Gel 60 plates (Merck, Darmstadt, Germany) after cleaning the plates with chloroform/methanol (2:1, vol/vol) (14). The lipid samples and a reference lipid mixture containing Cer(NS), Cer(NP), Cer(AS), palmitoleic acid, and cholesterol dissolved in chloroform/methanol (2:1, vol/vol) were applied in neighboring lanes by using a Camag Linomat IV (Camag, Muttenz, Switzerland) (24). The applied lipids were separated in developing chambers (Desaga, Heidelberg, Germany) by using a solvent system of Wertz *et al.* (14) (chloroform/methanol/glacial acetic acid, 190:9:1) and a modified solvent system of Lampe *et al.* (25) (hexane/diethylether/glacial acetic acid, 80:20:1.5). Migration distance was 6.8 cm.

*Isolation of separated ceramides for LC–MS analysis.* The separated ceramide classes were visualized under UV light after spraying the plates with 5% primulin in acetone/water (80:20, vol/vol) as described by Guey *et al.* (22). The separated ceramide bands were scraped off the plates using a spatula. The silica gel was extracted once with 1.5 mL hexane/ethanol (95:5, vol/vol), sonicated for 5 min, and centrifuged for 5 min at 8000 rpm. The supernatant was collected, and the extraction procedure was repeated with 1.5 mL hexane/diethylether (95:5, vol/vol). The combined solvent was filtered through a 0.2-µm syringe-filter (Gelman Sciences, Ann Arbor, MI) and evaporated under nitrogen. The residue was dissolved in 150 µL methanol containing 0.2 mM ammonium acetate, and 5 µL were applied for LC–MS-analysis.

## RESULTS AND DISCUSSION

*Investigation of ceramides by HPTLC and LC/MS.* HPTLC separations of ceramides on normal-phase plates are based on hydrophilic interactions between the hydroxyl groups of the silica gel and polar groups of the ceramides. As a result, different ceramide species migrate with different speeds and are separated according to their polarity. The method has frequently been used in the past to demonstrate alterations in the overall barrier lipid pattern or even of the ceramide pattern in aging, dryness or disease of human skin, and produced valuable results. However, although several samples can be separated on the same plate, the whole evaluation process including development, staining, and densitometric quantification can be automated to only a limited extent and therefore is time consuming.

RP separation of ceramides is based on hydrophobic interactions between the alkylated silica gel of the stationary phase and the hydrophobic chains (amide bond, fatty acid, and sphingoid base) of the ceramide. The use of direct coupling of RP-HPLC and MS enables a sensitive and selective separation of molecular subfractions of each ceramide species (26). The structure of every ceramide subfraction can be investigated by means of MS/MS fragmentation experiments (26,27).

Figure 1 shows the MS/MS fragment spectra and proposed fragment structures (negative ion mode) of a sphingosine-derived ceramide with nonhydroxylated amide-linked fatty acid, Cer(NS), (A) and α-hydroxylated amide-linked fatty acid, Cer(AS), (B).

Figure 2 shows the MS/MS fragment spectra and proposed fragment structures (negative ion mode) of a phytosphingosine (4-OH-sphinganine)-derived ceramide with nonhydroxylated amide-linked fatty acid, Cer(NP) (A) and α-hydroxylated amide-linked fatty acid, Cer(AP) (B).

Comparison of the fragment pattern of different ceramides with nonhydroxylated and α-hydroxylated amide bond fatty acids resulted in general fragmentation schemes of ceramides. The mass differences between the fragment ions arising from cleavage between the amide-linked acyl chain and the $C_{18}$ backbone chain resulted in a series of fragment ion masses with differences of 18, 25, and 16 Da. This was referred to as the 18-25-16 series of fragments. The 18-25-16 series of fragments was characteristic for sphingosine-derived ceramides, e.g., Cer(NS) and Cer(AS). The fragment ion located between the mass differences of 18 Da and 25 Da was always the fragment of the amide bond fatty acid.

The series 16-43-12 was characteristic for phytosphingosine (4-OH-sphinganine)-ceramides, e.g., Cer(NP) and Cer(AP);

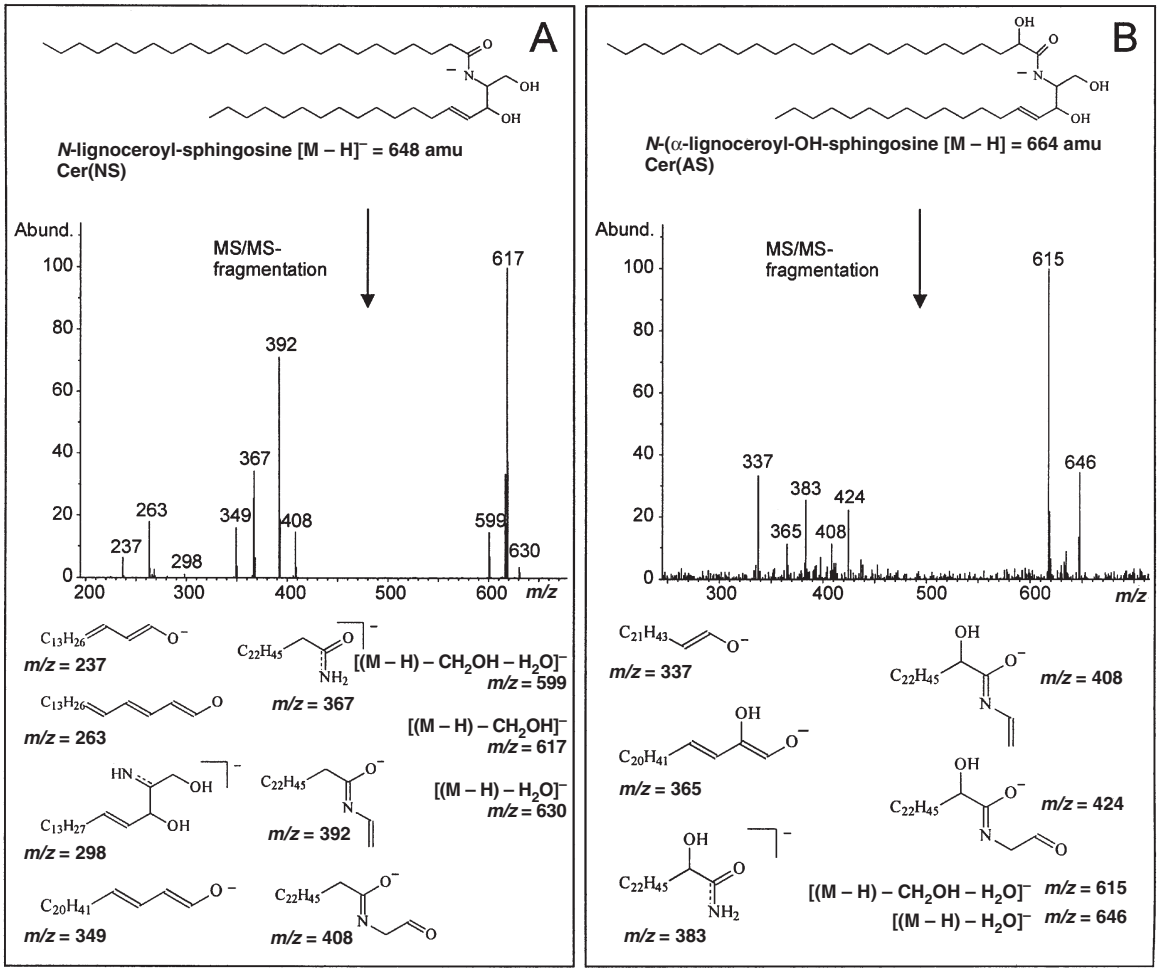


**FIG. 1.** Electrospray ionization–tandem mass spectrometry (ESI–MS/MS) negative ion mode fragment spectra of Cer(NS), (A), and Cer(AS), (B).

the amide bond fatty acid was always the fragment ion between the mass differences of 16 and 43 Da (Fig. 3).

This fragmentation scheme can be used as a tool to distinguish (i) ceramides containing sphingosine bases from phytosphingosine bases and (ii) ceramides containing α-OH-fatty acids from nonhydroxylated fatty acids. ω-Esterified ceramides *N*-(ω-OH-acyl)acyl-sphingosine [Cer(EOS)] and *N*-(ω-OH-acyl)acyl-6-OH-sphingosine [Cer(EOH)] did not show these kinds of fragmentation patterns. Nevertheless, structural information about these ceramides can be obtained by CID fragmentation spectra in the negative ion mode where the ω-esterified fatty acid splits off as an [(ω-fatty acid) – H]⁻- ion.

The use of LC/MS enables the selective analysis of many lipid samples overnight in an automated process using an autosampler. The resulting chromatograms can be processed using software-assisted evaluation procedures. The improved reproducibility of LC/MS compared to HPTLC is important if slight differences in the ceramide-pattern of different skintypes are to be evaluated and effects due to medical treatment of skin diseases and topical application of cosmetic formulations are to be demonstrated.

*Reinvestigation of isolated ceramide HPTLC bands by LC–MS.* Barrier lipids extracted from cyanoacrylate strippings of the human SC were first separated by HPTLC. A typical pattern of lipid bands on a HPTLC plate after charring is shown in Figure 4. LC–MS chromatograms of each ceramide band scraped off from the HPTLC plate showed a series of homologous subspecies. Identification of the ceramides was carried out by means of LC–MS and MS/MS and CID fragmentation experiments.

Although it is not possible to distinguish between variations of the chain length of either the amide-linked fatty acid or the sphingoid base from masses of intact molecular ions in the LC–MS chromatogram, we tried to describe the whole distribution of varying chain length by designation of the amide-linked fatty acids assuming a sphingoid chain length of $C_{18}$. The results are summarized in Table 1. The designation of the ceramides is in accordance with the nomenclature of Motta *et al.* (10) which was further modified by Robson *et al.* (15).

Our comparative analysis demonstrated that most of the ceramide bands on the HPTLC plate represented the molecular species corresponding to the assignment of Robson *et al.* (15) who described band 1 as Cer(EOS), band 2 as Cer(NS), band 3 as Cer(NP), band 4 as Cer(EOH), band 5 as Cer(AS), band 6 as Cer(AP) and band 7 as *N*-(α-OH-acyl)-6-OH-sphingosine. However, we discovered a few exceptions. First, cer-

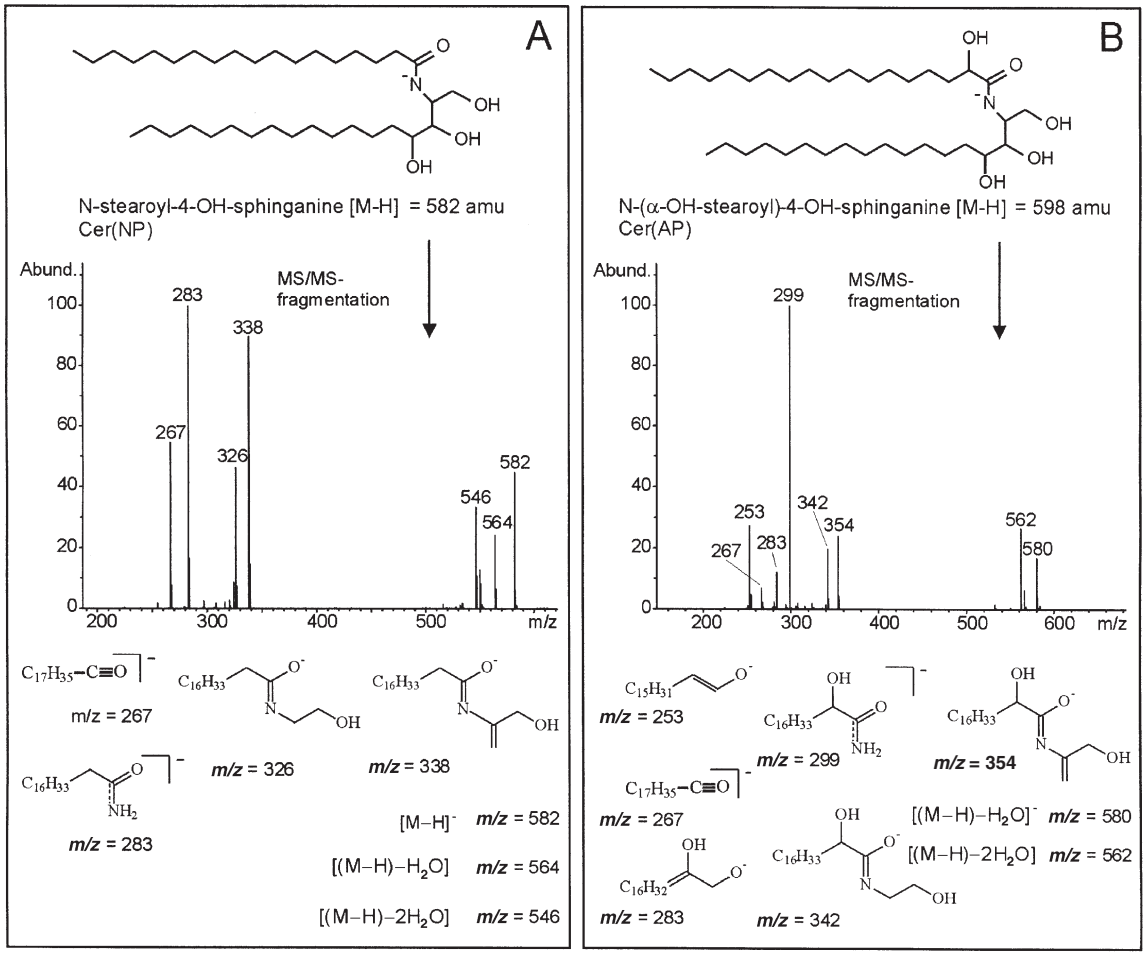


**FIG. 2.** ESI MS/MS negative ion mode fragment spectra of Cer(NP), (A), and Cer(AP), (B). For abbreviations see Figure 1.

amide band 2 comprises *N*-acyl-sphingosines, Cer(NS), as well as *N*-acyl-sphinganines. This is in agreement with the results of Wertz *et al.* (27). Second, apart from Cer(EOH), ceramide band 4 contained Cer(AS). Previous reports have ascribed band 5 to Cer(AS), which comigrates with the Cer(AS) standard (14,15). Moreover, in HPTLC-band 5 we detected *N*-acyl-6-OH-sphingosines, a relatively new class of ceramides described by Stewart *et al.* (16), Guey *et al.* (22), Vietzke *et al.* (26), and Bleck *et al.* (29).

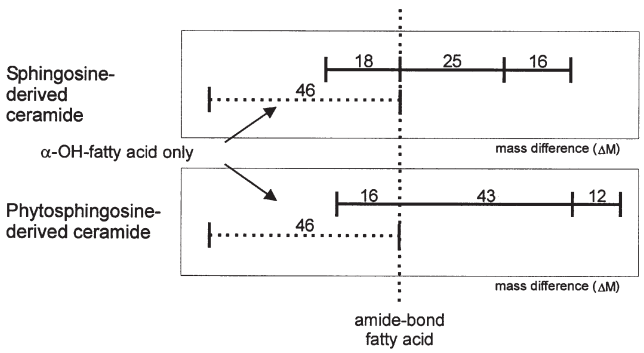


**FIG. 3.** General MS/MS fragmentation scheme of ceramides. For abbreviation see Figure 1.

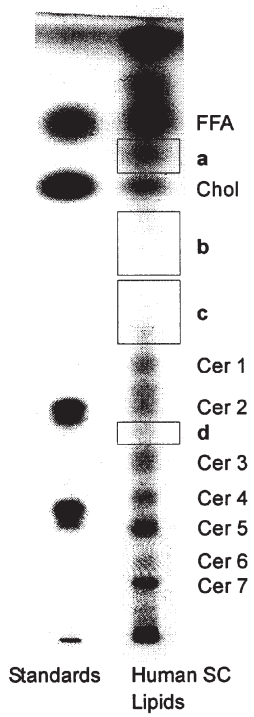


**FIG. 4.** Separation of human stratum corneum (SC) lipids by high-performance thin-layer chromatography. Bands Cer 1–7, ceramides; FFA, free fatty acids; Chol, cholesterol. Zones a–d: further investigated areas (see text).

**TABLE 1**
**Investigation by Means of Liquid Chromatography/Mass Spectroscopy (LC/MS) of Scraped-Off Ceramide Bands, Separated by High-Performance Thin-Layer Chromatography (HPTLC)**

| Scraped off HPTLC-based | Result of LC/MS-investigation | Ceramide-designation according to References 10,15 | Chain length of amidebond fatty acids (assumed chain length of sphingoid base: $C_{18}$) |
|---|---|---|---|
| Cer 1 | *N*-(ω-OH-acyl)acyl-sphingosine | Cer(EOS) | $C_{28}$–$C_{36}$ |
| Cer 2 | *N*-acyl-sphingosine | Cer(NS) | $C_{20}$–$C_{32}$ |
| | *N*-acyl-sphinganine | | $C_{22}$–$C_{36}$ |
| Cer 3 | *N*-acyl-4-OH-sphinganine | Cer(NP) | $C_{20}$–$C_{36}$ |
| Cer 4 | *N*-(ω-4-OH-acyl)acyl-6-OH-sphingosine | Cer(EOH) | $C_{28}$–$C_{36}$ |
| | *N*-(α-OH-acyl)-sphingosine | Cer(AS) | $C_{22}$–$C_{36}$ |
| Cer 5 | *N*-acyl-6-OH-sphingosine | Cer(NH) | $C_{22}$–$C_{34}$ |
| Cer 6 | *N*-(α-OH-acyl)-4-OH-sphinganine | Cer(AP) | $C_{20}$–$C_{32}$ |
| Cer 7 | *N*-(α-OH-acyl)-6-OH-sphingosine | Cer(AH) | $C_{22}$–$C_{32}$ |

Interestingly, LC–MS investigation of the scraped-off areas a, c, and d as well as the band of cholesterol (see Fig. 4) also shows ceramide-like lipids in small amounts. The molecular mass, the chromatographic behavior on RP-phases, and the CID fragmentation patterns in the spectra suggested the presence of further ω-esterified ceramides in the chromatogram containing ω-esterified saturated fatty acids and linoleic acid, respectively. One of these molecular species might even represent Cer(EOP), an ω-esterified ceramide that contains phytosphingosine as a sphingosine backbone and an ω-esterified linoleic acid. Remarkably, the glucosylated derivative of Cer(EOP) has already been identified by Hamanaka *et al.* (30). However, the assumptions made on the basis of the LC–MS data require further investigation to prove and verify the identification, e.g., analysis by NMR spectrometry.

However, the data presented here demonstrate that a combination of HPTLC and LC/MS enables a detailed analysis of the complex ceramide composition in human skin.

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METHOD

# Optimization of Methods and Treatment Conditions for Studying Effects of Fatty Acids on Cell Growth

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**ABSTRACT:** Antiproliferative properties of molecular regulators of lipid metabolism have been increasingly studied during recent years. Discussion is ongoing concerning optimal treatment conditions and assays used for monitoring proliferation and cytotoxicity. The objective of the present work was to optimize methods and treatment conditions used for studying antiproliferative effects of fatty acids and analogs, represented by palmitic acid (PA) and the β-oxidation-restricted fatty acid analog tetradecylthioacetic acid (TTA), in rat (BT4Cn) and human (D54Mg and GaMg) glioma cell lines. Changes in [$^3$H]thymidine incorporation preceded changes in cell number in TTA-treated glioma cell cultures, and the growth inhibition was more significantly expressed by [$^3$H]thymidine incorporation than cell number. Addition of bovine serum albumin decreased cellular fatty acid uptake and reduced the effects of TTA and PA on [$^3$H]thymidine incorporation. Determination of the antiproliferative effect of TTA in BT4Cn cells by MTT conversion and [$^3$H]thymidine incorporation yielded concordant results. TTA-mediated reduction in cell number corresponded to reduction in cellular protein and total DNA content in BT4Cn cells. Reduced growth potential in TTA-treated multicellular D54Mg and GaMg spheroids supported the findings from monolayer cultures. In conclusion, cell density, treatment period, fatty acid administration, and methods for growth determination may profoundly influence the outcome of cell growth experiments. Thus, experimental conditions should be carefully controlled when performing cell growth experiments, and effects on cell growth should preferably be confirmed by different methods.

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Anticancer properties of compounds modulating cellular lipid metabolism have been extensively studied during recent years. This category of compounds includes fatty acids such as essential fatty acids (EFA) (1–8) as well as more complex compounds such as fibrates and aromatic fatty acids (9,10). We have for several years investigated metabolic effects of a novel sulfur-substituted fatty acid analog tetradecylthioacetic acid (TTA; $CH_3$–$(CH_2)_{13}$–S–$CH_2$–COOH) that has major impacts on cellular metabolism (11,12). TTA itself cannot be β-oxidized but is, in other aspects, metabolized like ordinary fatty acids. The exact mechanism behind the antiproliferative effect of TTA is not fully elucidated, but it is thought to involve alterations in lipid metabolism (4,13) and changes in oxidative status (4,13,14). Increased cytosolic cytochrome c, indicating initiation of apoptotic cascades, was observed in glioma cells.

*In vitro* models have been useful experimental tools for studying the cytotoxic effects of fatty acids in cancer cells. However, the results from such studies have exhibited a certain degree of inconsistency concerning both type and magnitude of response. Differences in growth conditions, drug administration, treatment period, and the methods used for monitoring cancer cell growth may be reasons for such variations (15,16).

Commonly used methods for monitoring cell proliferation and cytotoxicity can be divided into two classes: (i) mass-based methods and (ii) metabolic-based methods. Mass-based methods are employed to quantify the amount of cellular material such as cell number, protein, or DNA. On the other hand, metabolic-based assays are used to measure metabolic activities that are closely related to the number of viable cells or proliferation rate, such as conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and incorporation of [$^3$H]thymidine. It has previously been concluded that different methods should be directly compared in the same settings when performing cytotoxicity assay (15). Care should be taken when designing these kinds of experiments since the reliability of some methods has been questioned (16,17).

The mode of fatty acid administration is likely to influence the cellular uptake of the fatty acids. The exact mechanisms behind cellular fatty acid uptake are not clearly understood, but the concept of fatty acid uptake consisting of two coexisting pathways, one passive and one carrier-mediated, is today generally accepted (see review in Ref. 18). In both pathways the concentration of unbound fatty acids seems to be of crucial importance, and the two pathways contribute differentially to net uptake depending on fatty acid/albumin ratios and cell types. Tissue-specific expression of putative fatty acid carriers further substantiates the notion that transport mechanisms may vary between tissues. Fatty acid transfer to the membrane is limited by tight fatty acid binding to high affin-

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Abbreviations: BSA, bovine serum albumin; DMSO, dimethylsulfoxide; EFA, essential fatty acids; $IC_{50}$, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBCS, new-born calf serum; PA, palmitic acid; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator activated receptor; TTA, tetradecylthioacetic acid.

ity sites on albumin. In blood, fatty acids are present in millimolar concentrations (0.1–1), and the fatty acid/albumin ratio varies between 0.1 and 1.0 in circulating plasma. The concentration of nonprotein bound fatty acids in human plasma is on the order of 5–10 nM (19). The poor solubility of fatty acids in water impedes their administration in cell culture medium. Supplementation of albumin in the culture medium may overcome this problem; however, albumin may artificially perturb the effects of fatty acids (6,20,21). Another interfering factor is that supplemented albumin may alter the availability of fatty acids to normal and tumor cells (22,23). In serum-containing cell culture medium, supplementation of albumin can be avoided since the sera contain proteins capable of binding and transporting fatty acids.

Cell density may affect cellular metabolism, differentiation, and proliferation (16). Along this line, expression of peroxisome proliferator-activated receptors (PPAR), which are intimately involved in the regulation of lipid metabolism, may be controlled in response to cell density as has been observed in BT4Cn rat glioma cells (Berge, K., Tronstad, K.J., Flindt, E.N., Rasmussen, T.H., Madsen, L., Kristiansen, K., and Berge, R., unpublished data). Thus, because of the differences between confluent and subconfluent cells, the initial cell density and growth period should be carefully controlled.

To optimize the methodology and treatment conditions in our research on anticancer effects of fatty acids and analogs we used one rat (BT4Cn) and two human (D54Mg and GaMg) glioma cell lines as models. It was of interest to determine the importance of treatment period, methods for growth determination, and fatty acid administration.

## EXPERIMENTAL PROCEDURES

*Chemicals.* TTA and [1-$^{14}$C]TTA were prepared at the Department of Chemistry, University of Bergen, as previously described (24). [$^{3}$H]Thymidine (TRA 310) was purchased from Amersham International (Amersham, United Kingdom). [1-$^{14}$C]Palmitic acid (PA; 50 mCi/mmol) was obtained from New England Nuclear (Boston, MA). All other chemicals and solvents were of reagent grade from common commercial sources.

*Cells.* The rat glioma cell line BT4Cn (25) and the human glioma cell lines D54Mg and GaMg (26) were routinely kept in a standard culture incubator (37°C, 5% $CO_2$ and 95% air). The cells were grown as monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% new-born calf serum (NBCS), L-glutamine (0.58 mg/mL), streptomycin (100 μg/mL), penicillin (100 IU/mL), and three times the prescribed concentration of nonessential amino acids (all from Sigma, St. Louis, MO). For cell counting analysis, cells were seeded in 25 cm$^2$ tissue culture flasks ($10^5$ cells/flask) or 24-well tissue culture plates ($8 \times 10^3$ cells/well). For [$^{3}$H]thymidine incorporation, $10^3$ cells/well were seeded in 96-well tissue culture plates. The cells were allowed to settle overnight before the fatty acid treatments were started.

*Cell growth.* Cell number was determined in trypsinized cell suspensions using a Coulter Z1 Counter (Coulter Electronics, Luton, United Kingdom). [$^{3}$H]Thymidine incorporation was measured after 4 h of incubation with 1.0 μCi/well of [$^{3}$H]thymidine. Following incubation, the cellular DNA was transferred to UniFilter™-96 GF/C™ using a Filtermate Harvester (Packard Instruments, Meriden, CT), before the nuclear radioactivity was measured using a TopCount NXT™ microplate scintillation counter (Packard Instruments). The MTT assay was performed essentially as described in Reference 27. Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) was used for protein measurement. Bovine serum albumin (BSA), dissolved in distilled water, was used as standard. DNA amounts were determined by fluorometry. Cell samples were harvested by trypsination and dissolved in 1 mL high salt harvest buffer (10 mM Tris, 2 mM EDTA, 2 M NaCl; pH 7.4). Samples were then sonicated and properly diluted in high salt buffer (2 mM Tris, 2 mM EDTA, 2 M NaCl; pH 7.4), and the DNA amount was measured on a Hoefer DyNA Quant 200 (Hoefer Scientific Instruments, San Francisco, CA) in high salt buffer containing 0.1 μg/mL 2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole (Hoechst No. 33258).

*Multicellular tumor spheroids.* Multicellular tumor spheroids were produced by the agar overlay culture method described by Yuhas *et al.* (28). In the beginning of each experiment spheroids were individually transferred into agar-coated 24-well tissue culture plates before the treatments were started. The diameters of the spheroids were microscopically measured. The spheroid volume was calculated, and fold volume increase was calculated by dividing the spheroid volume by the initial volume.

*Preparation of TTA and PA.* (i) TTA or PA was dissolved in 0.1 M NaOH at 80°C to a final concentration of 25 mM. The 25 mM TTA stock solution was diluted in NBCS (40°C) to a concentration of 2 mM. This fatty acid-supplemented NBCS was used in the preparation of growth medium. The control medium was prepared in the same way, replacing 25 mM TTA or PA with 0.1 M NaOH. (ii) TTA or PA (25 mM, prepared as described above) was added to a 21.17% (wt/vol) solution of EFA-free BSA in phosphate-buffered saline (PBS) (40°C), to give a final concentration of 6 mM TTA or PA, and 2.4 mM BSA. This resulted in a solution with a molar fatty acid/BSA ratio of 2.5:1, which was diluted in culture growth medium.

For studying the cellular uptake of TTA or PA with different vehicles, the following preparations were made: 25 mM [1-$^{14}$C]TTA (12.5 μCi/mL) in (i) 0.1 M NaOH, (ii) 0.1 M KOH, (iii) dimethylsulfoxide (DMSO)/ethanol (1:10, vol/vol), and (iv) 0.1 M KOH/ethanol (1:3, vol/vol). These solutions were diluted to the final concentrations in culture growth medium.

*Cellular uptake of [1-$^{14}$C]TTA and [1-$^{14}$C]PA.* Cells were seeded in 24-well tissue culture plates (70,000/well) and incubated overnight. The cells were then incubated for 9 h with 50, 100, 200, or 500 μM [1-$^{14}$C]TTA or [1-$^{14}$C]PA (prepared as described above). Following incubation, the medium was

removed, and the cells were washed with 1.5 mL PBS before they were harvested by scraping. After centrifugation (2,000 rpm, 5 min), the cells were suspended in cold distilled water. Radioactivity in the cell suspension was measured by liquid scintillation counting.

*Statistical analysis.* The data are presented as mean ± standard deviation (SD) of triplicates or quadruplicates. The results were evaluated by a two-sample variance Student's *t* test (two-tailed distribution). The level of significance was set at $P < 0.05$. The data presented are representative of the results collected in at least two experiments.

## RESULTS

*Treatment period.* Cell counting and [$^3$H]thymidine incorporation measure population size and DNA synthesis, respectively, and thus the two methods represent different approaches for monitoring cell growth. It was of interest to see how these two methods agreed as a measure of the antiproliferative properties of TTA. The cell number and [$^3$H]thymidine incorporation in BT4Cn cultures were determined after 2, 4, and 6 d of incubation (Figs. 1A,1B). In control cultures the cell number increased exponentially, and the cultures reached confluence after 6 d. The amount of cellular protein closely corresponded to cell number (Fig. 1C). The [$^3$H]-thymidine incorporation was increased on day 4 compared to day 2; however, it was reduced at confluence on day 6. As can be seen from Figure 1A, there was only a modest increase in cell number during the incubation period in presence of 200 μM TTA. The cell number was reduced to 33 and 11% in TTA-treated cultures compared to the control after 4 and 6 d, respectively. [$^3$H]Thymidine incorporation was nearly abolished after 2 d with TTA, and it remained low during the 6-d incubation period. Thus, the antiproliferative effect of TTA was evident from cell number determination after 4 d, whereas [$^3$H]thymidine incorporation was reduced after only 2 d with TTA.

Optimal experimental conditions may differ from cell line to cell line; thus we measured the effect of TTA on cell number and [$^3$H]thymidine incorporation in the two human glioma cell lines D54Mg and GaMg (Fig. 2). A time-dependent decrease in cell number was found in TTA-treated D54Mg and GaMg cultures, compared to control cultures. In common with BT4Cn cells, the inhibitory effect of TTA was evident in D54Mg and GaMg cells after 4 d. Also in these two human cell lines the antiproliferative effect of TTA was more significantly expressed by [$^3$H]thymidine incorporation than by cell number. After 2 d, the [$^3$H]thymidine incorporation was reduced to 4% of the control in both D54Mg and GaMg. A drastic decrease in [$^3$H]thymidine incorporation after 6 d, as seen in untreated BT4Cn cells (Fig. 1B), was not seen in untreated D54Mg and GaMg cells, since these cells were still subconfluent at this stage.

**TABLE 1**
**$IC_{50}$ Concentrations of TTA (μM)[a]**

| | Day 2 | Day 4 | Day 6 |
|---|---|---|---|
| D54Mg | 150 | 125 | 100 |
| BT4Cn | 175 | 125 | — |
| GaMg | 75 | 40 | 30 |

[a][$^3$H]Thymidine incorporation was measured in three glioma cell lines after tetradecylthioacetic acid (TTA) treatment. The values given are the approximate TTA concentrations extrapolated to give 50% ($IC_{50}$) decrease in [$^3$H]thymidine incorporation. BTCn, rat glioma cells; D54Mg and GaMg, human glioma cells.

It can be seen from Table 1 that the TTA concentration ($IC_{50}$) giving 50% reduction in [$^3$H]thymidine incorporation decreased as the treatment period was prolonged in all three cell lines.

*Fatty acid administration.* It is likely that the mode of fatty acid administration influences the cellular fatty acid uptake, which consequently affects the observed effects on cellular growth and metabolism. To study how the mode of administration affected cellular fatty acid uptake in glioma cells, we compared the cellular uptake of TTA (1 sulfur + 16 carbon atoms) and the natural β-oxidizable PA (16 carbon atoms) when they were bound or not to BSA (Figs. 3A,3B). BSA-binding reduced the cellular uptake of TTA, especially at higher concentrations (Fig. 3A). The cellular uptake of BSA-bound TTA only marginally responded to increasing TTA concentrations, compared to the uptake of unbound TTA, which

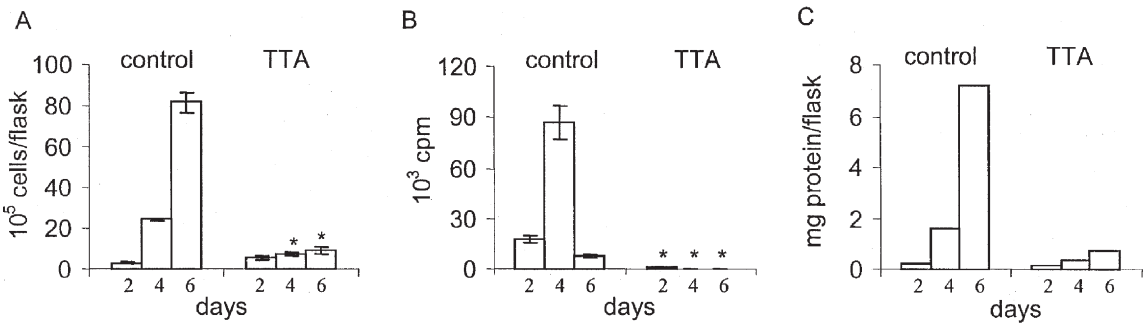


**FIG. 1.** Comparison of the effect of tetradecylthioacetic acid (TTA) on cell number (A), [$^3$H]thymidine incorporation (B), and protein content (C). BT4Cn rat glioma cells were treated, in triplicate or quadruplicate, for 2, 4, or 6 d with or without 200 μM TTA in the absence of bovine serum albumin (BSA). Cells (3500–4000/cm$^2$) were seeded in 25-cm$^2$ tissue culture flasks (cell number, protein content) or 96-well tissue culture plates ([$^3$H]thymidine incorporation). Samples within each group were pooled before cellular protein was measured. Vertical bars indicate standard deviation. *$P < 0.05$ compared to control.

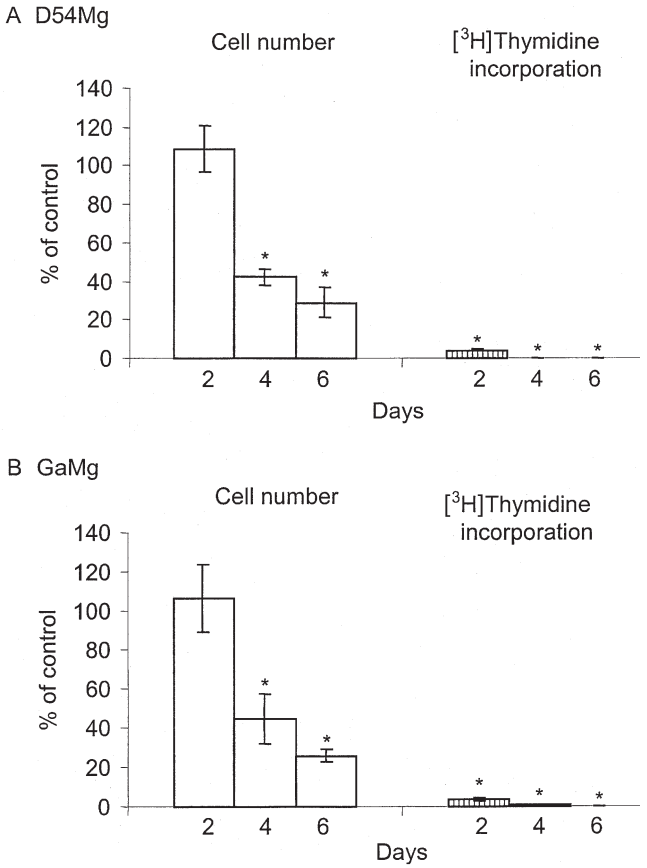


**FIG. 2.** Effects of TTA on cell number (open bar) and [$^3$H]thymidine incorporation (striped bar) in human glioma cell lines D54Mg (A) and GaMg (B) cell cultures. Cells (3500–4000/cm$^2$) were seeded in 24-well (cell number) or 96-well tissue culture plates ([$^3$H]thymidine incorporation). Three or four cultures in each group were treated for 2, 4, or 6 d with 200 μM TTA. Vertical bars indicate standard deviation. *$P < 0.05$ compared to control. For abbreviation see Figure 1.

strongly responded to increasing TTA doses. BSA binding also reduced the uptake of PA, but compared to TTA, the inhibitory effect was less pronounced (Fig. 3B). At concentrations of 200 and 500 μM, respectively, the uptake was reduced to 27 and 20% for BSA-bound TTA, and 54 and 36% for BSA-bound PA, compared to the unbound forms. Noncomplexed TTA and PA, at a concentration of 200 μM, gave a significantly higher cellular uptake than 500 μM of the BSA-bound forms. These data suggest that binding to BSA makes the fatty acids less available for cellular uptake. It is also worth noting that the uptake of BSA-complexed TTA was significantly lower than the uptake of BSA-complexed PA.

We also investigated the cellular uptake of noncomplexed TTA and PA using 0.1 M NaOH, 0.1 M KOH, KOH (0.1 M)/ethanol (1:3), or DMSO/ethanol (1:10) as solvents before addition to culture medium, as described in the Experimental Procedures section. Figures 3C and 3D show that there were only marginal differences between the preparations, and the cellular uptake of TTA and PA in BT4Cn cells increased with all four preparations in a dose-dependent manner. The use of 0.1 M NaOH as a vehicle seemed to give slightly higher uptake than observed with the other preparations. Interestingly, the cellular uptake of PA was significantly higher than the uptake of TTA in all preparations.

*Influence of BSA binding on antiproliferative properties of fatty acids.* Since BSA binding influenced the cellular uptake of TTA and PA, it is also likely to affect antiproliferative properties of these fatty acids. Although growth reduction was observed, BSA binding clearly attenuated the TTA-mediated reduction in [$^3$H]thymidine incorporation in BT4Cn, D54Mg, and GaMg cells compared to unbound TTA (Fig. 4). The [$^3$H]thymidine incorporation results from BT4Cn, D54Mg, and GaMg cells treated with unbound TTA have been presented previously (14), but are included here for comparison. The antiproliferative effect of TTA in BT4Cn cells was efficiently neutralized in BSA-supplemented cultures (Fig. 4A). Although PA retained antiproliferative properties in the presence of BSA, the PA concentration had to be increased significantly to get the same inhibition as with unbound PA (Fig. 4A). D54Mg cells were more sensitive to TTA than to PA in the absence of BSA, but the opposite was found in the presence of BSA as the antiproliferative effect of TTA was more affected than the antiproliferative effect of PA (Fig. 4B). Although unbound PA and TTA equally affected [$^3$H]thymidine incorporation in GaMg cells, the 50% inhibitory ($IC_{50}$) value of BSA-bound PA (approximately 150 μM) was lower than the $IC_{50}$ value of BSA-bound TTA (approximately 450 μM) (Fig. 4C). BSA alone significantly stimulated [$^3$H]thymidine incorporation in GaMg cells, whereas it had no effect on [$^3$H]thymidine incorporation in BT4Cn and D54Mg cells.

*[$^3$H]Thymidine incorporation and the MTT assay.* The MTT colorimetric assay measures the conversion of the orange-colored soluble MTT into purple insoluble formazan crystals by mitochondrial dehydrogenases, and it is used as a simple and inexpensive assay for monitoring survival and proliferation (27). To compare the MTT assay with [$^3$H]thymidine incorporation and to verify that these two assays reflect the number of BT4Cn cells, we compared the measurements that were obtained when the two assays were applied to BT4Cn cell cultures of different densities (0–100,000 cells per well) in 96-well tissue culture plates (Figs. 5A,5B). We found a linear correlation between cell number and MTT conversion in wells with up to 20,000 cells per well. In contrast, [$^3$H]thymidine incorporation correlated linearly with cell number up to at least 100,000 cells per well, which was the highest cell number tested. We also performed an experiment to compare the MTT assay with [$^3$H]thymidine incorporation in experiments with TTA. Figure 5C shows that both MTT conversion and [$^3$H]thymidine incorporation were similarly reduced in BT4Cn cells treated with 200 or 250 μM TTA for 40 h.

*Cell number, cellular protein, and total DNA content.* In conformity with cell number, cellular protein and total DNA content represent measures for the amount of cellular mass, and thus they can be used for monitoring cell growth. These three parameters were measured and compared in BT4Cn cell cultures grown for 4 or 8 d in 100 μM TTA. As expected, cell number, cellular protein and total DNA content were simi-

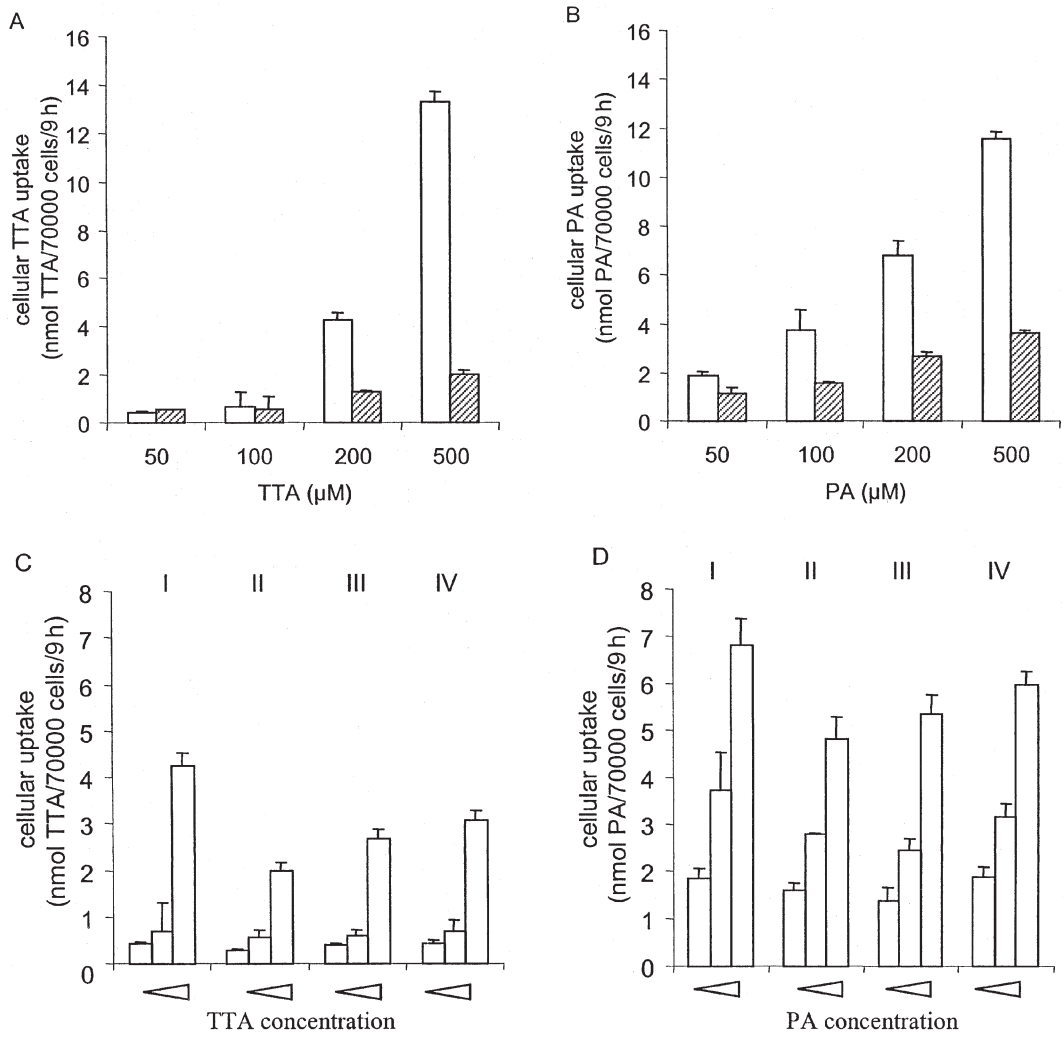


**FIG. 3.** Cellular uptake of TTA and palmitic acid (PA) in the presence of different vehicles. BT4Cn (70,000 cells per well) were seeded in 24-well tissue culture plates and incubated overnight before [1-$^{14}$C]-labeled TTA or PA was added in triplicate cultures. After 9 h of incubation the cells were washed, and the cellular radioactivity was measured. The cellular uptake of [1-$^{14}$C]TTA (A) and [1-$^{14}$C]PA (B) was measured in cells grown in culture medium with different concentrations of unbound (open bar) or BSA-bound (ruled bar) TTA or PA (vehicle: 0.1 M NaOH). The molar fatty acid/BSA ratio was 2.5:1. The cellular uptake of unbound [1-$^{14}$C] TTA (C) and [1-$^{14}$C] PA (D) was compared when they were dissolved in (I) 0.1 M NaOH, (II) 0.1 M KOH, (III) 0.1 M KOH/ethanol (1:3, vol/vol), or (IV) dimethylsulfoxide/ethanol (1:10, vol/vol) before addition to the growth medium (final concentration of TTA or PA: 50, 100, and 200 μM). Wedges indicate increasing concentrations. The data are given as mean ± SD. For abbreviations see Figure 1.

larly affected by the TTA treatment (Fig. 6). However, after 8 d of treatment, DNA content was more reduced than cell number and cellular protein.

*Multicellular tumor spheroids.* In contrast to two-dimensional monolayer models, multicellular spheroid models offer opportunities to investigate the behavior of three-dimensional tumor cell masses (28). Not all cell lines have the properties needed to develop spheroids, and consequently, this methodology has some limitations. In contrast to D54Mg and GaMg cells, BT4Cn cells were not able to form proper spheroids. The growth of D54Mg and GaMg spheroids decreased in the presence of 500 μM BSA-bound TTA compared to BSA alone (Figs. 7A,7B). A dose-dependent growth-inhibiting effect of TTA was observed in both cell lines (Figs. 7C,7D).

## DISCUSSION

We have evaluated and compared different protocols commonly used for the analysis of the effects of fatty acids on cellular proliferation. As expected, experimental design may profoundly influence the outcome of such experiments. Obviously, cell density has a major impact on the rate of [$^{3}$H]-thymidine incorporation, and hence this type of analysis is of limited value when analyzing the effects of fatty acid on post-confluent growth of transformed cells. Another problem associated with differences in degree of confluency is the fact that the expression of important regulators of lipid metabolism often changes according to growth phase. Thus, PPAR expression in rat BT4Cn glioma cells changes dramatically in

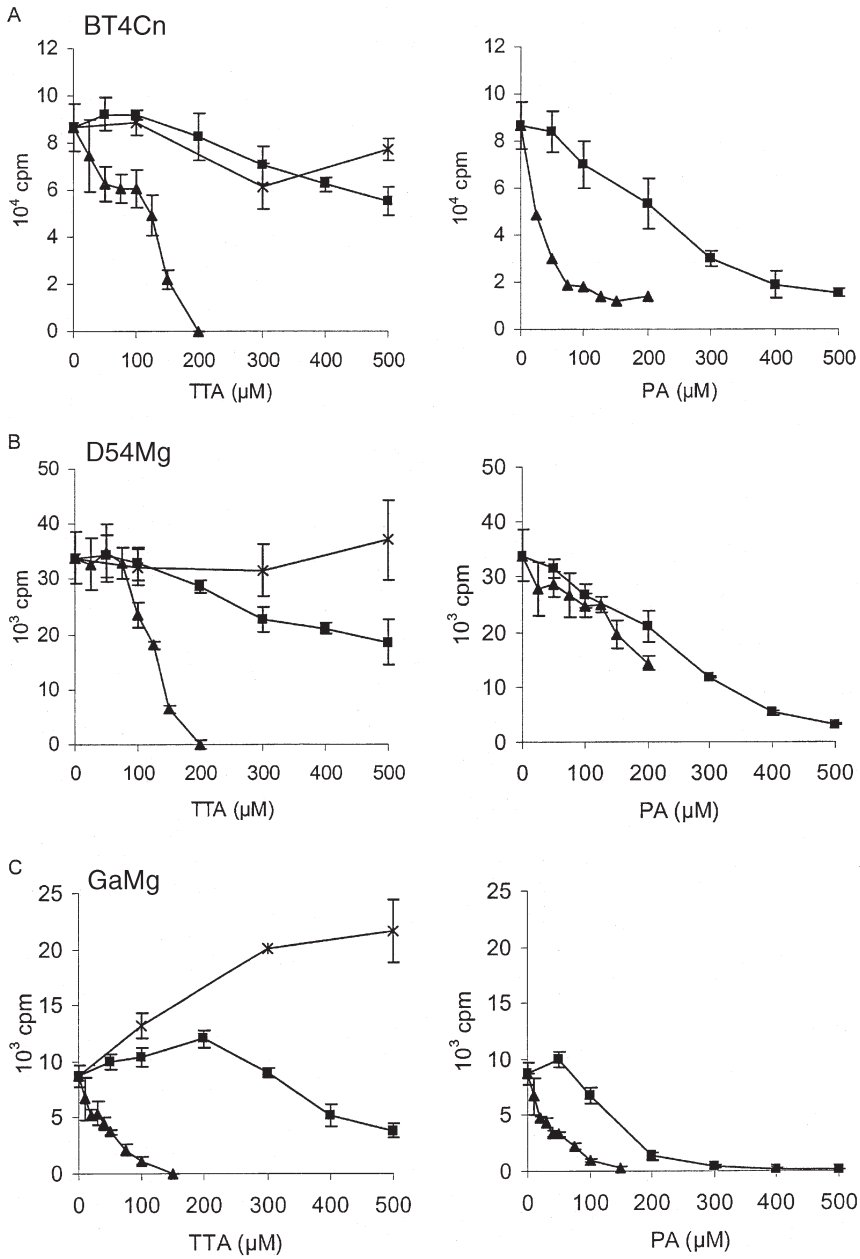


**FIG. 4.** Influence of BSA on antiproliferative effects of TTA and PA. BT4Cn (A), D54Mg (B), and GaMg (C) cells were grown in triplicate cultures for 4 d in different concentrations of TTA (left panel) or PA (right panel) in the absence or presence of BSA. Proliferation was measured by [$^3$H]thymidine incorporation in cultures treated with BSA alone (×) in concentrations corresponding to BSA concentrations in the fatty acid preparations, BSA-bound TTA or PA (■), and unbound TTA or PA (▲). The data (cpm) are presented as mean ± SD. For abbreviations see Figures 1 and 3.

response to cell density (Berge, K., Tronstad, K.J., Flindt, E.N., Rasmussen, T.H., Madsen, L., Kristiansen, K., and Berge, R., unpublished data). To get reliable and reproducible results, the degree of confluency should be carefully controlled, and measurements of postconfluent growth should be performed by mass-based methods since these are insensitive to metabolic changes caused by confluency.

Another parameter of importance is the duration of the treatment with fatty acids. Here, the $IC_{50}$ value, determined by [$^3$H]thymidine incorporation, decreased in conformity with prolonged periods of treatment in all three cell lines (Table 1). [$^3$H]Thymidine incorporation clearly was a more sensitive measure of the antiproliferative effect of TTA at the earlier stages than cell number in BT4Cn (Fig. 1), D54Mg (Fig. 2A), and GaMg (Fig. 2B) cells, possibly reflecting the ability of cells in M-, $G_2$- and late S-phase to undergo mito-

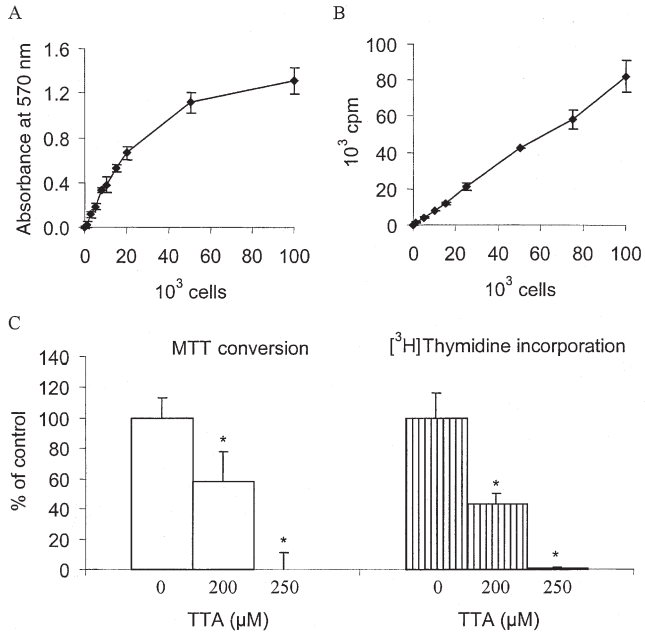


**FIG. 5.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion and [$^3$H]thymidine incorporation. Different numbers of BT4Cn cells were seeded in 96-well tissue culture plates. MTT reduction (A) and [$^3$H]thymidine incorporation (B) were measured as described in the Experimental Procedures section. (C) BT4Cn cells (1,000 per well) in 96-well tissue culture plates were treated for 40 h with TTA, followed by detection of MTT conversion (open bar) or [$^3$H]thymidine incorporation (striped bar). The measurements were performed in triplicate cultures. The results are presented as mean ± SD. *$P < 0.05$ compared to control. For abbreviation see Figure 1.

sis, although TTA leads to DNA-synthesis arrest. In this case the cell number will increase at first, but the TTA-mediated arrest in DNA-synthesis will inhibit further mitosis.

Both [$^3$H]thymidine incorporation and MTT assays should be performed under standardized conditions, as variation in labeling/incubation conditions and, in the case of MTT assays, formazan solubilization may lead to experimental variation. When studying anticancer effects of compounds that cause metabolic changes unrelated to growth, metabolic assays, such as the MTT assay, may give erroneous results in terms of cellular proliferation. Furthermore, it was reported that cells induced to differentiate and exhibit morphological changes indicative of preapoptosis, take up [$^3$H]thymidine nonspecifically (16). In spite of these sources of variation, there was no discrepancy between MTT conversion and [$^3$H]thymidine incorporation in BT4Cn cells treated for 40 h with TTA (Fig. 5C).

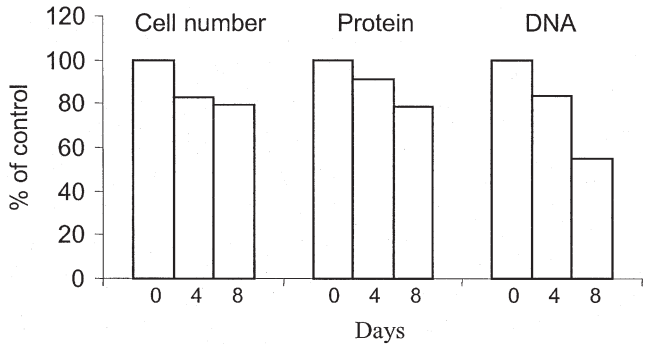


**FIG. 6.** Effects of TTA on cell number, cellular protein, and total DNA content. Cell number, cellular protein, and DNA content were determined in triplicate or quadruplicate BT4Cn cultures as described in the Experimental Procedures section after 2, 4, and 8 d of incubation in 100 μM TTA. The columns represent percentages of the respective controls. Standard deviations were less than 15%. Samples within each group were pooled before cellular protein was measured. For abbreviation see Figure 1.

Altered cellular metabolism mediated by TTA may change the amount of cellular protein. Furthermore, the production of apoptotic cellular fragments may lead to overestimation of cell number determined by an automatic particle counter. These factors may explain the small discrepancy between the measurements of cell number, cellular protein, and DNA content after 8 d of TTA treatment (Fig. 6).

Albumin has a very high affinity for fatty acids, which keeps the concentration of nonprotein bound fatty acids in human serum in the order of 5–10 nM (19). Dissociation of fatty acids from the albumin–fatty acid complex is thought to be facilitated by specific albumin-binding proteins, and both passive and active mechanisms are thought to be involved in cellular fatty acid uptake (18,29). In our experiments, the uptake of TTA and PA in BT4Cn glioma cells was significantly reduced when the fatty acids were bound to BSA, compared to nonbound TTA and PA (Figs. 3A,3B). In the fatty acid preparations without BSA supplementation, the serum still contained an abundance of fatty acid-binding proteins, including albumin, preventing the formation of micelles due to excessive concentration of the free fatty acid. Our results suggest that this provides more favorable conditions for fatty acid uptake.

Albumin supplementation has been reported to reduce the antiproliferative properties of polyunsaturated fatty acids in human and rat hepatoma (6,30). In the colon adenocarcinoma cell line HT29-D4, albumin addition induced cell growth accompanied with a low level of differentiation (20). However, in human MCF-7 breast cancer cells, proliferation was inhibited by albumin supplementation (21,31). In our experiments BSA significantly induced proliferation of GaMg, but not of BT4Cn and D54Mg cells. The differences in TTA and PA sensitivity in experiments with and without addition of BSA (Fig. 4) can be explained by fatty acid-specific changes in cellular uptake, since BSA seemed to affect the uptake of TTA more than the uptake of PA (Fig. 3). These data show that BSA is not a "silent factor," and it may mask fatty acid properties in these kinds of experiments. In preparations without BSA supplementation, there were only minor changes in cellular TTA and PA uptake between the tested fatty acid vehicles (Fig. 3).

The use of multicellular tumor spheroid cultures makes it possible to study three-dimensional growth *in vitro*. Furthermore, the treatment period can be extended compared to monolayer cell cultures since the multicellular tumor spheroids grow for a longer period and confluence effects are avoided. The spheroids have to be compact, since spheroid

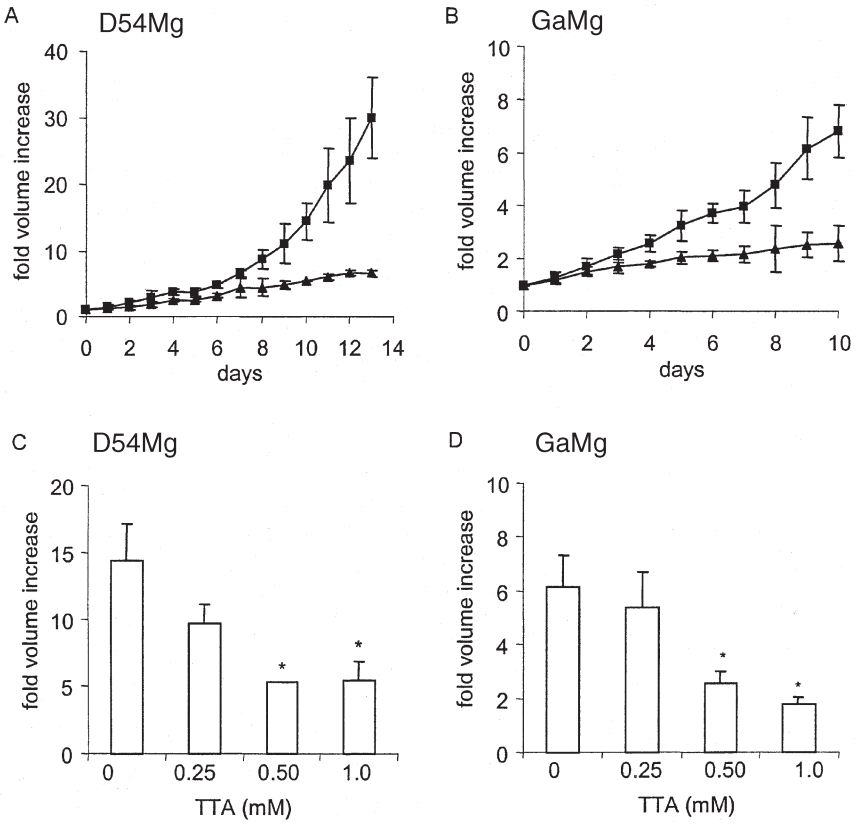


**FIG. 7.** Effects of TTA on the growth of multicellular tumor spheroids. D54Mg (A) and GaMg (B) multicellular tumor spheroids were treated in quadruplicate with BSA (control) (■) or 500 μM of BSA-bound TTA (▲). The diameters of the spheroids were microscopically measured. Fold volume increase was calculated by dividing the spheroid volume on initial spheroid volume. The effect of different doses of BSA-bound TTA on the growth of D54Mg (C) and GaMg (D) multicellular spheroids was measured after 10 and 9 d of incubation, respectively. The data are presented as mean ± SD. $^*P < 0.05$. For abbreviations see Figure 1.

splitting will complicate the microscopic growth determination. Thus, this methodology is restricted to those cell lines capable of making proper multicellular spheroids, and consequently, it has some limitations. In our experiments GaMg and D54Mg cells, but not BT4Cn cells, were capable of making proper multicellular spheroids. TTA was found to reduce the growth of both D54Mg and GaMg multicellular spheroids in a time- and dose-dependent manner (Fig. 7), which was in line with results from the monolayer experiments.

Enhanced radical generation and lipid peroxidation are commonly suggested mechanisms for the cytotoxic action of fatty acids on tumor cells. Consequently, since the redox situation in the cell cultures may be influenced, the levels of prooxidants and antioxidants in culture supplements are of importance in these kinds of experiments. Since the serum may vary from batch to batch with regard not only to levels of metals and antioxidants but also to growth factors, it is recommended to use serum from the same batch in a series of experiments and to test if the cellular response is altered when a new serum batch is employed. This advice should also be considered concerning BSA since it may contain substances, such as metals, that may influence the results.

In conclusion, during exponential growth of glioma cells, [$^3$H]thymidine incorporation was a more sensitive measure of the effects of TTA than measurements of cell number, whereas the reverse was found when the effect on postconfluent growth was determined. MTT conversion and [$^3$H]thymidine incorporation yielded concordant results in determination of the antiproliferative effects of TTA on BT4Cn cells. TTA-mediated reduction in cell number corresponded to the reduction in cellular protein and total DNA content in BT4Cn cells. Addition of BSA may significantly affect the effects of fatty acids on proliferation, as it both reduced the cellular fatty acid uptake and, in certain settings, exerted a growth-regulating function. The multicellular tumor spheroid technique is a valuable supplement providing additional opportunities in the studies of the antiproliferative properties of drugs.

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COMMUNICATION

# Changes in Conjugated Linoleic Acid Composition Within Samples Obtained from a Single Source

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**ABSTRACT:** Conjugated linoleic acid (CLA; 9*c*,11*t*-18:2) and CLA isomers have been reported, in animals, to exhibit a variety of health-related benefits. Silver ion high-performance liquid chromatography (Ag-HPLC) was found to provide better resolution of the isomers than gas chromatography. Most commercially available samples of CLA, prepared by base-catalyzed isomerization of linoleic acid (9*c*,12*c*-18:2), are composed of mixtures of four major isomers. While these isomers have been characterized, we found significant changes in CLA isomer ratios within samples obtained from the same producer/commercial supplier over a period of 1.5 yr. In the first sample, the four *cis/trans* isomers (8*t*,10*c*-18:2, 9*c*,11*t*-18:2, 10*t*,12*c*-18:2 and 11*c*,13*t*-18:2) were present in a ratio of approximately 1:2:2:1, while in the second sample they were present in almost equal proportions. If indeed certain daily levels of CLA intake are required to produce suggested health benefits in humans, changes in concentrations of specific CLA isomers could significantly impact these effects. Care must be taken to analyze the CLA used in human and animal studies.

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**KEY WORDS:** Conjugated, linoleic, CLA, composition, isomers, HPLC, GC

Conjugated linoleic acid (CLA; 9*c*,11*t*-18:2), present in dairy products and beef, and its isomers have been reported in animals to have anticarcinogenic, growth-promoting, antiatherogenic, antidiabetic, and lean body mass-enhancing properties (1–8). Most commercially available samples of CLA, prepared by base-catalyzed isomerization (9) of linoleic acid (9*c*,12*c*-18:2), are composed of mixtures of CLA isomers. While 9*c*,11*t*-18:2 is the primary CLA isomer found in nature (10), the "CLA" available in diet supplements is composed (11) primarily (>80% by weight) of four isomers (8*t*,10*c*-18:2, 9*c*,11*t*-18:2, 10*t*,12*c*-18:2, and 11*c*,13*t*-18:2).

Data from animal models have been used to suggest the 9*c*,11*t*-isomer is responsible for CLA's anticarcinogenic properties, whereas the 10*t*,12*c*-isomer is considered responsible for the observed weight loss/muscle-mass enhancement effects (12,13). The different CLA isomers present in commercially available diet supplements may thus have different health-related benefits, and other isomers (11*c*,13*t*-18:2, for example) may potentially be harmful (14). To determine the effects of specific CLA isomers, pure samples of each isomer are required. Yet CLA mixtures continue to be used (15).

We wish to report a second problem: significant batch-to-batch variations over time in CLA isomer ratios within samples obtained from the same source. Although researchers have noted significant variations in CLA composition (Ref. 16; from 0 to 75% CLA) in samples obtained from different suppliers, the existence of differences in composition of samples obtained from the same producer/commercial supplier has not been adequately addressed.

## MATERIALS AND METHODS

Composition data from CLA samples obtained *ca.* 1.5 yr apart (June 1998 and December 1999) from the same commercial supplier were compared. The samples were obtained, in fatty acid form, in gel capsules. In each instance, 3 × 1 g capsules were opened, the contents combined, and one 30-mg sample was removed. The fatty acids were converted to fatty acid methyl esters with diazomethane (17) and analyzed in duplicate utilizing silver ion high-performance liquid chromatography (Ag-HPLC) [two Varian ChromSpher Lipids (C) columns (Varian Chrompack B.V., Middelburg, The Netherlands) linked in series; flow rate of 1.0 mL/min of 0.1% acetonitrile in hexane; ultraviolet detection at 233 nm] and gas–liquid chromatography [GC; Varian 3400; 100 m SP2380 (C) capillary column (Supelco, Inc., Bellefonte, PA); flame-ionization detector].

## RESULTS AND DISCUSSION

The results are summarized in Table 1. Analysis by Ag-HPLC yielded much improved separation of the CLA isomers than we observed utilizing GC. With Ag-HPLC, the *cis/trans* isomers were separated into four peaks (six peaks in the year 1999 sample), the *trans/trans* isomers into six peaks, and the *cis/cis* isomers into four peaks, all resolved at >90%. When GC was utilized, the *trans/trans* isomers were resolved into only two peaks (the 11,13-/12,14-isomers; then the 7,9-, 8,10-, 9,11-, and 10,12-isomers), the *cis/trans* isomers into three peaks (9*c*,11*t*- and 8*t*,10*c*-18:2 eluted as one peak), and the *cis/trans* and *cis/cis* peak patterns tended to overlap. (Elution order was: *cis/trans*, *cis/cis*, then *trans/trans*.)

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Abbreviations: Ag-HPLC, silver ion high-performance liquid chromatography; CLA, conjugated linoleic acid; GC, gas–liquid chromatography.

**TABLE 1**
**Conjugated Linoleic Acid (CLA) Compositions**

| | Sample-Analyzed 6/1998 | | | | Sample-Analyzed 1/2000 | | | |
|---|---|---|---|---|---|---|---|---|
| | Gas chromatography | | Ag-HPLC | | Gas chromatography | | Ag HPLC | |
| CLA Isomer[a] | 1[b] | 2[b] | 1[b] | 2[b] | 1[b] | 2[b] | 1[b] | 2[b] |
| 12*t*,14*t*-18:2 | 0.8[c] | 0.7[c] | 0.3 | 0.2 | 1.3[c] | 1.3[c] | 0.6 | 0.6 |
| 11*t*,13*t*-18:2 | | | 0.6 | 0.6 | | | 1.5 | 1.4 |
| 10*t*,12*t*-18:2 | 6.0[d] | 6.1[d] | 2.1 | 2.3 | 7.1[d] | 7.1[d] | 3.2 | 3.0 |
| 9*t*,11*t*-18:2 | | | 1.9 | 1.9 | | | 3.0 | 2.5 |
| 8*t*,10*t*-18:2 | | | 0.3 | 0.3 | | | 1.4 | 1.0 |
| 7*t*,9*t*-18:2 | | | 0.2 | 0.2 | | | 0.9 | 0.8 |
| 13*c*,15*t*-18:2? | | | 0.0 | 0.0 | | | 0.6 | 0.8 |
| 12*t*,14*c*-18:2? | | | 0.0 | 0.0 | | | 0.2 | 0.4 |
| 11*c*,13*t*-18:2 | 16.9 | 17.1 | 16.6 | 15.9 | 23.6 | 24.0 | 20.6 | 21.4 |
| 10*t*,12*c*-18:2 | 31.2 | 30.6 | 31.4 | 31.7 | 22.1 | 22.2 | 23.5 | 23.5 |
| 9*c*,11*t*-18:2 | 39.0[e] | 38.1[e] | 27.1 | 26.9 | 35.7[e] | 36.4[e] | 20.2 | 19.6 |
| 8*t*,10*c*-18:2 | | | 14.0 | 14.5 | | | 18.6 | 17.9 |
| 11*c*,13*c*-18:2 | 3.9 | 4.6 | 0.9 | 0.8 | 1.9 | 0.7 | 1.5 | 1.6 |
| 10*c*,12*c*-18:2 | | | 2.1 | 2.4 | 3.9 | 2.0 | 1.7 | 2.3 |
| 9*c*,11*c*-18:2 | 2.2 | 2.8 | 1.9 | 1.9 | 2.4 | 3.9 | 1.2 | 1.8 |
| 8*c*,10*c*-18:2 | | | 0.6 | 0.4 | 2.0 | 2.4 | 1.3 | 1.4 |
| Total | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

| Total: | *trans/trans* | *cis/trans* | *cis/cis* | TOTAL CLA[f] | Total: | *trans/trans* | *cis/trans* | *cis/cis* | TOTAL CLA[f] |
|---|---|---|---|---|---|---|---|---|---|
| GC | 6.7 | 87.1 | 6.1 | 56.8 | GC | 8.4 | 81.4 | 10.1 | 62.2 |
| | 6.8 | 85.8 | 7.4 | 56.2 | | 8.6 | 82.6 | 8.9 | 61.0 |
| Ag-HPLC | 5.4 | 89.1 | 5.4 | | Ag-HPLC | 10.6 | 82.9 | 5.7 | |
| | 5.5 | 89.0 | 5.5 | | | 9.2 | 82.4 | 7.1 | |

[a]CLA isomers listed as eluted from silver-high-performance liquid chromatography (Ag-HPLC) as fatty acid methyl esters.
[b]Run number.
[c]Sum 11*t*,13*t*- and 12*t*,14*t*-18:2.
[d]Sum 7*t*,9*t*-, 8*t*,10*t*-, and 9*t*,11*t*-18:2.
[e]9*c*,11*t*- and 8*t*,10*c*-18:2 unresolved.
[f]Total CLA isomers (wt%) in sample. GC, gas–liquid chromatography.

Our "Total CLA" results (Table 1) determined by GC were in agreement with the "Total CLA" listed in the Material Safety Data Sheet (MSDS) included with the samples. We found the total percentage of conjugated isomers had increased *ca.* 10% by weight (GC data) from the year 1998 to the end of 1999 sample (16:0, 18:0, 9*c*-18:1, and 9*c*,12*c*-18:2 were also present at 35–40% by weight of total sample, with 9*c*-18:1 predominating at 23–25%). Between June 1998 and December 1999, the total percentage of *cis/cis* and *trans/trans* isomers increased slightly, and the % *cis/trans* totals decreased. The greatest change, however, was noted in the relative percentages of the four predominant *cis/trans* isomers (8*t*,10*c*-18:2, 9*c*,11*t*-18:2, 10*t*,12*c*-18:2, and 11*c*,13*t*-18:2). In the 1998 sample, the four *cis/trans* isomers were present in a ratio of approximately 1:2:2:1, but in the 1999 sample they were present in almost equal proportions. The presence of two other isomers (12*t*,14*c*- and 13*c*,15*t*-18:2?) in the 1999 sample is another indication that more "extreme" isomerization conditions were employed.

A quotation from a recent (18) paper, "CLA typically used in animal studies is prepared by alkali isomerization from pure linoleic acid, and contains more than 95% CLA, mainly *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers (85–90%) along with other minor isomers (*trans,trans* or *cis,cis*)" might thus not be applicable to CLA obtained from other producers/suppliers. And if, indeed, certain daily levels of CLA intake are required to produce the suggested health benefits in humans, changes in concentrations of specific CLA isomers could significantly influence these effects. Care must be taken to analyze the CLA used. "Total % CLA" should not be considered a sufficient label for these materials.

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# A New Δ7-Polyunsaturated Fatty Acid in *Taxus* spp. Seed Lipids, Dihomotaxoleic (7,11-20:2) Acid

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**ABSTRACT:** A previously undescribed fatty acid, all-*cis* 7,11-20:2 (dihomotaxoleic acid, DHT), has been characterized by gas chromatography–mass spectrometry as being present (approximately 0.1%) in seed oils of two Taxaceae containing high levels (11–16%) of taxoleic acid (all-*cis* 5,9-18:2). This compound was absent from oils of 10 other conifer genera, as well as from one member of Taxaceae containing very low amounts of taxoleic acid, suggesting that DHT is a taxoleic acid elongation product.

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In a recent reinvestigation of the seed oils from some species of the Taxaceae family, we have characterized a new Δ7-polyunsaturated fatty acid, dihomotaxoleic (DHT; *cis*-7,*cis*-11 20:2) acid, not reported previously (1–3). We wish here to comment on our gas chromatography–mass spectrometry (GC–MS) investigation and the possible origin of DHT acid in *Taxus chinensis* and *T. baccata* (Chinese and English yew, respectively) seed lipids.

A few Δ7-methylene-interrupted unsaturated fatty acids naturally occur in lipids in significant amounts, i.e., 16:3n-3 and 16:1n-9 acids in plant lipids or 22:5n-3 and 22:4n-6 acids in animal tissues. Aitzetmüller *et al.* (4) have characterized phlomic (7,8-20:2) acid, an allenic fatty acid, in *Phlomis* seed oil, by GC–MS of its methyl esters. Two other docosadienoates, 7,13-22:2 and 7,15-22:2 acids, have been reported in mollusks (5,6) and one octadecadienoate, 7,11-18:2 acid, in the sponge *Haliclona cinerea* (7). The 7,13-20:2 and 7,10,13-22:3 acids have been characterized by GC–MS of the trimethyl silyloxy derivatives in the liver lipids of rats raised on a fat-free diet (8). It was also observed that 5,11,14,17-20:4 acid, derived from intracranially injected 11,14,17-20:3 acid in developing rat brain, was elongated to 7,13,16,19-22:4 acid (9). A conjugated linoleic acid, *trans*-7,*cis*-9 18:2 acid, was identified in cow milk, cheese, beef, and human adipose tissue by Yurawecz *et al*. (10). Establishment of its structure was performed by GC–MS of the 4,4-dimethyloxazoline (DMOX) derivative. In a recent publication, Precht and Molkentin (11) reported on *cis*-7 16:1 acid occurring in human milk fat, and Christie *et al.* (12) gave the mass spectra for DMOX as well as picolinyl ester derivatives of a synthetic *cis*-7 18:1 acid.

The Taxaceae seed oils were available from an earlier investigation, and details of their source, extraction, and fatty acid composition are given elsewhere (3). Conditions for GC–MS are those described by Bhaskara *et al*. (13), but with a 30-m BPX-70 capillary column (SGE, Melbourne, Australia). The DMOX derivatives of fatty acids from the seed oils of *T. chinensis* and *T. baccata* were prepared by heating the oils directly with 2-amino-2-methyl-1-propanol according to Garrido and Medina (14), and examined by GC–MS. The DMOX spectra (Fig. 1) gave prominent molecular ions for DHT acid and dihomopinolenic (DHP) acid (*cis*-7,*cis*-11,*cis*-14 20:3) that confirmed both chain length and number of double bonds. Analysis of DHP acid from conifer seed oils as DMOX and picolinyl ester derivatives was previously performed by Wolff *et al*. (15). DHP acid had been characterized earlier in the seeds of a few pine species by Medvedev *et al*. (16), and in *Picea abies* wood extracts by Ekman (17). The mass spectrum of this fatty acid as a DMOX derivative showed an especially prominent ion at $m/z$ = 208, representing cleavage between C8 and C9, i.e., at the center of the di-methylene-interrupted ethylenic bond system (3,18). The Δ7-double bond was indicated by a gap of 12 amu between $m/z$ = 262 and 274. The spectrum of the DMOX derivative of DHT acid showed a similar cleavage for the di-methylene-interrupted ethylenic bond system. As such, the ion at $m/z$ = 208, as well as the molecular ion at $m/z$ = 361, allowed determination of the 7,11-20:2 acid structure. The geometrical *cis* configuration of both double bonds was defined by calculation of the equivalent chain length (ECL) and comparison with the ECL of *cis*-5,*cis*-11 20:2 acid, widely found in small quantities in conifer seed oils (19), which eluted just before DHT acid. Additionally, 7,11-20:2, *cis*-5,*cis*-11 20:2, and *cis*-5,*cis*-9 18:2 (taxoleic) acids co-eluted in the same fraction after argentation thin-layer chromatography (results not shown), indicating similar configurations of the two ethylenic bonds.

This unusual all-*cis* Δ7 di-methylene-interrupted fatty acid occurred in *T. chinensis* and *T. baccata* seed lipids at 0.13 and 0.06% of total fatty acids, respectively. Both species contained a high amount of taxoleic acid with 16.4 and 10.6% of total fatty acids for *T. chinensis* and *T. baccata,* respectively.

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Abbreviations: DHP, dihomopinolenic; DHT, dihomotaxoleic; DMOX, 4,4-dimethyloxazoline; ECL, equivalent chain length; GC–MS, gas chromatography–mass spectrometry.

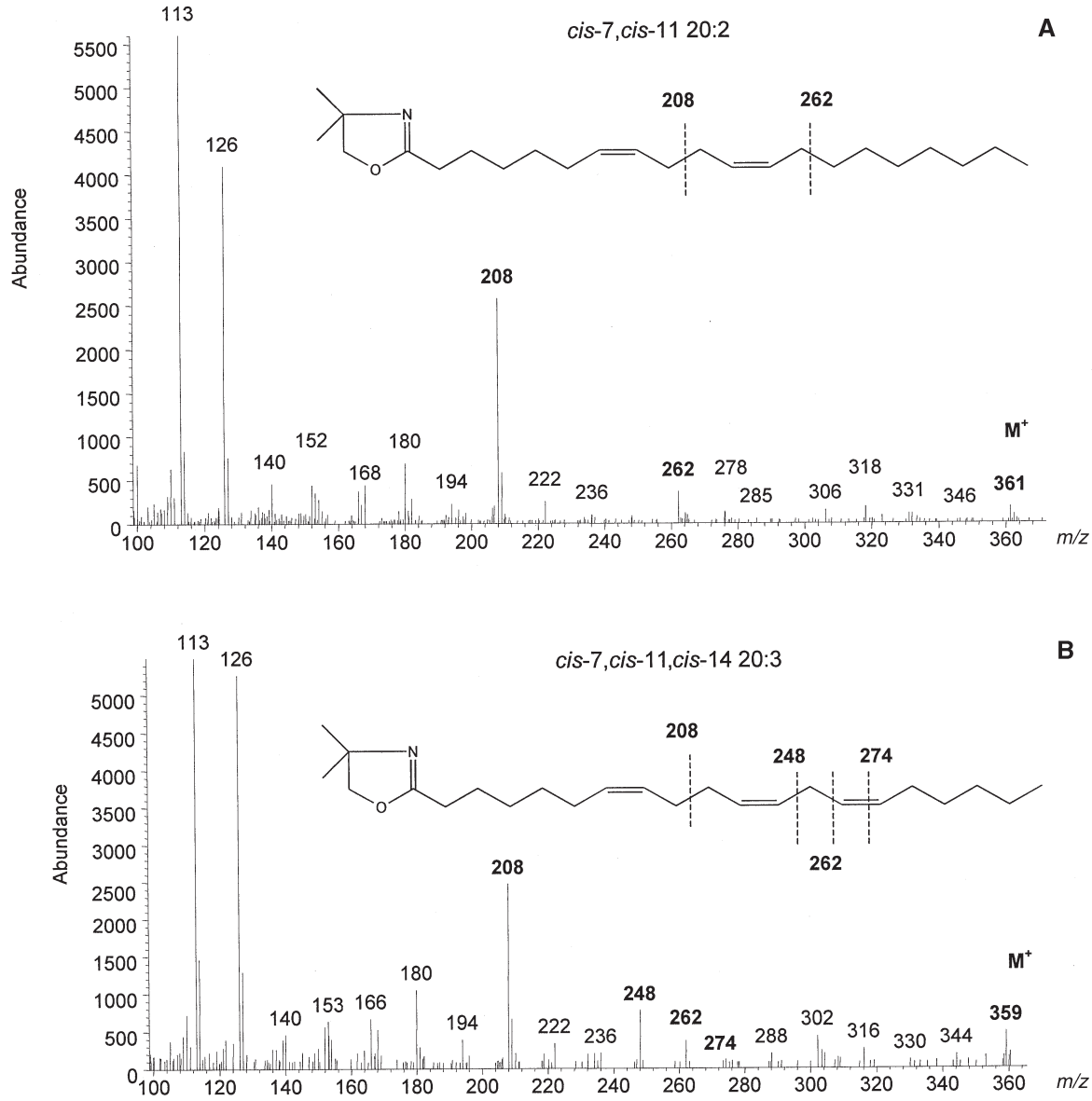


**FIG. 1.** Mass spectra of the 4,4-dimethyloxazoline derivatives of 7,11-eicosadienoate (A) and 7,11,14-eicosatrienoate (B) from *Taxus chinensis* seed lipids. Boldface numbers correspond to diagnostic ions.

These are the highest amounts found in conifers, and more generally, gymnosperm seed lipids. We did not find DHT acid in any other seed oil from several species of different conifer genera, i.e., *Pinus*, *Larix*, *Abies*, *Cedrus*, *Cephalotaxus*, *Nageia*, *Juniperus*, *Pseudolarix*, *Tsuga,* and *Hesperopeuce*, that contained at most 7.4% of taxoleic acid. *Torreya californica*, another member of the Taxaceae family, also is devoid of DHT acid, which can be explained by the fact that this species, in contrast to *Taxus* spp., contains only very minor amounts of taxoleic acid [0.03% (3)]. The coexistence of DHT acid and a high amount of taxoleic acid leads us to suggest that DHT acid could be the elongation product of taxoleic acid, in the same way as 7,11-18:2 acid could be the elongation product of 5,9-16:2 acid (7), and phlomic acid that of laballenic acid [(5,6-18:2) (4)].

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REVIEW

# Atmospheric Pressure Chemical Ionization Mass Spectrometry for Analysis of Lipids

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**ABSTRACT:** Atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) has proven to be a very valuable technique for analysis of lipids from a variety of classes. This instrumental method readily produces useful ions with gentle fragmentation from large neutral molecules such as triacylglycerols and carotenoids, which are often difficult to analyze using other techniques. Molecules that are easily ionized, such as phospholipids, produce molecular ions and diagnostically useful fragment ions that are complementary to those produced by methods such as electrospray ionization MS with collision-induced dissociation. The simplicity and versatility of APCI-MS make it an ideal tool for use in solving hitherto very difficult analytical problems.

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Mass spectrometry (MS) for analysis of liquid chromatographic effluent has long been a goal of chemists studying nonvolatile, often large molecules, which cannot be separated well, or at all, by gas chromatography. Many approaches have been developed to meet this objective, with particle beam, thermospray (TSP), continuous-flow fast atom bombardment (or the similar liquid secondary-ion MS, LSIMS), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) interfaces becoming most widely used (1). The recent book by Niessen (1) contains an excellent summary of the development and history of each, some of which is reflected here. Each approach has achieved varying degrees of success in allowing analysis of large molecules, and each has its own set of inherent drawbacks as well as benefits. Environmental and pharmaceutical samples have been the primary subjects of analysis, but lipids have also been analyzed extensively over the years by using each of these technologies as they were developed. TSP was the most popular commercially available liquid chromatography (LC)/MS interface for a period because of its relative simplicity, low background noise, and high flow rates allowed. But in recent years, TSP has been supplanted in popularity by two atmospheric pressure ionization (API) methods, ESI-MS and APCI-MS (1,2).

The first API source was developed in the 1970s by Horning *et al.* (3–6) at the Baylor College of Medicine (Houston, TX). The method described an ionization interface at atmospheric pressure, external to the high vacuum chamber of a modified Finnigan Model 1015 quadrupole mass spectrometer. Initially, a $^{63}$Ni foil was used as a source of electrons to perform ionization (3–5), but later a corona discharge electrode was used (5,6). This version with the corona discharge electrode became the model for modern commercially available APCI interfaces. Initially simply called API, this ionization method later become known as APCI to differentiate it from the other ionization source at atmospheric pressure, ESI. The full potential of APCI was not initially realized, and in the meantime the other emerging API source, ESI, was developed by Fenn and coworkers at Yale (New Haven, CT) (7). As ESI became recognized for its ability to identify the masses of large proteins (8), interest in API techniques increased. Improvements in commercial ESI sources, such as those made by Bruins *et al.* (9) to yield pneumatically assisted electrospray (called ionspray), allowed larger effluent flow rates than could previously be used. These improvements in ESI caused API methods to gain more widespread use. By the late 1980s and early 1990s, all major MS instrument manufacturers had introduced API sources, with most of them having both ESI and APCI interfaces. Although use of APCI is not yet as widespread as ESI, the number of reported applications of APCI-MS is burgeoning. A review of the applications of APCI-MS to analysis of lipids has appeared elsewhere (10). The number of recent applications reported in the literature warrants an updated chronicle of APCI-MS for lipid analysis.

APCI is a soft ionization technique, but unlike ESI, APCI usually does produce some degree of fragmentation that is useful for structural characterization. Very simple in its design, a typical APCI source has the following components, as shown in Figure 1: (i) a capillary out of which the LC effluent is sprayed through a nozzle by means of a concentric nebulizer gas surrounding the capillary, (ii) a heated vaporizer tube, concentric around the LC capillary outlet, which desolvates the analyte molecules, (iii) a corona discharge needle, which pro-

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Abbreviations: ACN, acyl carbon number; AMVN, azobis(2,4-dimethylvaleronitrile); APCI, atmospheric pressure chemical ionization; API, atmospheric pressure ionization; Br-MB, 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one; CID, collision-induced dissociation; cSFC, capillary supercritical fluid chromatography; DAG, diacylglycerol; ECN, equivalent carbon number; EIC, extracted ion chromatogram; ELSD, evaporative light-scattering detector; ESI, electrospray ionization; FA, fatty acid; FAME, fatty acid methyl ester; FI, flow injection; FID, flame-ionization detection; GC, gas chromatography; L, linoleic acid; LC, liquid chromatography; Ln, linolenic acid; LSIMS, liquid secondary ion mass spectrometry; MAG, monoacylglycerol; MS, mass spectrometry; MS/MS, tandem mass spectrometry; O, oleic acid; P, palmitic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; RP-HPLC, reversed-phase high-performance liquid chromatography; S, stearic acid; SFC, supercritical fluid chromatography; SIM, selected ion monitoring; TAG, triacylglycerol; THCA, 3α,7α,12α-trihydroxy-5β-cholestanic acid; TSP, thermospray; UV, ultraviolet; Vis, visible.

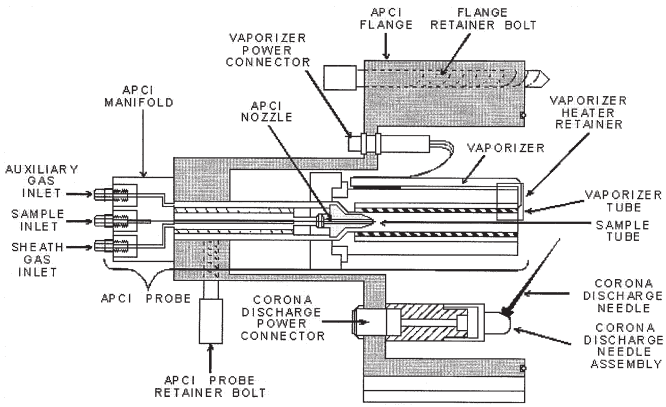


**FIG. 1.** Atmospheric pressure chemical ionization (APCI) source. (Reprinted with permission ThermoQuest Corp.)

duces ions from the molecular mist exiting the end of the heated vaporizer tube, and (iv) a pinhole entrance into an area of intermediate vacuum (~$10^{-3}$ Torr), followed by ion-focusing elements and skimmers that transport ions into the high-vacuum mass analyzer region. An auxiliary, or makeup, gas is often included concentric with the nebulizer gas to assist in desolvation. A countercurrent, or "curtain," gas is included in some designs to prevent clogging of the pinhole entrance by nonvolatile components, to assist in droplet evaporation, and to help break up solvent–ion clusters.

The primary reactions that lead to ion formation in a nitrogen atmosphere in the presence of water, at up to 4 Torr, were first studied by Good *et al.* (11). These reactions were recognized by Carroll *et al.* (4) to be the mechanism of hydronium ion formation in the APCI source. At atmospheric pressure, sufficient water is present that the principal ions present in the carrier gas without LC effluent or analyte are cluster ions of the type $H^+(H_2O)_n$. Figure 2 is a diagram of the ionization process for APCI. Spectra obtained using the $^{63}Ni$ β source were virtually identical to spectra obtained using a corona discharge needle, so the mechanism of ionization is assumed to be the same (6). At 200°C, with nitrogen as the carrier gas, and with only atmospheric water, the most abundant cluster is $(H_2O)_2H^+$, with $(H_2O)_3H^+$ and $H_3O^+$ also present. The $NO^+$ ion, $(H_2O)NO^+$, and $(H_2O)_2NO^+$ are also present. The following set of reactions result in the ionized water clusters responsible for ionization (3):

$$N_2 + e \rightarrow N_2^+ + 2e \quad [1]$$

$$N_2^+ + 2N_2 \rightarrow N_4^+ + N_2 \quad [2]$$

$$N_4^+ + H_2O \rightarrow H_2O^+ + 2N_2 \quad [3]$$

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH\cdot \quad [4]$$

$$H_3O^+ + H_2O + N_2 \rightarrow H^+(H_2O)_2 + N_2 \quad [5]$$

$$H^+(H_2O)_{n-1} + H_2O + N_2 \rightarrow H^+(H_2O)_n + N_2 \quad [6]$$

At atmospheric pressure in the presence of trace amounts of water and nitrogen gas, protonated molecular ions are formed by gas-phase ion–molecule reactions with water cluster ions. Thus, protonated molecular ions are the most common pseudomolecular ions observed using APCI-MS. However, the presence of chemicals in the LC effluent stream, or chemicals that are added as sheath liquid, can also participate in the chemical ionization reactions, as shown by applications below. The reactions and mechanisms observed under APCI conditions are nearly the same as those observed using TSP MS, direct insertion probe MS, desorption chemical ionization, and other soft ionization techniques. In positive ionization mode, the mechanisms are protonation, adduct formation, and charge transfer. In the negative ion mode, electron capture and anion attachment are the primary mechanisms of

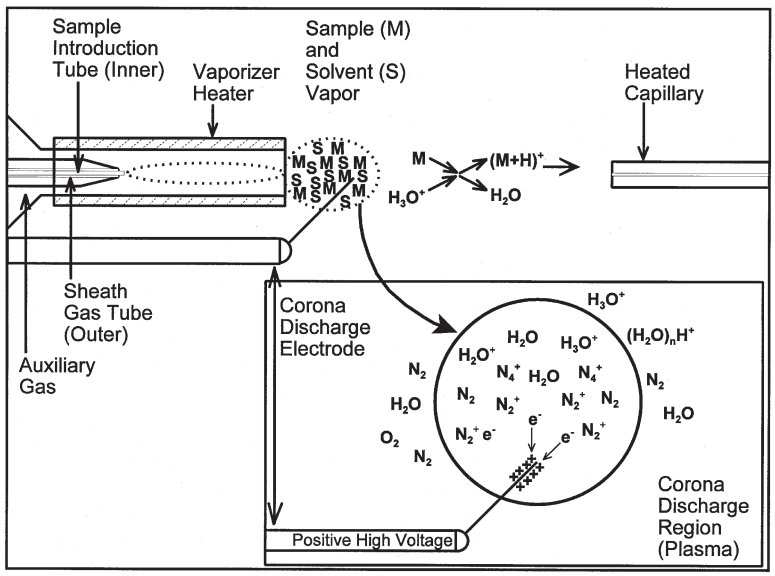


**FIG. 2.** Ionization process occurring in Atmospheric Pressure Chemical Ionization source. (Figure adapted from ThermoQuest Corp. operator's manual.)

ion formation (1) Most work on the APCI-MS analysis of lipids has been in positive ion mode, with ionization occurring according to the mechanisms above.

## FATTY ACID (FA) ANALYSIS

As mentioned, APCI was developed before ESI, but it took the popularity of ESI and the commercial availability of API interfaces to spur the use of APCI-MS. PE Sciex and Hitachi each introduced early API inlets to their machines in the mid-1980s. Through analysis of environmental and pharmaceutical chemicals, APCI became known as being suitable for analysis of neutral molecules of small to moderately large size. ESI was especially suited to charged or readily charged molecules, with quite large molecules such as proteins being characterized by their multiply charged ions. The Hitachi API interface with APCI source became commercially available in Japan and was used by Kusaka *et al.* (12) in 1988 for the first reversed-phase high-performance liquid chromatography (RP-HPLC)/APCI-MS analysis of a mixture of FA standards as their anilide derivatives. Anilide derivatives were formed because the API source did not provide sufficient sensitivity for methyl esters or the free acids. APCI was seen to yield virtually only molecular ions from the FA anilides. Ions at low mass arising from solvent clusters became relatively more abundant as the signal of the anilides decreased (i) with increasing fatty chain length, (ii) as a result of chromatographic peak broadening, and (iii) owing to the changing mobile phase composition. The potential use of LC/APCI-MS for qualitative and quantitative analysis of FA was demonstrated. Samples from 55 to 385 pg were injected to illustrate the potential for quantitative applications. Still, it was several more years until APCI-MS was further applied to analysis of lipids.

Ikeda and Kusaka (13) expanded their original work on FA anilides to a comparison of six amide-containing FA derivatives. In 1992, they reported a comparison of six amide derivatives useful for APCI-MS analysis of both hydroxy and non-hydroxy FA. Based on the slopes of calibration curves obtained for palmitamide derivatives, the sensitivities of the amides were found to increase in the following order: *N-n*-propyl amide (2.1) > anilide (1.0) > *N,N*-diethylamide (0.66) ≈ amide (0.62) > *N,N*-diphenylamide (0.44) > *N*-1-naphthylamide (0.06), where the numbers in parentheses represent the sensitivity relative to the original anilide derivative. The propyl amide derivative provided the greatest sensitivity, so this was used for further experiments. *N*-Propyl amide derivatives of hydroxy-containing FA were separated by RP-HPLC and detected using APCI-MS; typical APCI mass spectra are shown in Figure 3. The locations of the hydroxy groups had a distinct effect on the fragmentation of the FA under APCI conditions. FA standards with the hydroxy group in the middle of the chain (Δ12) produced primary fragments by loss of water from the protonated molecular ion $[M - H_2O + H]^+$, whereas the hydroxy group in the Δ2 position produced mostly protonated molecular ion, $[M + H]^+$. Prostaglandins and hydroxy FA from rat brain extracts were also analyzed.

Kusaka and Ikeda (14) then extended their analysis of FA derivatives to RP-HPLC/APCI-MS analysis of hydroxy and hydroperoxy FA standards and of bovine lecithin FA that underwent photo-oxidation. In this report, they produced a

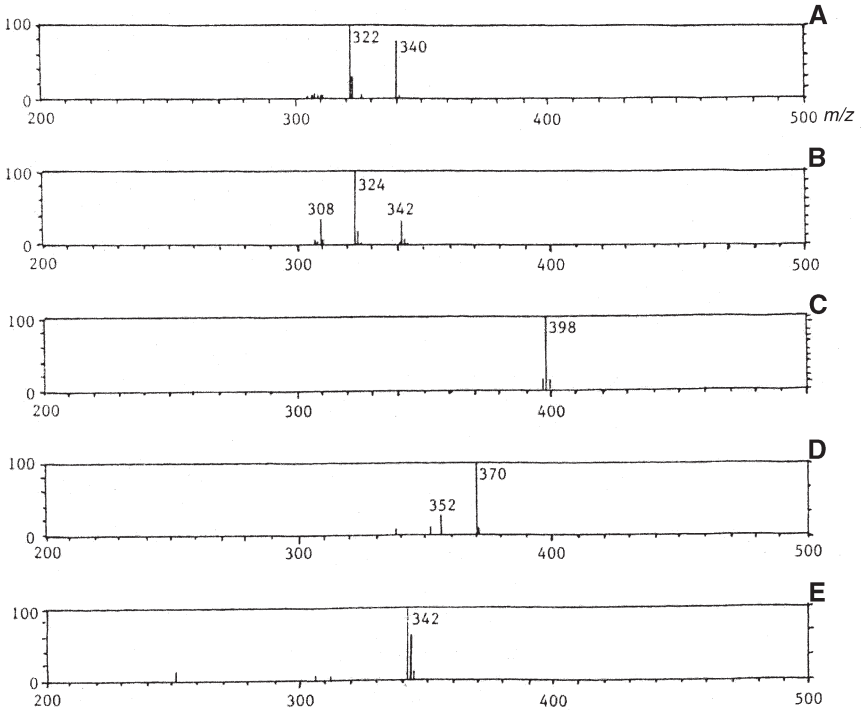


**FIG. 3.** Mass spectra of *N-n*-propylamide derivatives of hydroxy fatty acids. (A) 12-OH-$C_{18:1}$, (B) 12-OH-$C_{18:0}$, (C) 2-OH-$C_{22:0}$, (D) 2-OH-$C_{20:0}$, (E) 2-OH-$C_{18:0}$ (reprinted from Ref. 13 with permission from Elsevier Science).

fluorescent 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (Br-MB) derivative that allowed the LC effluent to be monitored by absorption at 355 nm, as well as detection by APCI-MS. Hydroperoxy FA were seen to produce no molecular ion, but instead to produce fragments representing loss of water, $[M - H_2O + H]^+$ (base peak), or representing loss of the entire hydroperoxy group, $[M - H_2O - O + H]^+$. Hydroxy FA produced a primary fragment representing loss of water, $[M - H_2O + H]^+$ (base peak), and a protonated molecular ion, $[M + H]^+$.

## TRIACYLGLYCEROLS (TAG)

Because TAG are large neutral molecules that are not amenable to gas chromatography (GC), better methods for detection of TAG separated by LC have long been sought. Whereas a host of derivatization methods is available for analysis of FA by GC and LC, there are relatively few methods for analysis of intact TAG. Thus, a technique like APCI-MS, which ionizes large neutral molecules in a broad range of mobile phases, is ideally suited for lipid analysis. In 1993, Tyrefors *et al.* (15) reported examples of APCI-MS spectra obtained from several classes of lipids after separation by a supercritical fluid chromatography (SFC) system using an open tubular column. The SFC system was attached to a Sciex API III tandem mass spectrometer *via* a lab-built column restrictor/interface system. Their results showed the separation of a FA methyl ester (FAME: either pentacosanoic acid methanoate or stearic acid methanoate), a TAG (trilaurin), cholesterol, and cholesterol palmitate. The data demonstrated the simple, uncluttered mass spectra that are characteristic of the ionization technique. Cholesterol yielded an $[M - 17]^+$ base peak (= $[M - H_2O + H]^+$), showing behavior similar to the alcohols reported by Kusaka *et al.* (12,13). Spectra of other molecules containing a hydroxy group [prostaglandin $F_{2\alpha}$ isopropanoate and bis(α-epoxyacrylate)] similarly exhibited loss of water from the protonated molecular mass. All of the spectra in the paper by Tyrefors *et al.* (15) exhibited a common characteristic. They each contained substantial water adducts in addition to, or instead of, protonated molecular ions. This was a result of the fact that the makeup gas used in the restrictor interface was synthetic air that was moistened by sparging through water before entering the oven where it was preheated to temperature. This demonstrated the effect of components in the nebulizer gas on the abundance of near-molecular ions produced during APCI. Similarly, components in the LC mobile phase can produce adduct ions with analyte molecules. Instrument manufacturer design differences may also lead to differences in proportions of near-molecular ions formed, but the published APCI spectra produced by most machines are remarkably similar, unless intended otherwise. Other authors have sparged carrier gas through solvents to improve the formation of high-mass ions, as discussed below.

Two years later, in 1995, Byrdwell and Emken (16) demonstrated the first RP-HPLC separation of a mixture of TAG with detection by APCI-MS. They used an RP-HPLC separation coupled to a Finnigan MAT SSQ 710C quadrupole mass spectrometer *via* an APCI interface for separation and identification of a mixture of synthetic TAG standards. The mass spectra produced by all of the TAG standards were simple and uncluttered, giving diacylglycerol (DAG) fragments and protonated molecular ions as primary peaks. It became apparent that the proportion of protonated molecular ion was dependent on the amount of unsaturation in the fatty acyl chains. TAG having the largest number of sites of unsaturation produced mostly a protonated molecular ion as a base peak, with a small abundance of DAG fragment ions. However, as the number of sites of unsaturation decreased, so did the amount of $[M + H]^+$. Fully saturated TAG gave virtually no protonated molecular ion, but instead gave only DAG fragment(s). Later results, such as the spectra in Figure 4, showed that spectra of TAG with more than four sites of unsaturation gave protonated molecular ions as base peaks, spectra of TAG with fewer than three sites of unsaturation had DAG fragment ions as base peaks, and TAG with three or four sites of unsaturation could have either of these ions as base peaks. The RP-HPLC/APCI-MS separation employed by Byrdwell and Emken (16) utilized propionitrile/hexane as the solvent gradient, and propionitrile adducts at $[M + 55]^+$ were reported. Water adducts were also reported, but spectra exhibited much lower abundances than the adducts observed by Tyrefors *et al.* (15) (because of the sparging through water).

Next, Neff and Byrdwell (17) reported the RP-HPLC/APCI-MS separation of natural mixtures of regular and genetically modified soybean oil TAG. These contained TAG with almost every combination of five FA, although only those present at > ~ 0.4% were reported. The chromatographic method used for the LC/MS in this report was later changed to an acetonitrile/methylene chloride separation to improve the separation quality, to eliminate propionitrile as solvent (due to adduct formation and the noxious nature of the solvent), and to match the method used with flame ionization-detection (FID) in their group. One, two, or three DAG fragment ions, and the protonated molecular ion, can result from a mixed-FA TAG. The tabulated APCI mass spectral results therein show the relative proportions (abundances) of the DAG fragment ions and protonated molecular ions. Some TAG with three sites of unsaturation have a $[M + H]^+$ ion as base peak [e.g., palmitic-linolenic-palmitic (PLnP)] while others with three sites have a DAG base peak (e.g., palmitic-oleic-linoleic (POL)]. Similarly, some TAG with four sites of unsaturation had $[M + H]^+$ ions as base peaks (e.g., LLP), while others had a DAG base peak (e.g., OLO). However, mass spectra of all TAG with less than three sites of unsaturation had DAG fragment ions as base peaks, while spectra of all TAG with more than four sites of unsaturation had $[M + H]^+$ ions as base peaks.

The next report by Neff and Byrdwell (18) of APCI-MS for analysis of TAG was on the TAG with naturally occurring functional groups contained in the specialty seed oils *Crepis alpina* and *Vernonia galamensis*. *Crepis alpina* contained crepenynic acid (*cis*-9-octadecen-12-ynoic acid), which had the alkyne functional group, and *V. galamensis* contained vernolic acid (*cis*-12,13-epoxy-*cis*-9-octadecenoic acid), which

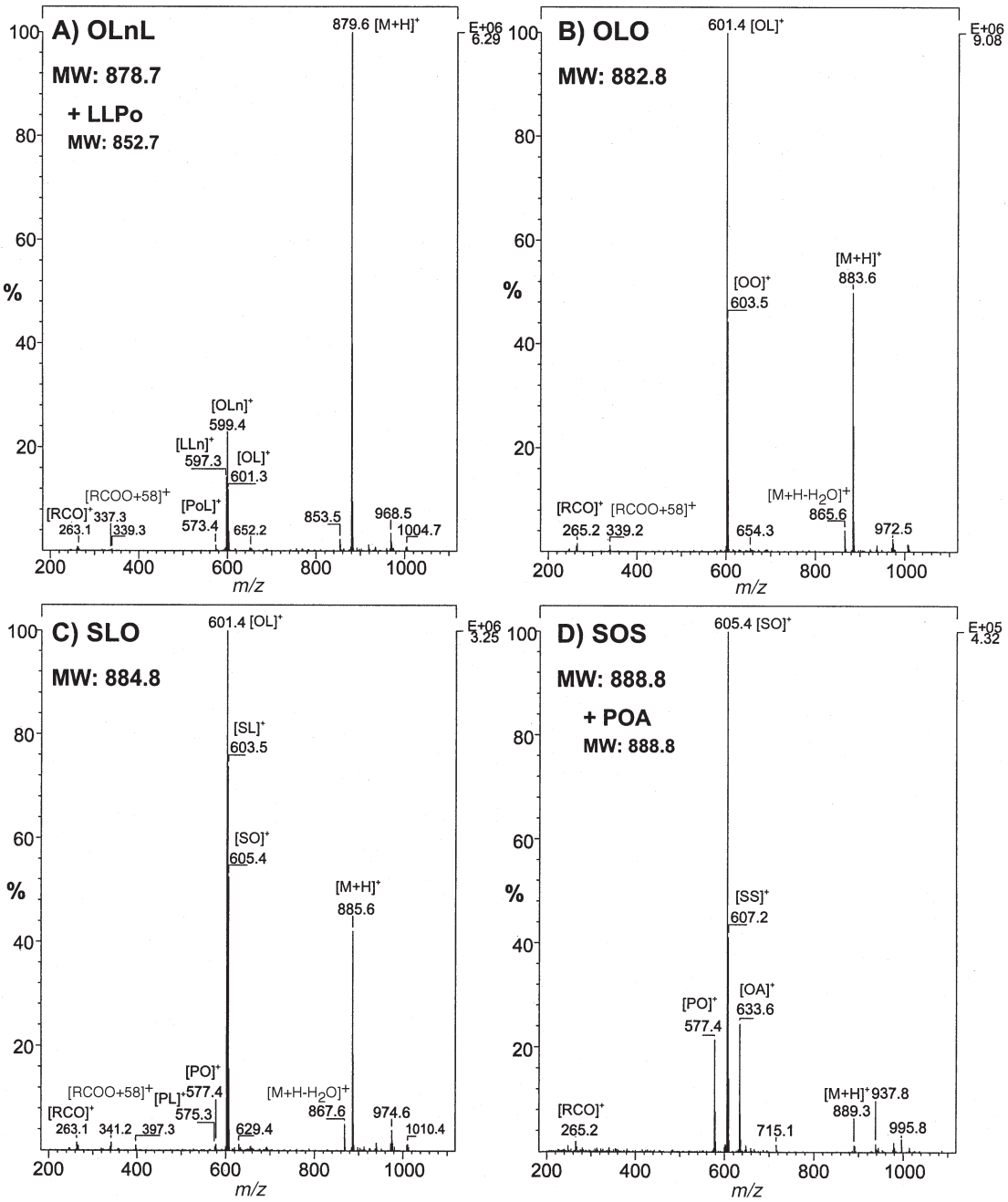


**FIG. 4.** Atmospheric pressure chemical ionization (APCI)-mass spectra of triacylglycerols in refined, bleached, deodorized soybean oil. (A) OLnL, (B) OLO, (C) SLO, (D) SOS. O, oleic acid; Ln, linolenic acid; S, stearic; L, linoleic; P, palmitic; A, arachidic; Po, palmitoleic.

contained an epoxide group methylene-interrupted from a double bond. These functional groups provided the opportunity to observe both complex fragmentation processes and adduct formation. The crepenynic acid-containing TAG behaved very much like any other unsaturated TAG. The spectra were simple, having a TAG protonated molecular ion as a base peak. Only small amounts of propionitrile and other adducts were observed from the crepenynic TAG. The vernolic acid-containing TAG, on the other hand, produced much more complicated spectra showing multiple fragmentation pathways, as well as adduct formation. The vernolic acid-containing TAG exhibited abundant protonated molecular ion peaks to allow molecular weight identification, and they also showed large peaks arising from loss of the epoxy groups through dehydration. The loss of 18 Da from the epoxides indicated that not only was the epoxy oxygen lost, which would leave a site of unsaturation, but also two other protons were removed, giving another site of unsaturation. This fragmentation occurred for the DAG fragments as well as the TAG molecular ions. In addition to dehydration, the epoxy-TAG underwent fragmentation by cleavage between the two carbons in the epoxide ring (intra-annular cleavage). Fragments appeared in which the epoxide oxygen left with the leaving fragment (hexanal), or in which the oxygen remained on the core TAG. The leaving

group containing the oxygen group then acted as a reactant to combine with another TAG molecule to produce adduct ions at $[M + 102]^+$. The epoxy groups appeared to have a stabilizing effect on the DAG fragments, because abundances of DAG fragments containing epoxy groups were larger than expected based on the number of sites of unsaturation.

## TAG POSITIONAL ISOMERS

Shortly after the initial reports of RP-HPLC/APCI-MS, Laakso and Voutilainen (19) demonstrated the application of silver ion (argentation) chromatography with APCI-MS detection using a Finnigan MAT TSQ 700 mass spectrometer. The silver ion chromatography method originally described by Christie (20) was combined with APCI-MS detection for analysis of seed oils containing both α- and γ-linolenic acids. Black currant, alpine currant, cloudberry, evening primrose, and borage oils were extracted and subjected to $Ag^+$-HPLC/APCI-MS analysis. The authors observed that the γ-linolenic acid isomer eluted prior to the α-isomer on the cation exchange column loaded with $Ag^+$ ions. Also, the position of the FA on the glycerol backbone was observed to affect the elution order on the column, with unsaturated FA in the 2-position causing the TAG to elute before the TAG having the same unsaturated FA in the 1- or 3-position. Because of the chromatographic resolution of positional isomers, differences in the mass spectra of the isomers could be observed. The authors found that the abundance of the 1,3-DAG (FA lost from the 2-position) was less than that of the 1,2- or 2,3-DAG, indicating that the ratio of DAG fragments could be used to deduce the positions of the acyl chains on regioisomers. In the mass spectrum of the TAG standard 1,3-dioleoyl-2-palmitoyl-*sn*-glycerol (OPO), the $[M - 16:0]^+$ ion was 8.5% of the abundance of the base peak, and for the 3-positional isomer, OOP, the abundance was 69.9% for the same fragment.

Around the same time, Mottram and Evershed (21) published results specifically to address the differences in mass spectra caused by different positions of FA on the glycerol backbone of TAG. They utilized loop injections made on a Finnigan MAT TSQ 700 with an APCI source to compare two types of TAG having the general form AAB vs. ABA. They showed that when B was in the 2-position, the ratio of the $[AA]^+$ to $[AB]^+$ DAG ions was much lower than when B was in the 1- or 3-position. For instance, POP would be expected to yield $[PO]^+$ and $[PP]^+$ ions in a two-to-one ratio, regardless of the positional placement of palmitic or oleic acid on the glycerol backbone. If the proportion of the $[PP]^+$ ion to $[PO]^+$ ion was substantially less than 1:2, or 50%, it meant that the $[PP]^+$ ion was energetically disfavored, by being the 1,3-isomer. The results by Mottram and Evershed showed that $[PP]^+/[PO]^+ = 0.20$, which is much less than the 0.50 which would occur if there were no discrimination between FA position. In the tabulated results reported by Neff and Byrdwell (17), the PPO in high-palmitic soybean oil exhibited a $[PP]^+$ fragment ion relative abundance of 16.8%, compared with the 50% relative abundance expected. Based on the results of Mottram and Evershed (21), this could be interpreted to mean that $[PP]^+$ was the 1,3-positional isomer, forming POP, since its fragment ion was energetically less favored. This low ratio is in agreement with the generally accepted trend that palmitic acid in vegetable oils is preferentially incorporated into the 1- and 3-positions. The very low relative abundance of the $[PP]^+$ fragment ion from PLP in the same results reported by Neff and Byrdwell, compared with the $[PL]^+$ fragment ion, also supports the general trend. Finally, the report by Mottram and Evershed showed that a TAG of the type ABC produced the smallest DAG abundance from the fragment produced by loss of the FA in the 2-position, or the AC fragment.

Mottram *et al.* (22) followed with a report showing the RP-HPLC/APCI-MS analysis of several seed, nut, and vegetable oils. They used the least-abundant DAG fragment ion to assign the most likely positional isomer identification. 2-Positional isomers were identified, without differentiation between the 1- and 3-positions. The most abundant isomers were identified for poppy seed, blackcurrant, evening primrose, hazelnut, rapeseed, olive, wheat germ, and soybean oils. A comparison with previous studies in which the soybean oil composition had been determined by evaporative light-scattering detector (ELSD) and by MS was given. Our own report, mentioned above, was included in the comparison. Our initial report (17), however, did not give a quantitative composition of soybean oils from APCI-MS, but instead stated that the semiquantitative results given there were from peaks in the ELSD chromatogram. As was stated therein, the ELSD detector is known to be nonlinear in its response to TAG. At the time the first report was published, a quantitative method was still being developed which could be used with confidence. The method for quantitation finally developed by Byrdwell *et al.* (23) was used by Mottram and Evershed (22), and the agreement with our results was good.

## QUANTITATIVE ANALYSIS

As mentioned in the initial reports on TAG, the relative abundances of DAG and protonated molecular ions were observed to vary dramatically with the number of sites of unsaturation in the TAG. And since protonated molecular ions have larger masses and are propagated through the mass spectrometer system less efficiently than DAG fragment ions, the TAG with more unsaturation (and strong $[M + H]^+$) yield less response per mole. This resulted in the observation that TAG with few or no sites of unsaturation were overrepresented during TAG quantification using APCI-MS, whereas those with a larger number of sites of unsaturation were underrepresented. A method for determining response factors was necessary. Byrdwell *et al.* (23) reported a comparison of several possible methods for response factor calculation. First it was shown that calibration curves with useful linear ranges could be constructed for individual TAG in a mixture of mono-FA synthetic TAG standards. The slopes of the calibration curves showed that the sensitivity was greater for TAG containing saturated FA (which yielded only DAG ions), followed by

monounsaturated FA, with the least sensitivity being exhibited by TAG having polyunsaturated FA (which gave abundant protonated molecular ions). However, because of the large number of TAG in a typical mixture (~30 to >100 TAG), a more practical means for determining TAG response factors was required. One approach shown was that response factors could be calculated from comparison of the raw APCI-MS data from a synthetic mixture of 35 TAG to its statistically known composition. The synthetic mixture was produced by random distribution of 5 FA. By setting the experimentally determined composition equal to the statistically known composition, a response factor for each TAG could be calculated. However, the response factors determined for the mixture were not generally applicable to samples with very different relative proportions of TAG. The next approach to quantitation used response factors calculated from a randomized sample, which were then applied to a normal, nonrandomized sample. This method worked quite well, because the response factors were calculated from a randomized oil sample (so its statistical composition could be known) that, ideally, had the exact same FA as the nonrandomized oil. This gave good results, but required the step of chemical randomization of the original oil sample to produce a calibration sample.

A fourth method for quantitation was also quite successful and was adopted as their standard method for quantitation of samples by APCI-MS (23). A method was developed that utilized response factors calculated for each FA, which were then multiplied together to produce response factors for each TAG. In APCI-MS extracted ion chromatograms (mass chromatograms extracted from the total, or reconstructed ion chromatogram), such as those shown in Figure 5, a peak appeared for each FA that was combined with a particular DAG fragment to form a TAG. The combination of the areas of all of the FA peaks in all of the DAG extracted ion chromatograms (EIC) plus the areas under the EIC of the protonated molecular ions allowed the calculation of a net FA composition from the mass data. The response of each FA in the EIC was similar to the behavior of mono-acid TAG: the smaller FA, and FA with fewer sites of unsaturation, gave more response than polyunsaturated FA. The FA composition calculated from the total areas from EIC was compared to the FA composition determined by calibrated GC-FID to determine a response factor for each FA. These FA response factors were then multiplied together to produce TAG response factors. This method had the benefit of reflecting the saturated/unsaturated dependence of FA and TAG, and also compensating for the isotope peak contribution of one FA to the peak area of another FA. Overstatement of the abundance of one FA could occur when its FA peak in a normal DAG EIC occurred at the same retention time as the isotope peak of a FA having one less site of unsaturation. For instance, the "L" peak in the EIC for $[PL]^+$ (*m/z* 575.5) gave an isotope peak in the $[PO]^+$ chromatogram (*m/z* 577.5) due to PLL molecules containing two $^{13}C$ isotopes or one $^{18}O$ isotope. The retention time of the "L" peak was similar to the "Ln" peak in the PO chromatogram, causing a contribution of the "L" LP isotope to the "Ln" PO abundance. Using the actual net FA composition from the mass data, which was normalized to the FA composition determined by GC-FID, effectively compensated for all saturation trends and isotope effects. Examples of normal and randomized soybean oil and normal and randomized lard demonstrated the efficacy of the last two approaches.

Byrdwell and Neff (24) followed up the initial report on quantitative analysis with a second report that described the same quantitation methods applied to the same samples mentioned above, but with the addition of normal and interesterified canola oil blends. Also described in this report was a mathematical model that attempted to compensate for the effect of saturation on the response factors of TAG, and the effect of chain length for 16- and 18-carbon chains, but which did not address the isotope contributions to peak areas. This report showed, more than the first, that the best methods for quantitative analysis of TAG were (i) use of response factors calculated using the FA composition obtained from the total areas from EIC normalized to the GC-FID FA composition, and (ii) use of a randomized oil to determine response factors for nonrandomized (or noninteresterified) oils. The combination of these reports showed that the final average relative error in the FA composition calculated from the TAG composition was very small compared to the FA composition determined by calibrated GC-FID when the GC-FID response factor normalization process was used. The average relative error was less than 5% for most normal seed oil samples, and was as low as 0.2% for randomized samples. For randomized or interesterified samples the error was lower than for nonrandomized samples, and was usually less than 2%. If the average relative error in the FA composition was larger than ~7%, then there was sufficient nonrandom distribution of FA to indicate that the TAG mixture was a blend, or had FA concentrated into a few TAG species present in higher proportions. Comparison of the TAG composition to the statistically expected composition allowed identification of which TAG were most responsible for the average relative error. A method for mathematically separating out the few TAG responsible for certain FA within a blend and applying separate response factors to the base oil vs. hardstock TAG was recently reported by Byrdwell, Neff, and List (25). This recent report also provided greater detail about the original quantitation method.

Mottram *et al.* (22) applied the method described above, with good agreement, to our results. Byrdwell and Neff (26) published another application of the method to normal, high-lauric, and high-stearic canola oils. Results were similar to those described above. TAG that contained FA present in small amounts were sometimes not reported in the quantitative results, although they could be unambiguously identified qualitatively.

Other groups have taken a simpler approach to quantitative analysis. Laakso (27) reported a study in which α- and γ-linolenic acid oils were separated using RP-HPLC/APCI-MS. Whereas the study focused primarily on qualitative identification of components, it did report unnormalized peak areas based on areas under peaks in the reconstructed (or total) ion chromatogram. No response factors were applied. However, since quantitation was dependent on the amount of unsatura-

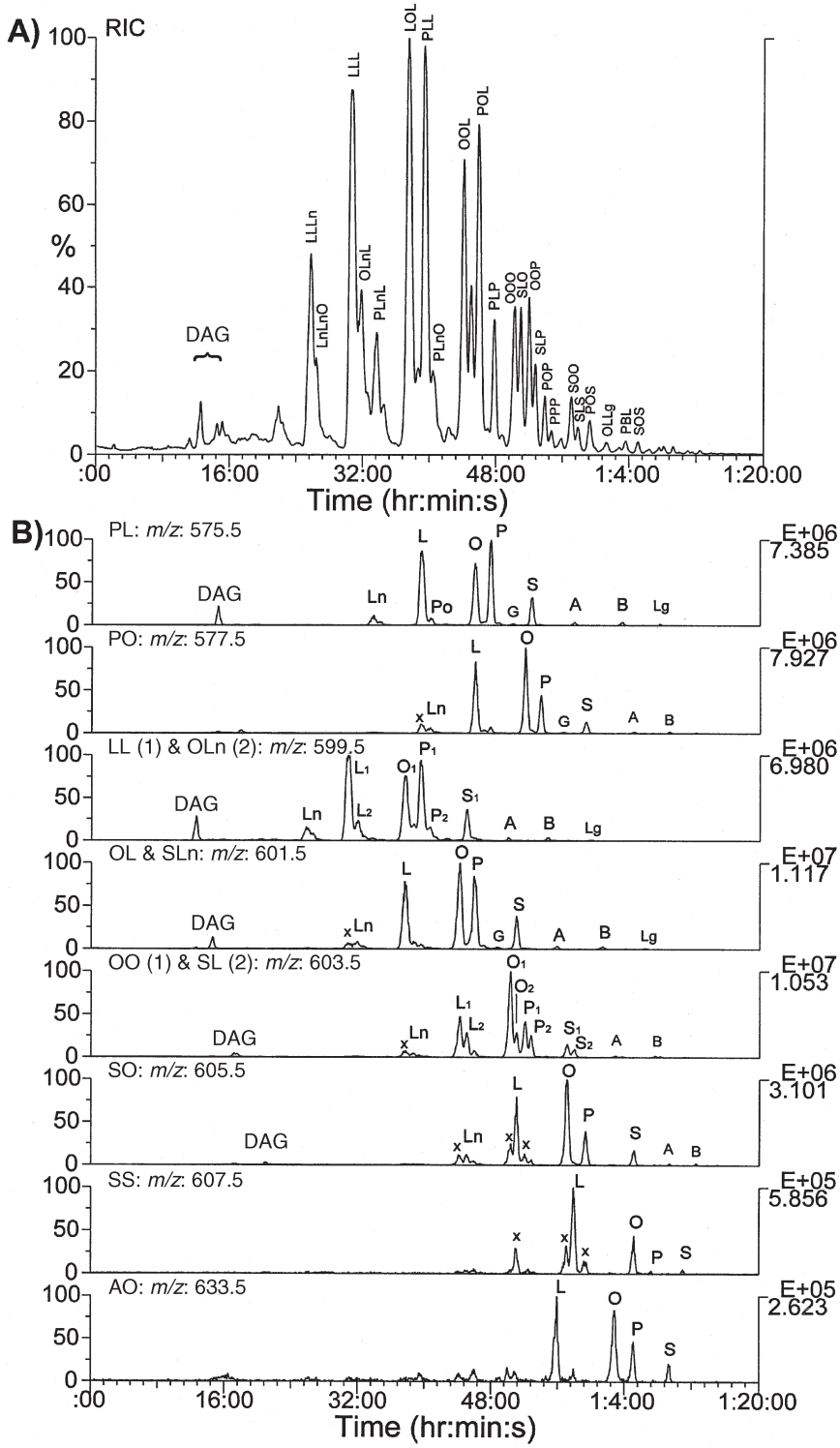


**FIG. 5.** (A) Reconstructed ion chromatogram (RIC) of refined, bleached, deodorized soybean oil. (B) Extracted ion chromatograms of diacylglycerol fragment ions. A, arachidic; B, behenic; Lg, lignoceric; G, gadoleic; DAG, diacylglycerol; x, abundance of 2 × $^{13}$C isotope of [M – 2] DAG; for other abbreviations see Figure 4.

tion in the TAG, if the amount of unsaturation did not vary much between TAG species (all having a similar degree of unsaturation), then the response factors would not vary dramatically between the TAG. The main thrust of the work was to show that RP-HPLC/APCI-MS was capable of separating α- and γ-linolenic acid isomers, as had been reported earlier by these authors using $Ag^+$-ion HPLC/APCI-MS. On the RP column, however, the α-linolenic acid isomer eluted before

the γ-linolenic acid isomer, all else being equal. This was the opposite of the behavior observed for similar isomers separated by $Ag^+$-ion HPLC/APCI-MS.

## OXYGEN FUNCTIONAL GROUP-CONTAINING TAG

Ikeda and Kusaka (13) first reported the APCI mass spectra of FA amides containing hydroxy groups. Later, Kusaka and Ikeda (14) reported the APCI-mass spectra of hydroxy and hydroperoxy acids as Br-MB derivatives. The hydroxy-containing FA underwent dehydration, for a loss of 18 Da, to form a $[M - H_2O + H]^+$ fragment (13,14). These spectra displayed abundant molecular ion, $[M + H]^+$, but the fragment formed by dehydration was large, and was the base peak in some spectra. A hydroperoxy FA (hydroperoxy eicosatetraenoic acid) was seen to exhibit a different mass spectrum from that given by the hydroxy FA (14). The hydroperoxy FA formed a fragment representing loss of $H_2O$ to give a $[M - H_2O + H]^+$ fragment, and a fragment representing loss of the entire hydroperoxy group to yield $[M - H_2O - O + H]^+$ fragments. Kusaka *et al.* (28) then reported mass spectra for normal plant TAG and the mass spectrum of a sample of hydroperoxidized stearic-oleic-linoleic (SOL). However, these results exhibited a mass difference of 2 Da compared to other reports.

More recently, Neff and Byrdwell (29) reported the RP-HPLC/APCI-MS analysis of TAG oxidation products formed by autoxidation of synthetic TAG standards (triolein, trilinolein, and trilinolenin). This report showed the APCI-MS mass spectra of mono- and bis-hydroperoxides, which were the most abundant oxidation products, and mono- and bis-epoxides, which were also formed in substantial amounts. Comparison of APCI mass spectra of autoxidation products to spectra obtained from vernolic acid in *V. galamensis* (18) showed that some of the epoxides that were formed during autoxidation were identical to the epoxides from vernolic acid. It was further seen that the hydroperoxide-containing TAG gave ions identical to the epoxides as primary fragmentation products. By comparison of the epoxides formed from hydroperoxides with those such as vernolic acid, two distinct mechanisms of epoxide fragmentation were described. The first fragmentation pattern occurred when the epoxide group was not next to a double bond (such as in vernolic acid). In this case the epoxide was lost as $H_2O$, $[M - H_2O + H]^+$, meaning the epoxide oxygen was lost to give a site of unsaturation, and two more hydrogens were also lost, to yield another site of unsaturation. This was in contrast to the fragments formed when the epoxide was next to a double bond. In such a case, the epoxide was lost with the loss of only 16 Da, $[M - O + H]^+$, giving only one more site of unsaturation where the epoxide had been. Epoxides produced fragments that allowed the position of the epoxide on the fatty chain to be determined. Two major chain-shortened fragments resulted from epoxides, one in which the epoxide ring cleaved, leaving the oxygen behind on the remaining shortened TAG, and one in which the epoxide ring cleaved and the oxygen left with the leaving fragment. Hydroperoxides formed multiple isomers, which were detected by the fragment ions produced. Hydroperoxides formed epoxides as primary fragments, $[M - H_2O + H]^+$, which then fragmented further according to the two mechanisms identified for epoxides, to form $[M - H_2O_2 + H]^+$ fragments and/or $[M - 2H_2O + H]^+$.

Byrdwell and Neff (30) then reported the HPLC/APCI-MS analysis of triolein oxidation products produced at frying temperature. The low molecular weight oxidation products were similar to those produced by autoxidation, with mass spectra being virtually identical to those reported for autoxidation products (29). Hydroperoxides and epoxides were the primary products, although a keto-TAG and chain-shortened TAG were also reported. Higher molecular weight components were also reported. Dimers formed by addition of two oxidized triolein molecules were observed, as well as several large molecules formed by losses of acyl chains from the dimers. Larger molecules formed by addition of chain fragments to an intact dimer were also indicated. Direct detection of these large oxidation products allowed direct characterization of the intact nonvolatile decomposition products that remain after loss of small volatile fragments lost during oil decomposition.

Byrdwell and Neff (31) then extended the analysis of oxidation products to the autoxidation products of normal and genetically modified canola oil varieties. Normal, high-stearic acid, and high-lauric acid canola oils were allowed to undergo autoxidation, and the product mixture was analyzed using RP-HPLC/APCI-MS. The same fragmentation mechanisms described for TAG model standards were exhibited by the TAG in the autoxidized oils. Hydroperoxides and epoxides were the primary intact oxidation products. Highly saturated epoxy-TAG were observed in the high-stearic and high-lauric acid canola oil oxidation product mixtures. Chain-shortened species produced by cleavage of an acyl chain at the site of oxidation were reported. TAG containing multiple functional groups were also indicated.

TAG in hydroxy-containing seed oils were also analyzed by Byrdwell and Neff (32) using RP-HPLC/APCI-MS. Characterization of hydroxy-TAG in castor oil and two species of *Lesquerella* allowed the fragmentation patterns of these TAG to be elucidated. As had been observed for the hydroxy-containing FA (13,14), hydroxy-containing TAG readily underwent loss of the hydroxy groups by dehydration to produce abundant $[M - H_2O + H]^+$ ions. Less of the molecular ion was observed for hydroxy TAG than was observed for hydroxy FA. A series of three adducts was reliably and reproducibly formed from the hydroxy-TAG separated using the methylene chloride/acetonitrile solvent system. These adducts allowed facile identification of the molecular weights of the hydroxy-TAG even in the absence of a strong protonated molecular ion. The adducts were formed by a combination of acetonitrile in the mobile phase with nitrogen carrier gas.

## SFC

As mentioned above, the initial report of the application of APCI-MS to a TAG was based on separation of a mixture of

different lipid classes by SFC with detection by APCI-MS (15). The report described a lab-built open tubular column SFC/MS interface to a commercial APCI ionization source on a Sciex API III tandem quadrupole mass spectrometer. Several years later, Schmeer *et al.* (33) reported the analysis of lipids from the seed arils of *Commiphora guillaumini*, a tree species native to western Madagascar. TAG and DAG were separated using SFC and identified by APCI-MS. In-source fragmentation caused by orifice voltages greater than 80 V was used to provide structural information. Mass spectra exhibited an $[M + 18]^+$ ion, which was identified as an ammonium adduct, $NH_4^+$, but which was likely the water adduct, as no source of $NH_4^+$ was given in the procedure. 1,2-Dioleoylglycerol, previously identified as an ant attractant, was identified in the seed arils.

In 1997, Manninen and Laakso (34,35) described a capillary SFC (cSFC) system that was connected to a commercial APCI source on a Finnigan MAT TSQ 700 tandem mass spectrometer. This system was used for the cSFC analysis of TAG in berry oils. One paper reported the compositions of cloudberry seed oil and sea buckthorn pulp and seed oil, extracted with carbon dioxide by SFC (34). A second paper reported the compositions of black currant and alpine currant seed oils, extracted with carbon dioxide by SFC. Both studies used two 10 m × 50 μm i.d. SB-Cyanopropyl-25 (25% cyanopropyl/25% phenyl/50% methylpolysiloxane) columns in series [the first study also used a 10 m × 50 μm i.d. SB-octyl-50 (50% octyl/50% methylpolysiloxane)]. Following up on their previous work identifying the regioisomers by using DAG ion fragment abundances, Manninen and Laakso tabulated the ion abundances for DAG fragments and protonated molecular ions first from two mixtures of regiospecific isomer standards, then from the seed oils just mentioned. Regiospecific structures were estimated based on 2-position isomer DAG abundances. cSFC gave a separation in which some peaks contained up to seven TAG, some of which could have overlapping DAG fragments. However, if coeluting TAG did not have overlapping DAG fragments, then the regioisomer could be assigned. The mechanism by which TAG mixtures were separated by SFC/APCI-MS was different from that by RP-HPLC/APCI-MS. The retention time was dictated by the number of carbons in the acyl chains (ACN, acyl carbon number) plus two times the number of double bonds, ACN + 2*n*. This meant that a TAG containing an 18:1 chain plus two other FA coeluted with a TAG having two FA the same, and a 20:0 chain in place of the 18:1. A monounsaturate was retained similarly to the next-longer saturate, or similar to the next-shorter di-unsaturate. This is in contrast to the elution order given by RP-HPLC. RP-HPLC produces a separation by increasing equivalent carbon number (ECN), which is the number of carbons in the acyl chains minus two times the number of double bonds, ACN − 2*n*. By RP-HPLC, a TAG containing a monounsaturated FA eluted at a similar time to the TAG containing two FA the same and the next-shortest saturated FA in place of the monounsaturate. The peaks appear to be better resolved by RP-HPLC, with partial and sometimes complete separation between TAG with the same ECN.

In the first report by Manninen and Laakso (34), a comparison among four different reactants incorporated into the sheath gas was given. The reactant ions were introduced into the sheath gas by bubbling through 50 mL of a reactant liquid in a 200-mL bottle. Methanol, isopropanol, water, and 0.5% $NH_4OH$ aqueous solution were compared. Methanol as reactant ion produced the best combination of abundant $[M + H]^+$ and DAG, $[M - RCOO]^+$, ions. Isopropanol produced the lowest abundances of protonated molecular ions and DAG fragment ions. Water was found to be a good alternative to methanol, although ion formation was somewhat less stable. Ammonium hydroxide was reported to produce mostly $[M + 18]^+$ ions, even from relatively saturated TAG, which normally produce little to no $[M + H]^+$ ion.

Laakso and Manninen (36) next applied cSFC to the complex mixture of milk fat TAG. The mixture was sufficiently complex that very few specific TAG structures could be identified. The coeluted TAG in the chromatographic peaks produced up to 30 $[M - RCOO]^+$ DAG fragment ions, and small $[M + H]^+$ abundances. The most notable result from this study was that the abundances of near-molecular ions was increased by addition of ammonium hydroxide as reactant ion. The sheath gas was bubbled through a solution of 0.5% ammonium hydroxide in methanol before being preheated prior to introduction into the APCI source.

Sjoberg and Markides (37) reported a new SFC/APCI-MS interface that allowed an easy transition between APCI and ESI modes. The interface was fabricated in their laboratory, starting with the commercial source for the PE-Sciex API III mass spectrometer. The researchers used a capillary drawn to a small diameter to act as an integral SFC restrictor. A restrictor tip heating wire was used to minimize the adiabatic cooling caused by the expanding supercritical fluid carrier gas. The interface was designed to provide improved performance for the low flow rates associated with microscale analyses. In addition to polypropylene glycol, the authors used a mixture of FAME, trilaurin, cholesterol palmitate, cholesterol, and others to demonstrate the efficacy of their interface design. They later reported (38) optimization of the parameters used for the new interface design, with slight modifications, including optimal sheath liquid and mobile phase flow rates, capillary and restrictor dimensions and positions, corona needle position, and reagent solvent incorporated into the makeup liquid. The degree of fragmentation in mass spectra from APCI and ESI ionization was reported to be essentially the same, producing similar ratios of $[M + H]^+$ to $[M - RCOO]^+$ ions. Sodiated adducts under ESI conditions produced less fragmentation than protonated molecular ions under APCI conditions.

## OTHER FA AND TAG APPLICATIONS

The references cited above demonstrated the first applications and provided the precedent for analysis of FA and TAG using LC/APCI-MS. As the technique has gained recognition for its versatility and ease of use, the number of applications has increased.

Siegel *et al.* (39) reported a combination APCI/ESI source designed for low flow rates, which allowed operation in several modes. ESI only, APCI only, mixed ESI-APCI (in which both ESI and APCI operated simultaneously), and alternating ESI and APCI modes were available. The ESI and APCI modes had different optimal carrier gas temperatures, effluent compositions, and other parameters, but good results could be obtained from both ionization modes by employing intermediate ("compromised") parameters between the optimal values for each. Spectra of neutral molecules such as methyl stearate were shown to demonstrate the effects of varying different parameters.

Adas *et al.* (40) reported HPLC-APCI-MS for analysis of hydroxylated metabolites of elaidic and oleic acids in human and rat liver microsomes. The negative ion APCI-MS mass spectra exhibited strong $[M - H]^-$ ions as base peaks.

As already mentioned, formation of ammonium adducts increased the amount of near-molecular ions formed from saturated TAG in the APCI source. In 1998, Mochida *et al.* (41) reported the effect of ammonia addition to improve the amount of near-molecular ion formation from oleic acid hydroperoxides. An ammoniated molecular ion became the base peak when ammonia was incorporated into the LC mobile phase. The effects on the formation of the $[M + NH_4]^+$ ions of changing the nebulizer temperature, solvent flow rate, and drift voltages were investigated.

An interesting and thorough report of the application of LC/APCI-MS was reported by Shibayama *et al.* (42). They demonstrated the analysis of monoacylglycerols (MAG), DAG, TAG, and FA standards and components in the residue exuded from a painting in the National Gallery of Art, and in extracts of dried oil paint films. Their report demonstrated the utility of LC/APCI-MS for analysis of several lipid classes in artists' materials and its use in art conservation.

Holcapek *et al.* (43) compared three detectors: ultraviolet (UV) detection, ELSD, and APCI-MS, for analysis of rapeseed oil used for biodiesel production. As a first approximation, quantification was restricted to components containing the most abundant three FA, oleic acid (O), linoleic acid (L), and linolenic acid (Ln). As was previously demonstrated (23) under APCI-MS conditions individual TAG produced calibration curves with wide linear ranges, having sensitivities (slopes of the calibration curves) dependent on the degree of unsaturation. The ELSD was shown to produce distinctly nonlinear calibration curves. Quantitation was performed by producing correction factors obtained by averaging the slopes of the calibration curves for OOO, LLL, and LnLnLn.

Parcerisa *et al.* (44) recently reported analysis of olive and hazelnut oil mixtures by HPLC/APCI-MS. They used areas under peaks in the total ion chromatograms (TIC) of the oil mixtures to determine TAG compositions, which were then subjected to one-way analysis of variance. This statistical treatment allowed comparison of pure oils to oil mixtures. Statistically significant differences in the percentages of specific TAG could be identified, showing the potential use of HPLC-APCI-MS for detection of adulterated oils. Tocopherols and sterols were analyzed by gas–liquid chromatography of their *O*-trimethylsilyl derivatives. These nonsaponifiable components were identified using FID, after identification of retention times by conventional GC–MS with electron impact ionization.

Mu and Hoy (45) utilized RP-HPLC/APCI-MS on a Hewlett-Packard LC/MSD to separate and characterize TAG molecular species from rat lymph samples. Capillary-skimmer fragmentation and nebulizer heater temperature were optimized for maximal sensitivity. Ammonium acetate was added postcolumn to enhance the formation of pseudomolecular ions, especially of saturates. Animals were fed structured lipids or safflower oil, and the change in the composition of lymph TAG was monitored over time. Incorporation of caprylic acid into endogenous lipids was observed. In a related study, Mu, Sillen, and Høy (46) reported the characterization of DAG and TAG in a structured lipid sample. In this report, the authors emphasized the differences in the mass spectra obtained from DAG vs. TAG. They reported that DAG produced greater abundances of MAG-related fragment ions than TAG, which could aid in the identification of DAG.

Recently, Rezanka (47) demonstrated preparative $Ag^+$ ion chromatography followed by analytical-scale RP-HPLC/APCI-MS for analysis of the FAME of very long chain polyunsaturated FA in freshwater crustacean species. They used APCI-MS to identify 70 FA, 50 of which had chain lengths of 24 carbons or more, extending up to 40 carbons. It was reported that the ratio of the intensities of representative ions could be employed to localize the position of the double bonds in positional isomers. A second report by Rezanka (48) described the analysis of the FAME of polyunsaturated FA. Fragmentation of the FAME was enhanced by increasing the voltage between the nozzle and the skimmer cone. An increase in the number of sites of unsaturation in the FA led to larger correction factors used for quantitative analysis. The correction factors decreased with increasing carbon chain length.

Bylund *et al.* (49) reported analysis of oxygenated products produced by the enzymatic oxidation of arachidonic and linoleic acids. They reported negative ion tandem mass spectrometry (MS/MS) spectra of hydroxylated products, and identified diagnostically useful fragments produced in the Finnigan MAT LCQ ion trap mass spectrometer. APCI and ESI were reported to yield very similar results; and for most mass spectra presented, which ionization method was employed was not clear. Localization of the positions of oxygen functional groups was possible in many cases based on the fragmentation patterns observed for the various isomers.

## PHOSPHOLIPIDS

ESI-MS has arguably become the method of choice for phospholipid analysis. However, since ESI-MS normally produces no fragmentation, a mass spectrometer capable of MS/MS or $MS^n$ is sometimes considered necessary to produce fragmentation for complete structural elucidation. Alternatively, in-source or near-source fragmentation can be used to produce nonspecific fragment ions. The fragments produced by

ESI-MS with upfront fragmentation are not necessarily the same as those produced by APCI-MS. For example, ESI-MS produces only head group ions for some phospholipids in positive ion mode, whereas APCI-MS produces diagnostically useful fragments from the same phospholipids. APCI-MS typically produces some protonated or near-molecular ions with gentle fragmentation, which leads to structurally diagnostic fragment ions. However, some phospholipids produce such small abundances of molecular ions under APCI-MS conditions that identification of all molecular species can be problematic. In such cases the molecular weight information provided by ESI-MS can be invaluable. Thus, ESI-MS and APCI-MS techniques are both useful and complementary for analysis of phospholipids and other lipid molecules.

Karlsson *et al.* (50) showed positive and negative ion mass spectra of the pure standard distearoyl (di-18:0) phosphatidylcholine (PC) to demonstrate that near-molecular ions, DAG and FA fragments resulted from APCI-MS analysis of a phospholipid. They chose to utilize ESI-MS for the remainder of their study, however, because of its greater sensitivity.

Byrdwell and Borchman (51) reported HPLC/APCI-MS using an amine column for separation and characterization of the sphingolipids and other phospholipids of human eye lens membrane extracts. APCI-MS was shown to produce mostly fragments formed by loss of the phosphate-containing head group from the sphingolipids, with small abundances of protonated molecular ions. Because of the small amount of near-molecular ion formed from sphingolipids, the discrimination due to differences in acyl chain length was assumed to be less than the discrimination observed for TAG. Semiquantitative results for the sphingolipids were presented without the use of response factors. PC, on the other hand, produced little protonated molecular ion from a concentrated sample, but a protonated molecular ion as base peak from a human lens extract containing only a small amount of PC. A more thorough treatment of the quantitative analysis of phospholipids using APCI-MS is necessary. Discrimination between classes is expected, which will require the use of response factors. Within a class, discrimination is expected to be much less.

Byrdwell (52) extended the initial identification of phospholipids using HPLC/APCI-MS by employing two mass spectrometers and two other detectors for analysis of sphingolipid, glycerophospholipid, and plasmalogen molecular species. Byrdwell employed a single-quadrupole mass spectrometer with an APCI source connected in parallel to a triple-quadrupole mass spectrometer with an ESI source. An ELSD was attached as an auxiliary detector to the single quadrupole mass spectrometer, while a UV-visible (Vis) detector was attached to the triple quadrupole mass spectrometer. This "dual parallel mass spectrometer" arrangement produced data from four detectors simultaneously—two mass spectrometers, the ELSD, and the UV-Vis—from the same column effluent. Typical data for phospholipid standards from this arrangement are shown in Figure 6. The triple quadrupole mass spectrometer was programmed to automatically switch from full-scan mode to MS/MS [with the most abundant ion(s) as parents] when the signal passed a threshold. APCI-MS was shown to produce mostly protonated molecular ions from phosphatidylethanolamine and its plasmalogen. Protonated molecular ions were observed from PC and PC plasmalogen, but DAG fragment ions were the base peaks from PC. The sphingolipids produced smaller abundances of protonated molecular ions than PC, with fragments produced by loss of the head group appearing as base peaks. These results demonstrated the discrimination between phospholipid classes that occurred during APCI. Also shown in this report were strong ammonium adducts of TAG formed from the ammonium hydroxide electrolyte included in the mobile phase to produce a stable current during ESI-MS. The TAG ammonium adducts greatly increased the amount of near-molecular ion formation observed from TAG with few sites of unsaturation, similar to observations reported by others (34). Also, when insufficient aqueous component was included in the mobile phase, sodiated adducts were formed from both phospholipids and TAG under ESI-MS conditions. The ESI-MS mass spectra of TAG showed substantial sodiated adducts in the high-mass region and normal DAG fragments at lower masses.

Karlsson *et al.* (53) reported the analysis of sphingolipids from bovine milk, brain, erythrocytes, and chicken egg yolk using both APCI and ESI. APCI in-source fragmentation was followed by MS/MS analysis of the ceramide ion ($[M - PO_4(CH_2)_2N(CH_3)_3 + H]^+$) produced in the source. Both ESI and APCI produced only phosphorylcholine as product ions when the protonated molecular ion was chosen as the precursor ion. But when the ceramide fragment ion was selected as the precursor using APCI, fragments resulted that allowed characterization of both the long-chain base backbone and the amide-linked fatty acyl chain. Although the APCI method exhibited markedly lower sensitivity than the ESI method, the APCI spectra provided structural information that could not be obtained using ESI. Isobaric fragments were shown that potentially could lead to ambiguity in identification of the long-chain base, but the use of accompanying fragments could be used to make specific structural assignments.

Qiu *et al.* (54) reported the analysis of phospholipids in the archaebacterium *Natronobacterium magadii*. They demonstrated positive and negative ion mass spectra of distearoyl phosphatidylglycerol (PG) and diphytanoyl (3,7,11,15-tetramethyl 16:0) PC. In negative ion mode, PG gave a molecular anion (phosphate not protonated), but in positive mode, the distearoyl DAG fragment was the primary peak. In positive ion mode, PC gave a DAG fragment ion as base peak, with $[RCOO + 58]^+$, protonated molecular ion (phosphate protonated), and near-molecular ions also present. Diether PG methyl phosphate analogs extracted from *N. magadii* that contained 20:0 (phytanyl) and 25:0 (sesterphytanyl) FA were identified by these authors. Deprotonated molecular ions produced in negative ion mode allowed molecular weight confirmation. There was no fragmentation within the fatty chains, so the methyl-substituted molecular and DAG ions were isobaric with straight-chain FA.

Ceramides, which are nonphosphate-containing interme-

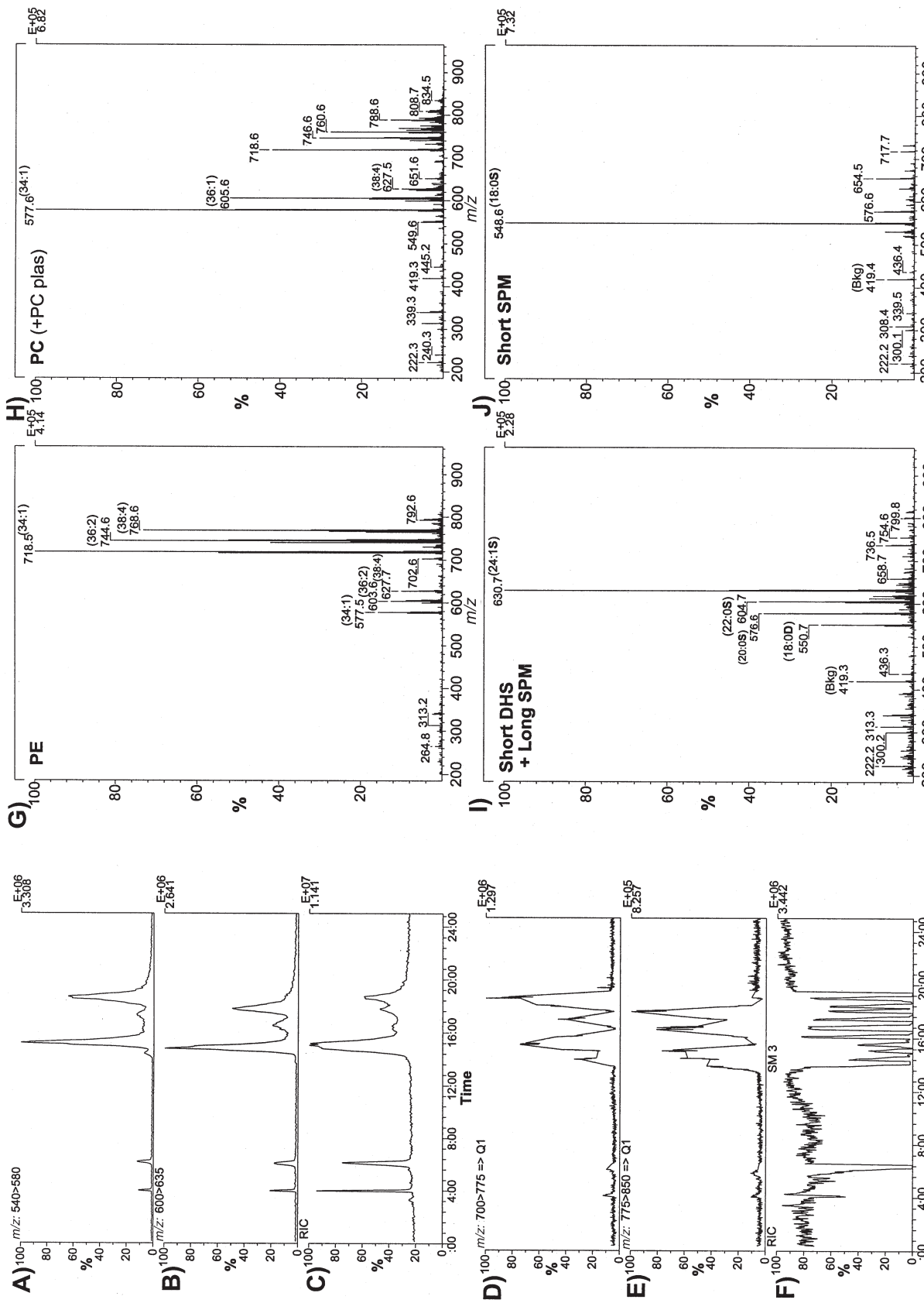


**FIG. 6.** (A) APCI-mass spectrometry (MS) extracted ion chromatograms (EIC) of short-chain phospholipids and (B) long-chain phospholipids; (C) APCI-MS RIC of phospholipids; (D) electrospray ionization (ESI)-MS EIC obtained in parallel with APCI-MS (filtered to show full scan mode); (E) ESI-MS EIC of short-chain phospholipids; (F) ESI-MS RIC of phospholipids (with cycling between full scan and MS/MS modes); (G) APCI-MS mass spectrum of phosphatidylethanolamine (PE) molecular species; (H) phosphatidylcholine (PC) + PC plasmalogen; (I) short-chain dihydrosphingomyelin (DHS) coeluted with long-chain sphingomyelin (SPM); (J) short-chain SPM molecular species. In (I) and (J), BKg is background, as in background contaminant. D (as in 18:00) refers to DHS, and S (as in 24:15) refers to SPM. These figures show that short-chain DHS species (e.g., 18:0) co-eluted with long-chain SPM (e.g., 24:1), but were easily differentiable by mass.See Figures 1 and 5 for other abbreviations.

diates in the biosynthesis of sphingolipids, were analyzed by Couch *et al.* (55) using HPLC with detection by APCI-MS. They reported the analysis of both underivatized and perbenzoylated ceramide standards and ceramides from human leukemic cells using detection by APCI-MS and UV-Vis. Selected ion monitoring was used on the VG (Manchester, United Kingdom) Platform II mass spectrometer to improve the sensitivity. Low skimmer cone voltages produced protonated molecular ions as base peaks, while higher cone voltages resulted in the fragment formed by loss of water (18 Da) as base peak. The highest cone voltage resulted in spectra in which a fragment representing loss of the fatty acyl chain was the base peak. The perbenzoylated derivatives produced spectra in which the primary fragment was formed by loss of a benzoic acid group.

## CAROTENOIDS AND RELATED MOLECULES

Carotenoids are large, nonvolatile, thermally labile tetraterpene molecules that have generally presented a number of difficulties for analysis. In 1996, Van Breeman *et al.* (56) reported the use of RP-HPLC/APCI-MS with a $C_{30}$ column for analysis of carotenoids in plant extracts. Their work demonstrated that APCI-MS produced both molecular ions and protonated molecular ions in positive ion mode, but in negative ion mode, molecular ions and deprotonated molecular ions were formed. The carrier gas (nebulizer gas) temperature could be adjusted to produce larger abundances of protonated molecular ions (below 400°C) or larger abundances of molecular ions (above 400°C). The hydroxy-containing xanthophyll lutein produced a base peak resulting from loss of water, similar to the behavior observed for other hydroxy-containing lipids, as already mentioned. Because only near-molecular ions were produced under APCI conditions, "in-source collision-induced dissociation (CID)" was used to increase the degree of fragmentation so as to provide structurally diagnostic fragment ions. This was accomplished by increasing the voltage between the capillary and the skimmer of the Hewlett-Packard HP 5989B MS Engine. Both positive and negative ion APCI-MS resulted in calibration curves with linear ranges spanning three orders of magnitude, with the lower limit of detection given in the negative ion mode. Thus, negative ion mode was used for quantification of β-carotene in extracts of fresh vs. heat-processed sweet potatoes.

Around the same time, Clarke *et al.* (57) reported the use of HPLC/APCI-MS for analysis of nine carotenoids from United Kingdom total-diet survey samples. The VG Platform single quadrupole mass spectrometer used for this study produced usable spectra only in positive ion mode. Seven of the compounds identified in this study produced protonated molecular ions as base peaks, while neoxanthin and lutein produced fragments resulting from the loss of water as base peaks. All of the hydroxy-containing carotenoids produced substantial $[M - H_2O + H]^+$ fragment ions, which, as mentioned, was the base peak for neoxanthin and lutein. These two compounds also showed substantial $[M - 2H_2O + H]^+$ fragments. In contrast to the work reported by van Breeman *et al.* (56), nonprotonated molecular ions were not observed under APCI conditions but were observed using ESI with high cone voltages. Hydroxy-containing carotenoids were detected with greater sensitivity than nonhydroxy components. Calibration curves exhibited sufficient linearity to allow quantitation of the carotenoids in total dietary study samples.

Liebler and McClure (58) described the analysis by APCI-MS of oxidation products from β-carotene with alkyl, alkoxyl, and alkylperoxyl free radicals formed by thermolysis with azobis(2,4-dimethylvaleronitrile) (AMVN). The primary oxidation products were epoxy and peroxy products, with the epoxy products representing the majority. Also formed were products formed from β-carotene with either AMVN radical fragments, AMVN-derived alkoxyl radical fragments, or AMVN-derived peroxyl radical fragments (collectively referred to by the authors as "substitution products"). Another group of ions was formed by combination of two of these radical fragments with a β-carotene molecule, referred to as "addition products." Daughter ion spectra and precursor ion spectra from CID of the oxidation products allowed structural elucidation of β-carotene-radical adducts and provided direct evidence for the reactions responsible for the antioxidant behavior of β-carotene.

Tang *et al.* (59) utilized flow injection (FI) APCI-MS for analysis of collected chromatographic fractions of β-carotene. They analyzed deuterium-labeled ($d_8$) β-carotene standard and in human serum extracts by FI/APCI-MS and then monitored its conversion to retinol using electron capture negative chemical ionization MS. Integration of the protonated molecular ion of labeled β-carotene produced a linear calibration curve (log-log plot), which was used to identify isotopic enrichment down to ~10% enrichment.

Hagiwara *et al.* (60) described the quantitative analysis of carotenoids in vegetable juice by RP-HPLC/APCI-MS on a $C_{18}$ column using selected ion monitoring (SIM) with cholesteroyl benzoate as internal standard. By using methanol as the mobile phase, mass spectra exhibited strong protonated molecular ions with little fragmentation, which were used for quantitative analysis. The reproducibility of the experiments was improved by using an iron corona discharge needle and by directing to waste the chromatographic effluent that came off the column prior to analyte elution (minimizing the effect of stains on the interface). After demonstrating low coefficients of variation and linear calibration curves, the concentrations of lycopene, α-carotene, and β-carotene were identified in eight commercially available vegetable juices.

Hagiwara *et al.* (61) later reported the application of RP-HPLC/APCI-MS to serum carotenoids. Unlike the vegetable juices, the hexane extracts of serum samples contained cholesterol-related components that co-eluted with the analytes, which fouled the interface. The mobile phase was changed to 70% methanol/30% acetonitrile to delay elution of the interferents. An automatic switching valve was used to admit only peaks containing the carotenoids into the mass spectrometer, while sending all other components to waste.

These changes, along with using squalene as the internal standard, resulted in a method useful for determination of the carotenoids in human serum. Sample mass spectra demonstrating the abundant protonated and nonprotonated molecular ions are shown in Figure 7.

Van Breeman *et al.* (62) reported a method for analysis of retinol (a diterpene that has a structure equal to one-half of a β-carotene molecule, plus the alcohol group) and retinyl palmitate in the hexane extracts of human serum samples. As with their method for carotenoid analysis (56), the method reported for the retinoids used a $C_{30}$ RP-HPLC system coupled to APCI-MS. Retinoids were analyzed intact, without derivatization, based on SIM of the mass of the retinyl moiety at *m/z* 269, the base peak which resulted from loss of water from

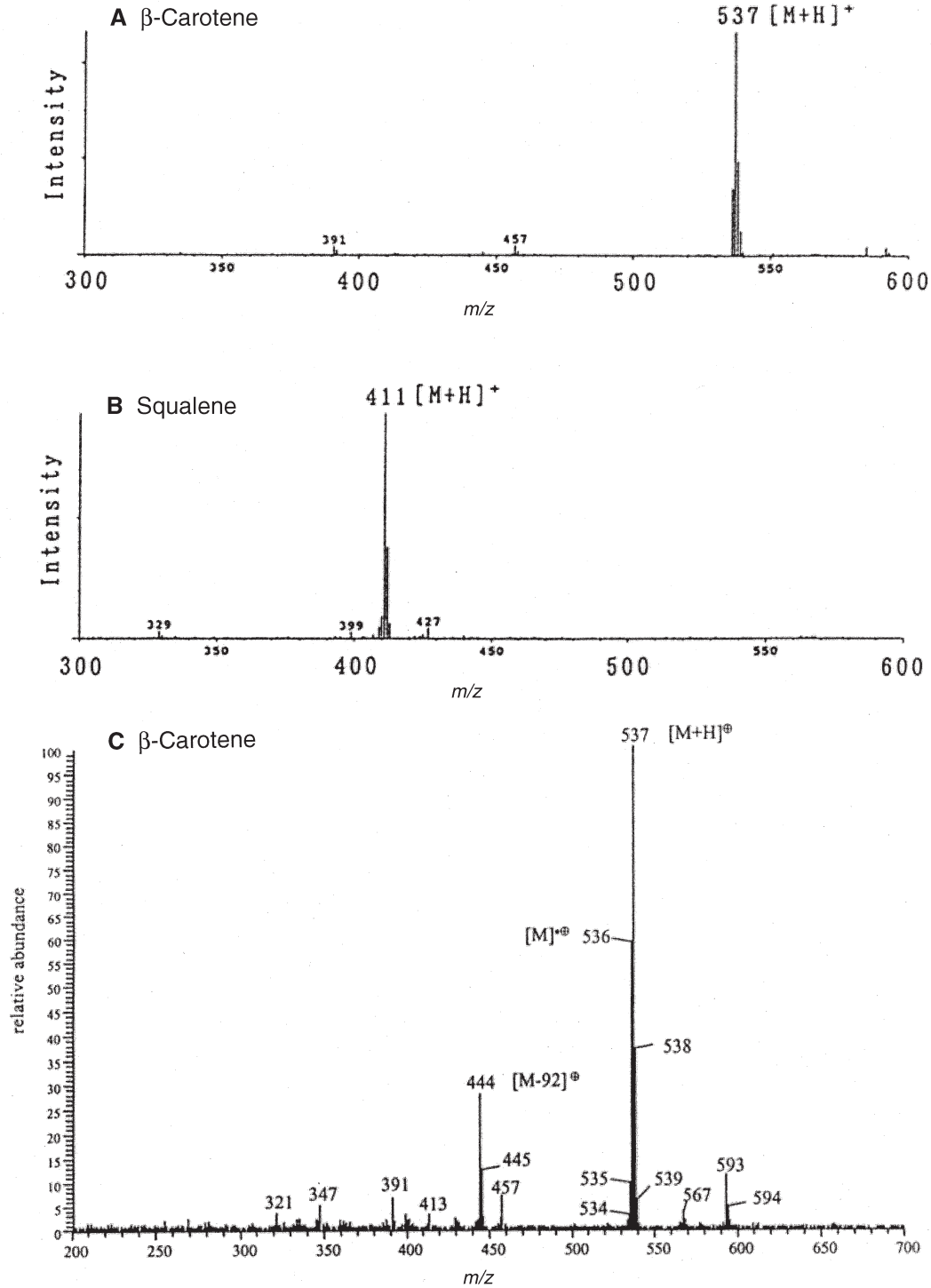


**FIG. 7.** APCI-MS mass spectra of carotenoids. (A) β-Carotene and (B) squalene from Reference 61; and (C) β-carotene from Reference 64. (Reprinted with permission from Elsevier Science.) See Figures 1 and 6 for abbreviations.

retinol, loss of the palmitic acid from retinyl palmitate, and loss of the acetate group from the internal standard, retinyl acetate. APCI-MS was shown to be superior to ESI-MS for retinoid analysis because APCI-MS exhibited linear response over several orders of magnitude.

More recently, Wang *et al.* (63) reported a method for the quantification of the bioavailability of $^{13}C_{10}$-labeled retinyl palmitate and β-carotene, and the bioconversion of β-carotene to retinol in Indonesian children. Building upon previous work (56,62), they showed that RP-HPLC/APCI-MS was a suitable analytical technique for monitoring physiologically relevant doses administered in a human intervention study. APCI-MS exhibited a wider linear dynamic range than ESI-MS. This wide dynamic range allowed determination of the amounts of low levels of isotope-labeled species in the presence of higher concentrations of endogenous compounds circulating in the serum, which coeluted chromatographically. SIM was used to produce detection limits below 1 pmol for both compounds.

A recent report by Lacker *et al.* (64) utilized a $C_{30}$ RP-HPLC/APCI-MS method for analysis of carotenoid standards and carotenoids in vegetable juice. Quantitation of β-carotene was based on the $[M + H]^+$ ion at *m/z* 537, whereas the $M^+$ ion was also present at an abundance more than half that of the $[M + H]^+$ base peak, as shown in Figure 7. Dioxygenated carotene was reported to elute at a short retention time, resulting in a base peak of *m/z* 565. *Cis* and *trans* isomers were differentiated by their UV-Vis spectra. Five carotenoids in a mixture and also carotenoids in a vegetable juice extract were separated and characterized by their $[M + H]^+$ ions.

Another class of lipids, which have structural similarities to carotenoids but which are not polyunsaturated and which contain the hydrocarbon chains ether-linked to a glycerol backbone, are tetraether lipids. A report of glycerol dialkyl glycerol tetraethers from the archaea *Sulfolobus solfataricus* and *Metallosphaera sedula* and two sediment samples was recently published (65). These large nonpolar molecules produced protonated molecular ions as base peaks and allowed characterization of previously unidentified tetraethers. Several of the tetraethers contained multiple cyclopentane rings and were analyzed intact, without derivatization (although acetylated derivatives were produced in small amounts as a by-product of the purification procedure). Protonated molecular ions in the range near 1300 Da highlighted the effectiveness of APCI-MS for ionization of large neutral molecules, which are difficult to ionize using other methods.

## CHOLESTEROL, STEROIDS, AND RELATED COMPOUNDS

The emphasis herein is primarily on lipids containing FA chains and acyl-like chains such as carotenoids. Cholesterol and structurally related steroids are also lipophilic molecules that are often associated with lipid systems, so these will be mentioned, but only briefly. A large number of articles have been published demonstrating the use of APCI-MS for analysis of steroid molecules. The number of such reports has grown such that a separate review of APCI-MS of these biomolecules is warranted. A review of LC/MS of steroids (66) and a review of the analysis of neurosteroids (67) recently appeared in Japanese journals. Therefore, this review will present only a few of these applications, to provide a starting point for researchers interested in pursuing this field.

Cholesterol has been mentioned in several of the reports just cited. It has either been part of a test mixture (15,37), been added as internal standard for analysis of other types of lipids (58), or been extracted with other lipid classes (49). Cholesterol produces a mass spectrum under APCI-MS conditions that consists of a base peak at 369, representing loss of the hydroxy group and giving a $[M - H_2O + H]^+$, or $[M - 17]^+$ peak. An APCI-MS analysis of cholesterol oxidation products has also been reported (68).

An early and quite extensive report of the use of LC/APCI-MS for steroid analysis was that of Kobayashi *et al.* (69). They reported the fragmentation characteristics of 60 steroids and the effects of nebulizer temperature and drift voltage on fragment abundances. They showed that protonated molecular ions were the base peaks formed by the majority of steroids, although a number of the steroids exhibited a near-molecular ion arising from loss of $H_2O$ from a protonated molecular ion. This fragmentation is now recognized as typical for hydroxy-containing compounds analyzed using APCI-MS. They also reported the formation of adducts with acetonitrile by some steroids.

Two other more recent reports also present extensive results for numerous steroids: Ma and Kim (70) reported results for 29 steroids, while Joos and van Ryckeghem (71) reported results for 36 steroids. Ma and Kim compared results obtained by APCI-MS with those obtained by ESI-MS. APCI was less sensitive for steroid analysis than ESI, but the slight fragmentation produced by APCI could assist in structural elucidation. The $[M + Na]^+$ near-molecular ions produced during ESI led to the best sensitivity in that ionization mode. Joos and van Ryckeghem reported results for fragmentation of steroids in multiple reaction monitoring mode. They reported optimized cone voltages and collision energies for each steroid.

Many other applications of APCI-MS analysis to steroids have been reported. Some of them are listed below. Analysis of brassinosteroids has been described by Gamoh *et al.* (72,73). Huopalahti and Henion (74) described LC/APCI-MS analysis of steroids after supercritical fluid extraction. Analyses of corticosteroids (75), ecdysteroids (76), and azasteroids (77) have been reported. Sjoberg and Markides (78) reported energy-resolved CID of geometric and positional isomers of steroids using both upfront (sometimes called "in source"—a misnomer) and conventional CID on a tandem mass spectrometer. Tuomola *et al.* (79) used SFC with APCI-MS for analysis of androstenone in pig fat, while Nakajima *et al.* (80) analyzed dehydroepiandrosterone in biological samples. Ikegawa *et al.* (81) used negative ion LC/APCI-MS to monitor dehydrogenation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) CoA thioester by THCA-CoA oxidases. Shimada and Muka (82) used LC/APCI isotope dilution MS

for analysis of derivatized pregnenolone and its 3-stearate in rat brains. Rule and Henion (83) used a semiautomated robotic 96-well solid-phase extraction sample preparation technique combined with APCI/MS/MS for analysis of equilenin and progesterone, using $d_4$-estrone as an internal standard.

## CONCLUSION

APCI-MS has become recognized for its ability to ionize large neutral molecules that are not amenable to ionization by most other techniques. Its versatility and ease of use is quickly making it a highly desirable method for lipid analysis. Naturally, the number of publications reporting its application is growing quickly as its popularity grows. Commercially available APCI sources are now readily available from most MS instrument manufacturers, usually in combination with the other API technique, ESI.

The spectra produced from APCI are simple, with a protonated molecular ion as the most common base peak, with minimal yet sufficient fragmentation to allow structural elucidation. For TAG and phospholipids, fragment ratios can often be used to determine the most likely positional isomers. Lower mass fragments ($[RCOO + 58]^+$ and acylium ions, $[RCO]^+$) can be used to identify individual fatty acyl chains to distinguish between isobaric DAG fragments. A wide variety of chemical ionization reagents are available to maximize near-molecule ion formation or sensitivity. Although the sensitivity of APCI-MS is usually less than that of ESI-MS, the structural information to be gleaned from the modest fragmentation is well worth the tradeoff in sensitivity. Some lipids containing oxygen functional groups (hydroperoxides and epoxides) yield information-rich fragmentation patterns that can allow not only the functional group but also sometimes their positions to be determined. However, some lipids containing oxygen functional groups yield such a paucity of pseudomolecular ions that identification of the molecular weight can be problematic. Examples include hydroxy-containing TAG, which readily lose $H_2O$, and oxoacylglycerols (aldehydes and ketones). In such cases, ESI can be an indispensable complement to APCI. A "dual parallel mass spectrometer" arrangement that combines these two ionization methods has already been demonstrated, and work is currently underway to apply this approach to highly complex mixtures of TAG oxidation products.

In the future, LC/APCI-MS should continue to gain more widespread usage and acceptance. Additional functional groups will be characterized, and their fragmentation patterns recognized. Also, the improvement of results by utilization and optimization of better chemical ionization reagents should continue. Although other ionization techniques have briefly flourished and then receded to give way to more advanced developments, it seems likely that API methods (both APCI and ESI) will endure well into the foreseeable future. They could likely endure long enough to participate in the never-ending push toward miniaturization, which continually produces smaller and more portable instrumentation. Handheld APCI and ESI mass spectrometers are not unfathomable. For now, those who already recognize the versatility and ease of use of API techniques will continue to solve difficult analytical problems that previously resisted facile instrumental solutions.

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# Tolerance and Incorporation of a High-Dose Eicosapentaenoic Acid Diester Emulsion by Patients with Pancreatic Cancer Cachexia

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**ABSTRACT:** Chemotherapy and radiotherapy offer little benefit to patients with advanced pancreatic cancer. Eicosapentaenoic acid (EPA) has anticancer effects both *in vitro* and in animal models. The dose of EPA that can be administered to cancer patients has previously been limited by the low purity of available preparations and the tolerability of large capsules. A high-purity preparation of EPA as a 20% oil-in-water diester emulsion allowed a small study of the tolerance, incorporation, and effects of EPA in high doses in five patients with advanced pancreatic cancer. Patients underwent assessment at baseline and every 4 wk thereafter. All patients managed to tolerate a dose providing 18 g EPA per day, with doses between 9 and 27 g daily being taken for at least a month. Dosage was limited by a sensation of fullness, cramping abdominal pain, steatorrhea, and nausea. All such symptoms were controlled by dose reduction or pancreatic enzyme supplements. No other adverse effects attributable to the trial agent were observed. Plasma phospholipid EPA content increased from around 1% at baseline to 10% at 4 wk and 20% at 8 wk. Incorporation of EPA into red blood cell phospholipids reached levels of around 10%. The present study has shown that a novel, high-purity, EPA diester emulsion can be tolerated at a dose providing around 18 g EPA per day with side-effects being easily controlled. The acceptibility of large doses of oral EPA should allow larger controlled clinical studies into potential anticancer effects of EPA.

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There has been increasing interest in the properties of n-3 fatty acids in oncology in recent years. The n-3 polyunsaturated fatty acids in general and eicosapentaenoic acid (EPA) in particular have been shown to reduce the growth rates of malignant cell lines in culture (1–4). The administration of fish oil that is rich in EPA and of EPA itself to immunocompromised animals bearing human tumor xenografts derived from breast (5–9), lung (10,11), prostate (12), and colon (13,14) cancer cell lines has been shown to retard tumor growth. Fish oil (containing EPA) and higher-purity EPA preparations have been administered to human cancer patients and have been suggested to produce an attenuation of cachexia in pancreatic cancer patients (15–17) and a prolongation in survival in a mixed group of cancer patients (18). These effects in human disease have been attributed to the anti-inflammatory and immunomodulatory effects of EPA, to direct effects on novel mediators of cachexia (19), and to a possible influence on tumor growth.

Conventional cytotoxic chemotherapy in pancreatic cancer is of limited effectiveness. A modest prolongation in survival can be achieved in some cases, but side effects are substantial and objective response rates are seen in less than 25% (20,21). It is therefore clear that a new approach to the management of these patients is required. Until now trials using EPA have mainly focused on its anticachectic potential. Doses used in studies of EPA in cancer have ranged from 2 to 6 g daily in capsule form (15,16,18). In such studies with low-dose EPA, no objective tumor responses have been reported. Thus, to test the true potential of EPA as an anticancer drug, it would seem logical to develop new formulations that might allow dose escalation to see if there is a dose-response curve. The relatively low EPA content of mixed fish oil preparations and the poor appetite and early satiety of these patients has limited the dose of EPA that may be tolerated in capsules. The availability of a liquid emulsion containing a 95% EPA diester that can be swallowed by the patient allowed the present small study of the tolerability and incorporation of high-dose EPA.

## MATERIALS AND METHODS

*Patients.* After local ethical committee approval, five patients with a histological ($n$ = 4) or unequivocal operative ($n$ = 1) diagnosis of unresectable pancreatic adenocarcinoma were enrolled. Patients had a life expectancy of over 2 mon, a Karnofsky performance status of 70, and a history of weight loss at enrollment. Patients were excluded if they had received surgery or endoscopic stenting during the previous 4 wk, had other active medical conditions, or another malignancy. No patients had received radiotherapy or chemotherapy. On enrollment none of the patients was jaundiced, pyrexial, severely anemic, or had clinical or radiological evidence of infection; and none was taking steroid drugs. All patients had adequate pain control at the time of study. Four patients were taking pancreatic enzyme supplements at enrollment. Two patients had previously taken the mixed fish oil preparation MaxEpa® (Seven Seas Ltd., Hull, England) at a dose

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

**TABLE 1**
**Patient Characteristics Prior to Intervention[a]**

| Patient | MaxEpa® taken | Other relevant medications | Percentage weight loss | Lymphocyte count ($\times 10^9$/L) | Serum albumin (g/L) |
|---|---|---|---|---|---|
| 1 | Yes (7 mon) | Pancreatin, domperidone | 15 | 1.6 | 42 |
| 2 | Yes (6 wk) | Pancreatin | 17 | 1.5 | 38 |
| 3 | No | Pancreatin | 16 | 0.7 | 41 |
| 4 | No | Pancreatin | 9 | 2.4 | 34 |
| 5 | No | Diclofenac | 8 | 2.0 | 34 |

[a]MaxEpa, manufactured by Seven Seas Ltd., Hull, England.

providing around 1 g EPA daily. Further details of the enrolled patients are shown in Table 1.

*Trial preparation.* The preparation administered was a 20% w/w emulsion of 1,3-di-(*Z,Z,Z,Z,Z*-eicosa-5,8,11,14,17-pentaenoyloxy)-propane, a diester of 95% EPA with propane-1,3-diol, in water, flavored with peppermint (Scotia Pharmaceuticals, Stirling, United Kingdom). The preparation provided 18 g EPA per 100 mL of emulsion and was stored in 200-mL amber glass bottles under nitrogen at 4°C. The fatty acid composition of the trial preparation was as follows: 20:5n-3, 93.8%; 20:4n-6, 3.3%; other, 3.0%.

*Protocol.* The study was an open-label, dose-escalation study. At enrollment patients gave written informed consent and were weighed in light clothing on a beam scale (Avery, Birmingham, United Kingdom). Pre-illness stable weight and Karnofsky performance status were documented. Mid upper arm circumference and triceps skinfold thickness were measured, and a venous blood sample was taken for measurement of routine full blood count, clotting, urea, electrolytes, and liver function. Blood was also collected for characterization of plasma and red cell phospholipid fatty acids. Subjects were given a diary in which to record the dose of emulsion taken and the number of doses taken per day. They were also asked to record any symptoms experienced. Subjects were asked to consume 25 mL of the emulsion (equivalent to 4.5 g EPA) daily for 2 wk. Subjects were contacted by telephone at this point, and if tolerability was satisfactory they were asked to increase the dose to 50 mL per day (9 g EPA). After 4 wk subjects returned to the clinic for a repeat of the baseline assessments and were asked to increase the dose to 100 mL per day (18 g EPA). At 6 wk after further telephone contact the dose was increased to 200 mL daily (36 g EPA). After 8 wk subjects underwent assessment once more. The emulsion was continued thereafter at the choice of the patient, and assessments were repeated every 4 wk. Patients were permitted to lower the dose taken at their discretion throughout the study.

*Fatty acid analysis.* EDTA-anticoagulated venous blood samples were centrifuged at 2500 rpm for 15 min and the plasma was removed. The layer of platelets and white cells was removed from above the red cell layer and discarded. Red cells were washed twice in phosphate buffered saline. Plasma and red cells were stored at −70°C until subsequent analysis. Lipids were isolated and purified by the method of Folch *et al.* (22). Lipids were extracted from plasma and red blood cells by homogenizing in 20 vol of chloroform/methanol (2:1vol/vol). After separation by adding a saline solution, the chloroform layer containing the lipid extract was removed, and phospholipids were separated by thin-layer chromotography in hexane/diethyl ether/acetic acid (80:20:1 by vol). Samples were then saponified in 5% potassium hydroxide in methanol. The solution was acidified with hydrochloric acid, and the fatty acids were methylated with boron trifluoride in methanol (12% w/w) with heating to 90°C for 30 min; the solutions were then extracted with hexane. Samples were analyzed using a Hewlett-Packard 5880 gas chromatograph with a 2-m glass column of 2-mm internal diameter packed with gp 10% SP-2330 (Supelco, Bellefontaine, PA) on 100/120 Chromosorb WAW with a level 4 integrator. The column was operated under a temperature program with an initial temperature of 165°C, increasing to 195°C, maintained for 25 min, at a rate of 2°C/min with a gas flow of 30 mL/min of helium through the column. Injection port temperature was 200°C and flame-ionization detector temperature 220°C. The fatty acid methyl esters were identified by their retention times based on the use of authentic standards from Nu-Chek-Prep (Elysian, MN). Samples were analyzed in quadruplicate.

Red cell fatty acids are reported at baseline and 8 wk only.

## RESULTS

The five patients were able to take the study emulsion for the planned 8-wk study period, and all patients chose to continue it after the formal 8-wk study period had ended.

Two patients managed a maximum tolerated dose of 100 mL/d (18 g EPA), one 150 mL/d (27 g EPA), and two 200 mL/d (36 g EPA). This maximum dose was often only tolerated for around 1 wk when patients cut the dose to one with which they were comfortable and were able to tolerate for over a month. One patient settled on 50 mL/d (9 g EPA), two around 75 mL/d (13.5 g EPA), one on 100 mL/d (18 g EPA) and one on 150 mL/d (27 g EPA). Lower doses of 25 and 50 mL/d were taken once daily by the patients. Above 50 mL/d, two or three doses were generally taken daily although one patient took up to 150 mL daily in a single daily dose.

The dosage was limited by a sensation of fullness in three patients, steatorrhea in two patients, nausea in one patient, and cramping abdominal pain in two patients. All symptoms settled with dose reduction and pancreatic enzyme supplementation as appropriate. All patients required pancreatic enzyme supplementation by the end of the study period.

**TABLE 2**
**EPA Emulsion Consumption and Fatty Acid Incorporation in Five Evaluable Patients with Advanced Pancreatic Cancer**

| Period of EPA consumption (wk) | Total EPA consumed (g) | Plasma phospholipids | | | | | | Red blood cell phospholipids | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 18:2n-6 | 20:3n-6 | 20:4n-6 | 20:5n-3 | 22:5n-3 | 22:6n-3 | 18:2n-6 | 20:3n-6 | 20:4n-6 | 20:5n-3 | 22:5n-3 | 22:6n-3 |
| Patient 1 | | | | | | | | | | | | | |
| 0 | (MaxEpa) | 18.2 | 2.6 | 7.3 | 4.3 | 1.6 | 5.3 | 9.0 | 1.5 | 11.8 | 3.5 | 4.1 | 7.2 |
| 4 | 198 | 18.7 | 1.8 | 7.2 | 9.2 | 2.5 | 3.9 | | | | | | |
| 8 | +603 | 19.1 | 1.3 | 8.5 | 13.6 | 3.4 | 3.5 | 8.1 | 1.0 | 11.1 | 7.3 | 6.0 | 5.0 |
| Patient 2 | | | | | | | | | | | | | |
| 0 | (MaxEpa) | 18.6 | 3.0 | 9.2 | 8.2 | 1.9 | 9.2 | 9.5 | 2.0 | 18.0 | 4.2 | 3.3 | 7.5 |
| 4 | 189 | 11.1 | 1.8 | 6.4 | 16.8 | 5.1 | 4.6 | | | | | | |
| 8 | +603 | 9.1 | 1.0 | 6.6 | 22.6 | 6.8 | 3.4 | 6.3 | 1.1 | 13.3 | 13.7 | 7.8 | 5.2 |
| Patient 3 | | | | | | | | | | | | | |
| 0 | | 20.9 | 4.6 | 11.6 | 1.5 | 1.3 | 5.2 | 5.7 | 0.7 | 3.7 | 0.2 | 0.4 | 1.6 |
| 4 | 189 | 15.8 | 2.9 | 11.7 | 8.4 | 2.6 | 4.4 | | | | | | |
| 8 | +252 | 14.3 | 3.1 | 10.1 | 8.9 | 3.0 | 4.6 | 5.9 | 1.2 | 9.8 | 2.9 | 3.7 | 3.8 |
| Patient 4 | | | | | | | | | | | | | |
| 0 | | 23.3 | 2.7 | 8.7 | 1.5 | 1.4 | 5.1 | 9.6 | 1.6 | 15.2 | 0.9 | 2.9 | 7.5 |
| 4 | 207 | 12.1 | 1.0 | 6.4 | 17.6 | 5.2 | 3.4 | | | | | | |
| 8 | +378 | 15.7 | 1.0 | 6.5 | 20.8 | 5.7 | 3.7 | 6.9 | 0.4 | 3.3 | 2.0 | 1.3 | 1.2 |
| Patient 5 | | | | | | | | | | | | | |
| 0 | | 23.1 | 3.2 | 11.6 | 0.8 | 0.9 | 4.2 | 11.7 | 1.9 | 19.9 | 0.8 | 2.7 | 7.2 |
| 4 | 162 | 16.0 | 2.7 | 8.7 | 10.3 | 2.2 | 3.7 | | | | | | |
| 8 | +468 | 13.1 | 1.2 | 8.0 | 18.5 | 3.0 | 2.3 | 9.0 | 1.1 | 12.7 | 10.2 | 4.9 | 5.1 |

Patient survival from the time of study enrollment was 116, 160, 191, 203, and 232 d. The four longest-surviving subjects continued to take the EPA emulsion until the week of their demise. All patients died from progression of their disease. No consistent changes were noted in measured biochemical and hematological parameters. In particular, no patient had any change in clotting or platelet count outside the normal range. Prior to enrollment, patients had been losing weight at a median rate of 1.2 kg/mon (range 0.6–1.9). Two patients gained weight over the 8-wk study period (2 and 2.5 kg) and three patients continued to lose weight (0.9, 1.1 and 2.6 kg). Parallel changes in anthropometry were noted. Karnofsky performance status was stable or improved in all five patients over the study period.

Results of fatty acid analysis of plasma and red blood cell phospholipids are shown in Table 2. Large increases in the percentage of EPA in plasma phospholipids were achieved, from a median of 1.5 at baseline in those who had not previously received fish oil supplementation to 10.3% (range 8.4–17.6%) at 4 wk and 18.5% (range 13.6–22.6%) at 8 wk. Owing to the small number of patients included in this study, it was not possible to establish a clear relationship between the quantity of emulsion consumed and the EPA level achieved over this period. After 4 mon of consuming the supplement, three subjects underwent further measurement of plasma phospholipid fatty acids revealing EPA levels of 10.7, 15.3, and 20.3% at a long-term daily dose of emulsion of approximately 75 mL (13.5 g EPA), 100 mL (18 g EPA), and 150 mL (27 g EPA), respectively. Incorporation of EPA into red cell phospholipids was less marked, from an initial median of 0.8% in those who had not previously received fish oil to 7.3% (range 2.0–13.7%) at 8 wk. After 4 mon in the three sampled patients EPA levels were 10.5, 7.2, and 14.8%.

## DISCUSSION

The present study has demonstrated that large doses of EPA may be tolerated by advanced cancer patients in emulsion form. A dose of 100 mL (18 g EPA) was tolerated by most patients for a substantial period. Such a dose would require the consumption of twenty 1-g capsules of high-purity EPA or around 100 1-g capsules of fish oil daily. Eighteen fish oil capsules daily have been taken for several months by patients with advanced cancer and cardiovascular disease (15,18,23), and doses of around 6 g/d of high-purity EPA have been taken by those with cancer (16). A liquid fish oil preparation has been taken at a dose of 50 mL daily in cardiovascular disease without side effects (24,25). The dose tolerated in the present study thus represents a large increase in the dose that may be administered easily.

The side effects observed in the present study are perhaps predictable considering the intervention group and the product administered. Patients with pancreatic cancer frequently suffer from malabsorption, particularly of fat, due to a combination of organ destruction and duct blockage (26). The administration of substantial doses of EPA increased lipid consumption, resulting in symptoms of malabsorption in all patients. However, these were easily controlled by pancreatic enzyme supplements and dose reduction if necessary.

No adverse events related to bleeding were observed, and all clotting measurements remained within the normal range. It has been suggested that the formation of thromboxanes and

prostaglandins from EPA rather than arachidonic acid may result in a bleeding tendency due to their potential effect in prolonging bleeding time and inhibiting platelet function (27). However, only one study has found this to be a clinically significant problem with an increased rate of epistaxis in hyperlipidemic adolescents given fish oil (28).

Although EPA has been shown to affect human tumor growth in animal models, this could not be assessed from the present study without a control group. All patients in the present study died from progress of their cancer despite the consumption of a large quantity of EPA over several months. Clearly, EPA alone is not a cure for cancer, but further controlled studies may be worthwhile to assess an effect on tumor growth. It has been suggested that in cell culture EPA will increase the sensitivity of leukemia cells to the cytotoxic drug adriamycin (29); thus combination therapy may be of benefit.

Diol lipids similar to the trial preparation are found in the normal diet in small quantities (30). The results of fatty acid analysis of plasma and red cell phospholipids suggest that the trial preparation was well absorbed. Several previous studies in healthy volunteers and those with cardiovascular disease have suggested that, after 4 wk of supplementation with fish oil or EPA providing 2–10 g EPA daily, plasma phospholipid EPA will rise to around 6% (23–25). We have reported similar EPA incorporation after the provision of around 2 g EPA daily in a fish oil preparation to patients with pancreatic cancer (15,17). A plasma phospholipid EPA of around 10% was achieved in patients with pancreatic cancer after consumption of 6 g high-purity EPA daily for 4 wk (16). In Eskimos with a high intake of n-3 polyunsaturated fatty acids, EPA has been reported to make up around 7% of plasma phospholipids (31). A mixed group of 22 cancer patients was reported as tolerating a dose of around 21 1-g fish oil capsules (providing approximately 8 g EPA) daily for about 1 mon (32). We are not aware of previous reports of the level of incorporation of EPA achieved in the present study with EPA representing around 20% of plasma phospholipids after 8 wk of supplementation. Similar large increases in the proportion of EPA in red blood cell membrane phospholipids were observed in the present study although these took around 2 mon to be seen and did not reach the same levels as in plasma phospholipids. The reason for this was not clear. There was also an increase in the proportion of docosapentaenoic acid in phospholipids in the present study, however, levels of the other main n-3 fatty acid docosahexaenoic acid fell in keeping with the known lack of appreciable conversion of EPA to DHA in humans consuming high amounts of n-6 fatty acids (33).

In summary, the present study has demonstrated that doses of around 100 mL daily of a novel emulsion preparation of EPA (providing around 18 g EPA daily) can be tolerated by weight-losing patients with advanced pancreatic cancer. Such large doses appear to be absorbed and result in previously unreported levels of EPA incorporation into plasma phospholipids of around 20% after an 8-wk dose escalation period. Such tolerance and incorporation suggest that this preparation may be used in clinical studies to explore further the potential antitumor effects of EPA previously established in *in vitro* and in animal models.

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# Effects of Carp and Tuna Oils on 5-Fluorouracil-Induced Antitumor Activity and Side Effects in Sarcoma 180-Bearing Mice

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**ABSTRACT:** In this study, we examined the effects of fish oils on 5-fluorouracil (5-FU)-induced antitumor activity in mice. First, we examined the antitumor activity of the oral administration of two fish oils (carp oil and tuna oil) in sarcoma 180-bearing mice. Carp oil (0.2 and 0.4 mL/mouse) and tuna oil (0.2 and 0.4 mL/mouse) had no effects on tumor growth. Next, we examined the combined effects of 5-FU plus two fish oils (carp oil and tuna oil) on the antitumor activity and side effects compared to the effects of 5-FU alone (12.5 mg/kg/d). We found that carp oil (0.4 mL/mouse) or tuna oil (0.2 or 0.4 mL/mouse) enhanced the ability of 5-FU (12.5 mg/kg/d) to prevent tumor growth, without increasing side effects such as myelotoxicity and immunocompetent organ toxicity. Tuna oil (0.2 mL/mouse) slightly reduced body weight as compared to the effects of 5-FU alone and water alone (control). The area under the curve (AUC) (0–120 min) of blood 5-FU levels was reduced by the oral co-administration of 5-FU with carp oil or tuna oil. Apparent $T_{max}$ was shortened by the oral co-administration of 5-FU with carp oil or tuna oil. On the other hand, AUC (0–4 h) of 5-FU incorporation into tumor RNA fraction was not affected by the oral co-administration of 5-FU with carp oil or tuna oil.

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Fish oils that contain high amounts of the n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) have been suggested to decrease the risk of development of cardiovascular disease (1). The freshwater carp has long been used in Korea, China, and Japan as a health food. In ancient Chinese medicine, carp was eaten as a diuretic and as a remedy for eye fatigue. In Japan, carp meat, oil, and blood have traditionally been consumed as tonics. The fatty acid composition of carp oil has not been reported. But freshwater fish usually are not rich in n-3 polyunsaturated fatty acids (PUFA). Tuna fish is also widely eaten, and it is rich in n-3 PUFA such as EPA and DHA (2). EPA and DHA reportedly have antitumor activities in tumor-bearing animals *in vivo* and inhibit tumor cell growth *in vitro* (3–6), and inhibit tumor metastasis and angiogenesis (7,8). Thus, there are suggestions that fatty acids found in fish have inhibitory effects on tumor growth. Therefore, to clarify whether fish oils have antitumor activity, we examined the effects of fish oils with high and low n-3 PUFA content on tumor growth in sarcoma 180-bearing mice, and compared their effects on the antitumor activity of 5-fluorouracil (5-FU). 5-FU was first synthesized by Duschinskey *et al*. (9) and has been used extensively in the treatment of certain types of cancer (10–13). It is well known that gastrointestinal toxicity (14), immunotoxicity (15–17), and myelotoxicity (18) are caused by the administration of high doses of 5-FU *via* its phosphorylation. In this study, we examined the antitumor activity and side effects of the combination of 5-FU and two fish oils (from freshwater carp and saltwater tuna) in sarcoma 180-bearing mice.

## MATERIALS AND METHODS

*Materials.* Carp oil, and oil prepared from the orbital adipose tissue of tuna, were supplied by Carp Food Co. (Tottori, Japan) and Maruho Co. (Tokyo, Japan), respectively. 5-FU was purchased from Wako Pure Chemical Inc. (Osaka, Japan). 5-[6-$^3$H]Fluorouracil (specific activity: 462.5 GBq/mmol) was purchased from NEN Life Science Products Inc. (Boston, MA). Other chemicals were of reagent grade. Dulbecco's modified Eagle's medium was purchased from Nissui Co. (Tokyo, Japan). Sarcoma 180 was maintained in the laboratory of the Second Department of Medical Biochemistry, School of Medicine, Ehime University. Sarcoma 180 was cultured for 7 or 10 d and used in Experiments 1 and 2, respectively.

*Analysis of fatty acids in carp oil and tuna oil.* Fatty acid composition was determined according to the method of Nelson *et al*. (19). Briefly, the total lipid extracts were transmethylated at 90°C for 2 h by adding 7% methanol/HCl (5 mL). The fatty acid methyl esters were then extracted with *n*-hexane and analyzed by gas–liquid chromatography (GC-14B, Shimadzu Co., Kyoto, Japan) under the following conditions: column: 0.25 mm i.d. × 25 m length (capillary column, Shinwa Chemical Industries Ltd., Tokyo, Japan); carrier gas flow rate: 1.5 mL/min (helium), column temperature: 250°C; injection and flame-ionization detector temperatures: 200°C.

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Abbreviations: AUC, area under the curve; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; 5-FU, 5-fluorouracil; HPLC, high-performance liquid chromatography; PCA, perchloric acid; PUFA, polyunsaturated fatty acid; T/C, (mean tumor volume in drug-treated animals)/(mean tumor volume in sarcoma 180-bearing mice).

The compositions of fatty acids in the carp and tuna oils are shown in Table 1.

*Animals.* Male ICR strain mice (6 wk old) were obtained from Clea Japan Co. (Osaka, Japan). ICR mice were housed in a room maintained at 25 ± 1°C with 60% relative humidity and given free access to food and water. The room was illuminated for 12 h per day starting at 7:00 A.M. The treatment of animals was performed according to the ethical guideline of the Animal Center, School of Medicine, Ehime University.

*Measurement of antitumor activity of fish oil, 5-FU, and 5-FU plus fish oils in sarcoma 180-bearing mice.* The antitumor activity of two fish oils, 5-FU, or 5-FU plus fish oils, was studied in two separate experiments. *(i) Experiment 1.* Solid-type sarcoma 180 was prepared by subcutaneous transplantation of $1 \times 10^6$ cells into the right abdomen of mice on day 0. One of the two fish oils (0.2 or 0.4 mL/mouse) was orally administered daily for 14 consecutive days, starting 12 h after implantation of tumor cells. Control mice were given distilled water solution alone on the same schedule. The volume of tumor growth was determined by direct measurement with calipers and calculated by the formula (length × width$^2$/2) every 2 or 3 d. On day 15, blood was obtained by venous puncture under anesthesia with diethyl ether, and then tumor was removed and weighed for evaluation of antitumor activity. *(ii) Experiment 2.* 5-FU (12.5 mg/kg/d) or 5-FU (12.5 mg/kg) plus one of the fish oils (0.2 or 0.4 mL/mouse) was orally administered daily for 14 consecutive days, starting 12 h after implantation of tumor cells cultured for 10 d. The following procedures were performed by the same methods described above.

*Side effects such as myelotoxicity, immunotoxicity and gastrointestinal toxicity of fish oils (Experiment 1), 5-FU, and 5-FU plus fish oils (Experiment 2) in sarcoma 180-bearing mice.* On day 15, blood was obtained by venous puncture under anesthesia with diethylether, and then small intestine, liver, epididymal adipose tissue, spleen, and thymus were removed and weighed. Blood samples were collected into chilled test tubes containing heparin, and the numbers of leukocytes, red cells, and platelets were measured using a Coulter Counter (Japan Scientific Instruments Co. Ltd., Tokyo, Japan).

*Measurement of 5-FU in blood of sarcoma 180-bearing mice.* 5-FU (12.5 mg/kg), 5-FU plus carp oil (0.4 mL/mouse), or 5-FU plus tuna oil (0.2 mL/mouse) was administered orally to mice. Blood taken by venous puncture under anesthesia 5, 15, 30, 60, 90 and 120 min after the administration of 5-FU, 5-FU plus carp oil, or 5-FU plus tuna oil was centrifuged at 1500 × *g* for 10 min at 4°C to separate the plasma. The plasma sample (0.4 mL) was shaken with 3 mL of chloroform for 10 min. The mixture was centrifuged at 1500 × *g* for 10 min at 4°C, and the organic phase was removed. 5-FU in the remaining aqueous layer was extracted twice with 4 mL of ethyl acetate, and the ethyl acetate extract was concentrated at 40°C under a stream of nitrogen gas. Then the residues were dissolved in distilled water, and the 5-FU contents were determined by reversed-phase high-performance liquid chromatography (HPLC) under the following chromatographic conditions: monitoring wavelength, 280 nm; flow rate, 1 mL/min; the internal standard, uracil, was added to the blood sample prior to the extraction; mobile phase, 5 mM tetrabutylammonium hydroxide solution containing 2% methanol adjusted to about pH 5 with dilute formic acid. Recovery of added 5-FU from the blood was 95%.

*Measurement of 5-FU incorporation into RNA fractions of tumor tissues in sarcoma 180-bearing mice.* [6-$^3$H]5-FU (12.5 mg/kg; 18.5 MBq/kg), [6-$^3$H]5-FU (12.5 mg/kg; 18.5 MBq/kg) plus carp oil (0.4 mL/mouse), or [6-$^3$H]5-FU plus tuna oil (0.2 mL/mouse) was administered to sarcoma 180-bearing mice on day 8 after tumor implantation. The mice were killed 0.25, 0.5, 1.0, 1.5, 2.0 and 4 h after the oral administration of [6-$^3$H]5-FU, [6-$^3$H]5-FU plus carp oil, or [6-$^3$H]5-FU plus tuna oil, and then their tumor tissues were removed and promptly frozen and stored at −80°C until use. Tumor tissue (500 mg) was homogenized with 5 vol of cold 10% perchloric acid (PCA) and centrifuged at 1500 × *g* for 10 min at 4°C. The radioactivity incorporated into the RNA fraction present in the PCA-precipitable material was extracted using a slight modification of the method of Schneider (20) for determination of the amount of 5-FU incorporated into RNA. [6-$^3$H]5-FU incorporation into RNA was determined by liquid scintillation counting.

*Statistical analysis.* All values are expressed as mean ± standard error (SE). Statistical analysis was performed with Dunnet's test to determine significance using Super ANOVA Software. The areas under the curve (AUC) were calculated using PK for Mac version 2.0 Software (Meiji Seika Pharmaceutical Research Center, Tokyo, Japan).

**TABLE 1**
**Fatty Acid Components of Carp Oil and Tuna Oil**

| Fatty acid composition (%) | Carp oil | Tuna oil |
|---|---|---|
| Myristic acid (14:0) | 2.0 | — |
| Palmitic acid (16:0) | 23.8 | 17.9 |
| Palmitoleic acid (16:1n-7) | 7.8 | — |
| Stearic acid (18:0) | 3.3 | 3.8 |
| Oleic acid (18:1n-9) | 36.6 | 17.5 |
| Linoleic acid (18:2n-6) | 18.2 | 3.7 |
| Arachidonic acid (20:4n-6) | — | 1.9 |
| Eicosapentaenoic acid (EPA) (20:5n-3) | 0.9 | 7.7 |
| Docosahexaenoic acid (DHA) (22:6n-3) | 2.3 | 26.1 |
| Others | 5.1 | 21.4 |

## RESULTS

*Antitumor and side effects of two fish oils (carp oil and tuna oil) in sarcoma 180-bearing mice (Experiment 1).* As shown in Figure 1 and Table 2 (Experiment 1), carp oil and tuna oil had no effect on tumor growth and final tumor weight in sarcoma 180-bearing mice. The weight of spleen and thymus was not affected by the administration of carp oil (0.2 and 0.4 mL/mouse) or tuna oil (0.2 and 0.4 mL/mouse) in sarcoma 180-bearing mice (Table 3). The weight of liver, adipose tissue, and small intestine and the number of blood cells (leukocyte, red cell, and platelet) were not affected by the administration of two fish oils (data not shown).

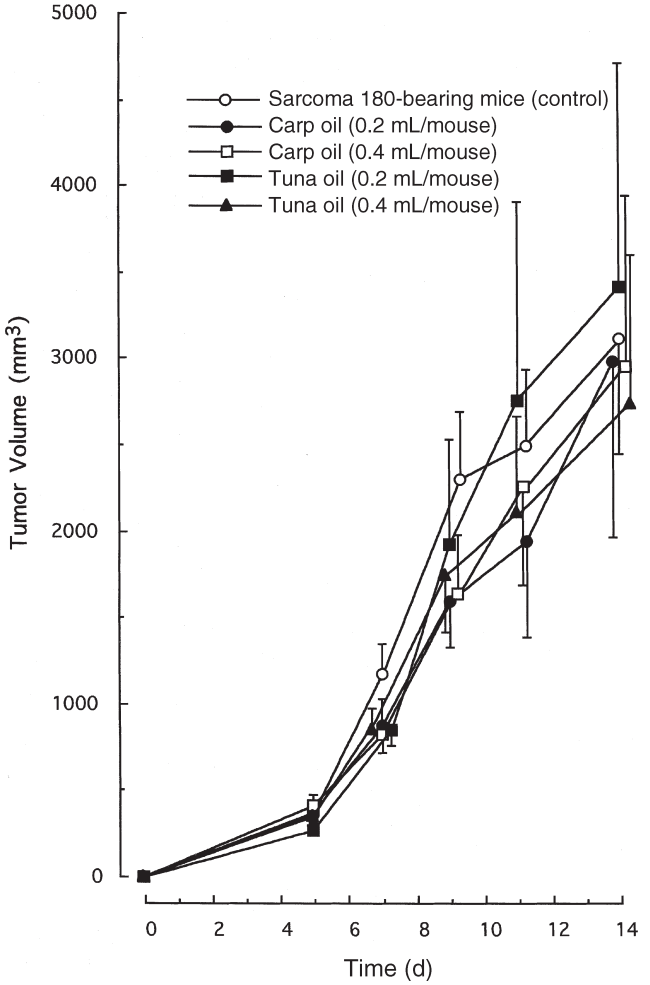



**FIG. 1.** Effects of carp oil and tuna oil on tumor growth in sarcoma 180-bearing mice (Experiment 1). Results are expressed as mean ± SE of 10 mice in each group.

*Antitumor and side effects of the combination of 5-FU (12.5 mg/kg/d) and carp oil or tuna oil (Experiment 2).* When 5-FU (12.5 mg/kg) was administered to sarcoma 180-bearing mice for 14 consecutive days, antitumor activities [T/C, (mean tumor volume in drug-treated animals/mean tumor volume in sarcoma 180-bearing mice (control)] were 40.9, 69.0, 61.4, 67.6, and 58.4 %, respectively, on days 5, 7, 10, 12 and 14 (Fig. 2). The T/C of final tumor weight was 64.5% (Fig. 3). In addition, the side effects such as myelotoxicity, immunocompetent organ toxicity and reduction of body weight did not occur. As shown in Figure 4, body weight was not affected by the combination of 5-FU (12.5 mg/kg/d) plus carp oil (0.2 or 0.4 mL/mouse). On the other hand, 5-FU plus tuna oil (0.2 mL/mouse) slightly reduced the body weight, as compared with sarcoma 180-bearing mice (control group) (Fig. 4). Tumor growth was inhibited by the combination of 5-FU plus carp oil (0.4 mL/mouse) (Figs. 2A and 3), and T/C of tumor volume for 5-FU plus carp oil (0.4 mL/mouse) were 41.6, 66.6, 54.8, 54.8, and 29.3%, respectively, on days 5, 7, 10, 12, and 14. The T/C of final tumor weight for 5-FU plus carp oil (0.4 mL) was 24.6%. Thus, the combination of carp oil plus 5-FU enhanced the antitumor activity induced by 5-FU alone. The T/C of tumor volume for the co-administration of 5-FU plus tuna oil (0.2 mL/mouse) were 25.9, 40.1, 39.6, 44.3, and 37.4%, respectively, on days 5, 7, 10, 12, and 14. Furthermore, the T/C of tumor volume for 5-FU plus tuna oil (0.4 mL/mouse) were 13.9, 26.4, 35.5, 34.5, and 27.4 %, respectively, on days 5, 7, 10, 12 and 14. The final T/C of tumor weight for the 5-FU plus tuna oil (0.2 mL) and 5-FU plus tuna oil (0.4 mL/mouse) were 24.5 and 17.6%, respectively. Thus, the dose of 0.2 and 0.4 mL/mouse of tuna oil plus 5-FU enhanced the antitumor activity induced by 5-FU alone. As shown in Table 3 (Experiment 2), the weight of spleen and thymus was not affected by the combination of 5-FU plus

**TABLE 2**
**Effects of Carp Oil and Tuna Oil on the Weights of Body and Tumor in Sarcoma 180-Bearing Mice in Experiment 1[a]**

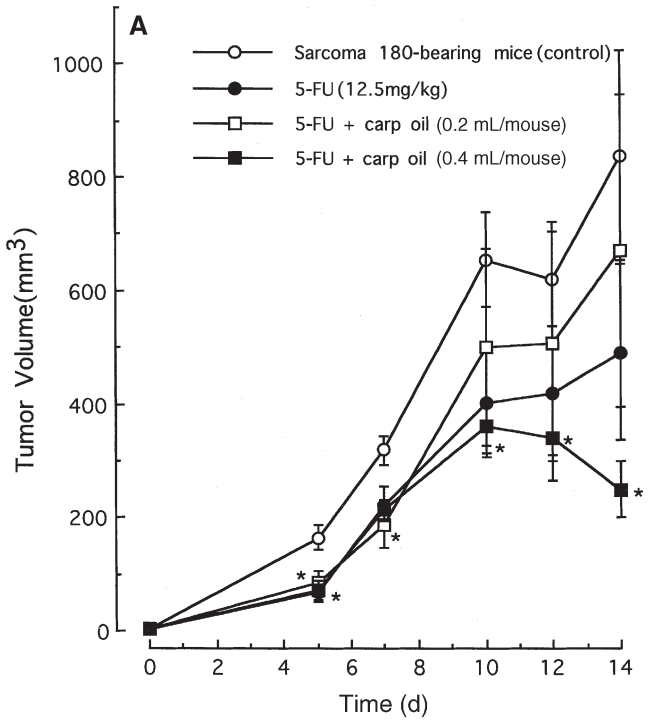
| | Mean ± SE | |
|---|---|---|
| | Final body weight (g) | Final tumor weight (g) (T/C) |
| Sarcoma 180-bearing mice (control) | 35.5 ± 1.06 | 3.29 ± 0.78 (100%) |
| Carp oil | | |
| (0.2 mL/mouse) | 36.8 ± 0.89 | 2.26 ± 0.54 (68.7%) |
| (0.4 mL/mouse) | 37.5 ± 0.55 | 2.40 ± 0.77 (72.9%) |
| Tuna oil | | |
| (0.2 mL/mouse) | 38.7 ± 0.68 | 2.54 ± 0.80 (77.2%) |
| (0.4 mL/mouse) | 36.1 ± 1.21 | 2.59 ± 0.73 (78.7%) |

[a]Results are expressed as mean ± SE of 10 mice in each group. T/C, (mean tumor volume in drug treated animals)/(mean tumor volume in sarcoma 180-bearing mice).

**TABLE 3**
**Effects of Carp Oil, Tuna Oil, and the Combination of 5-FU plus Carp Oil or Tuna Oil on the Weights of Spleen and Thymus Sarcoma 180-Bearing Mice[a]**

| | | Mean ± SE | |
|---|---|---|---|
| | Animal no. | Spleen (mg) | Thymus (mg) |
| | Experiment 1 | | |
| Sarcoma 180-bearing mice (control) | 10 | 193.9 ± 20.7 | 53.53 ± 8.76 |
| Carp oil | | | |
| (0.2 mL/mouse) | 10 | 186.6 ± 16.4 | 53.45 ± 2.44 |
| (0.4 mL/mouse) | 10 | 153.9 ± 16.5 | 50.18 ± 4.74 |
| Tuna oil | | | |
| (0.2 mL/mouse) | 10 | 169.6 ± 12.2 | 52.81± 6.60 |
| (0.4 mL/mouse) | 10 | 184.5 ± 11.4 | 55.44 ± 5.34 |
| | Experiment 2 | | |
| Sarcoma 180-bearing mice (control) | 10 | 190.0 ± 30.5 | 47.95 ± 5.42 |
| 5-FU(12.5 mg/kg) | 10 | 149.0 ± 16.8 | 50.56 ± 3.43 |
| 5-FU + carp oil | | | |
| (0.2 mL/mouse) | 10 | 159.0 ± 23.1 | 36.69 ± 4.19 |
| (0.4 mL/mouse) | 10 | 158.0 ± 10.3 | 49.53 ± 3.74 |
| 5-FU + tuna oil | | | |
| (0.2 mL/mouse) | 10 | 132.0 ± 4.90 | 42.23 ± 6.00 |
| (0.4 mL/mouse) | 9 | 152.2 ± 9.25 | 50.33 ± 3.61 |

[a]Results are expressed as mean ± SE of 9 or 10 mice in each group. 5-FU, 5-fluorouracil.



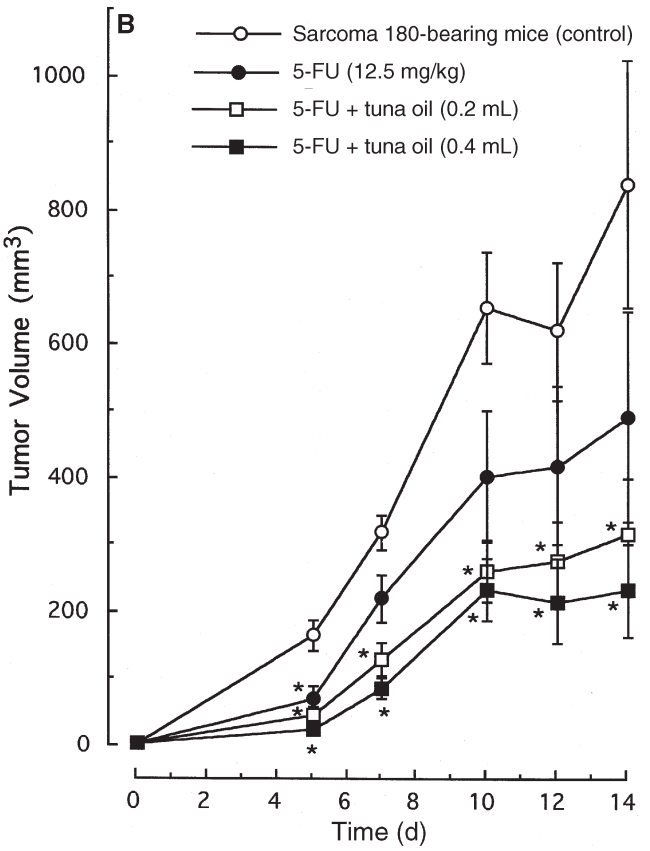




**FIG. 2.** Effects of the combination of 5-fluorouracil (5-FU) (A) plus carp oil and of 5-FU plus tuna oil (B) on tumor growth in sarcoma 180-bearing mice (Experiment 2). Results are expressed as mean ± SE of (A) 10 mice in each group and of (B) 9 or 10 mice in each group.*Significantly different from sarcoma 180-bearing mice (control), $P < 0.05$.

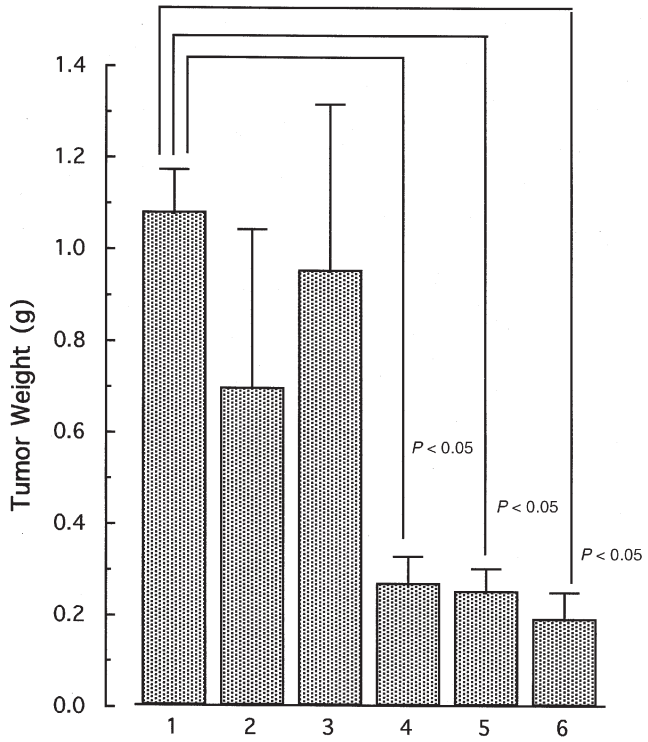



**FIG. 3.** Effects of the combination of 5-FU plus carp oil or tuna oil on tumor weight on day 15 in sarcoma 180-bearing mice (Experiment 2). Results are expressed as mean ± SE of 9 or 10 mice in each group. 1 = Sarcoma 180-bearing mice (control); 2 = 5-FU (12.5 mg/kg); 3 = 5-FU plus carp oil (0.2 mL/mouse); 4 = 5-FU plus carp oil (0.4 mL/mouse); 5 = 5-FU plus tuna oil (0.2 mL/mouse); 6 = 5-FU plus tuna oil (0.4 mL/mouse). See Figure 2 for abbreviation.

carp oil or 5-FU plus tuna oil (Table 3). The weight of liver, adipose tissue, and small intestine, and the number of leukocytes, red cells, and platelets were not affected by the combination of 5-FU plus two fish oils (data not shown).

*5-FU levels in the plasma of sarcoma 180-bearing mice after oral co-administration of 5-FU plus carp oil or 5-FU plus tuna oil.* As shown in Figure 5, the 5-FU levels in the blood of mice were about 133.8 ± 27.5 and 285.0 ± 19.3 ng/mL, respectively, at 5 and 15 min after the oral administration of 5-FU (12.5 mg/kg) and then decreased rapidly. Apparent $C_{max}$ and AUC (0–120 min) for the 5-FU administration were 285.0 ± 19.3 ng/mL and 138.16 ng·h/mL, respectively. On the other hand, the blood 5-FU levels after the co-administration of 5-FU plus carp oil (0.4 mL/mouse) were 99.7 ± 42.4, 48.9 ± 15.6, 39.9 ± 17.2, and 18.6 ± 9.75 ng/mL, respectively, at 5, 15, 30, and 60 min (Fig. 5). The apparent $C_{max}$ and AUC (0–120 min) for the 5-FU plus carp oil co-administration were 99.7 ± 42.4 ng/mL and 48.29 ng·h/mL, respectively. The blood 5-FU levels after the co-administration of 5-FU plus tuna oil (0.2 mL/mouse) were 376.4 ± 172.1, 182.6 ± 113.0, 33.9 ± 2.10, and 22.8 ± 5.73 ng/mL, respectively, at 5, 15, 30, and 60 min (Fig. 5). The apparent $C_{max}$ and AUC (0–120 min) for the 5-FU plus tuna oil were 376.4 ± 172.1 ng/mL and 97.83 ng·h/mL (Fig. 5). Thus, the apparent $T_{max}$ of the blood 5-FU was rapidly enhanced by the oral

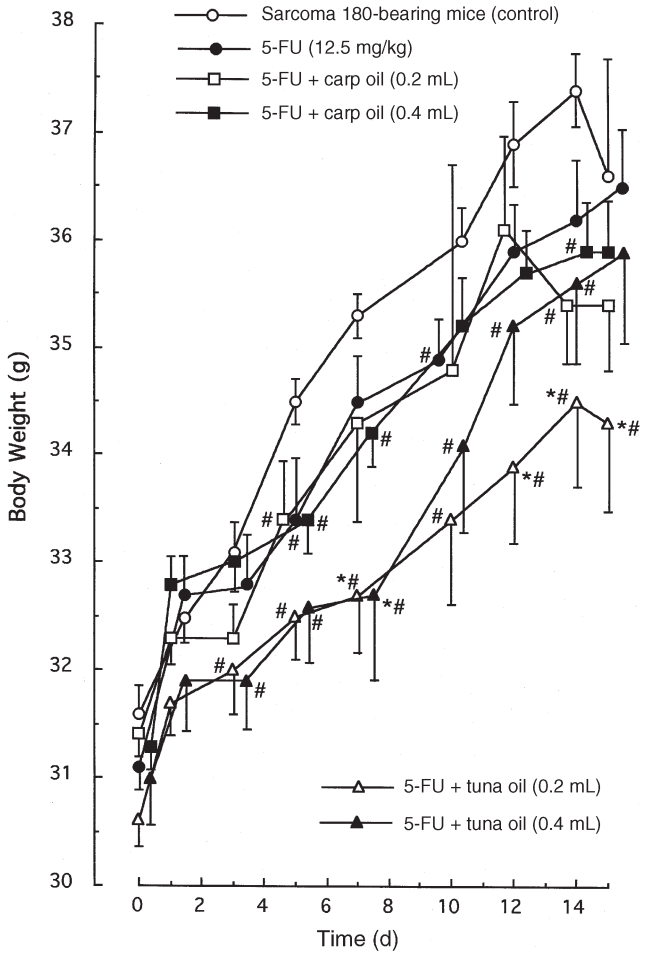



**FIG. 4.** Effects of the combination of 5-FU plus carp oil or 5-FU plus tuna oil on body weight of sarcoma 180-bearing mice (Experiment 2). Results are expressed as mean ± SE of 9 or 10 mice in each group. #Significantly different from sarcoma 180-bearing mice (control), $P < 0.05$. *Significantly different from 5-FU-treated group, $P < 0.05$. See Figure 2 for abbreviation.

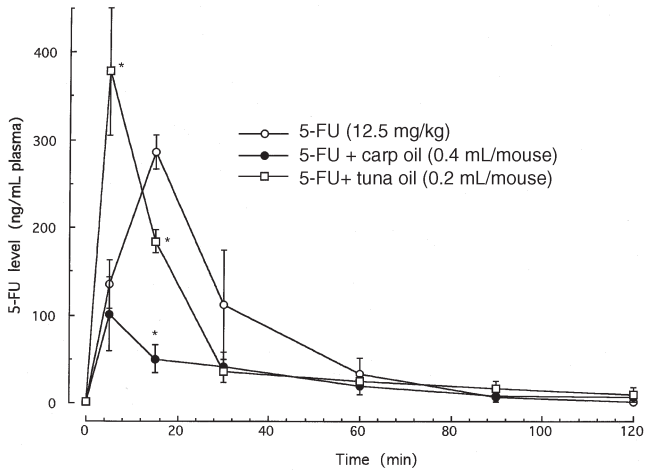



**FIG. 5.** 5-FU levels in the plasma of mice after oral co-administration of 5-FU plus carp oil or 5-FU plus tuna oil. Results are expressed as mean ± SE of five mice in each group. *Significantly different from the administration of 5-FU alone, $P < 0.05$. See Figure 2 for abbreviation.

co-administration of 5-FU with carp oil or tuna oil as compared with that of 5-FU alone. Apparent $C_{max}$ and AUC (0–120 min) of the blood 5-FU levels were significantly ($P < 0.05$) reduced by the oral co-administration of 5-FU with carp oil. In contrast, apparent $C_{max}$ of the 5-FU levels were significantly ($P < 0.05$) increased by the oral co-administration of 5-FU with tuna oil, while AUC (0–120 min) of the blood 5-FU levels after co-administration of 5-FU plus tuna oil slightly decreased as compared to that of 5-FU alone.

*Effects of fish oils on 5-FU incorporation into RNA fractions of tumor tissue in sarcoma 180-bearing mice.* As shown in Figure 6, 5-FU incorporation into RNA fractions of tumor tissue was about 2.35, 3.31, 5.34, 4.59, and 3.62 ($\times 10^3$) dpm/g tumor tissue, respectively, at 15 min, 30 min, 1 h, 2 h, and 4 h after the oral administration of [6-$^3$H]5-FU (12.5 mg/kg; 18.5 MBq/kg). The apparent $T_{max}$, $C_{max}$, and AUC (0–4 h) for the [6-$^3$H]5-FU administration were 1 h, 5.34 ± 1.84 ($\times 10^3$) dpm/g tumor tissue, and 16.70 ($\times 10^3$) dpm·h/g tumor tissue, respectively. The 5-FU incorporation into RNA fractions after the oral co-administration of [6-$^3$H]5-FU plus carp oil (0.4 mL) was 1.10, 1.12, 6.22, 4.73, and 4.07 ($\times 10^3$) dpm/g tumor tissue, respectively, at 15 min, 30 min, 1 h, 2 h, and 4 h (Fig. 6). The apparent $T_{max}$, $C_{max}$, and AUC (0–4 h) for the [6-$^3$H]5-FU plus carp oil were 1 h, 6.22 ± 1.39 ($\times 10^3$) dpm/g tumor tissue, and 17.25 ($\times 10^3$) dpm·h/g tumor tissue, respectively. The 5-FU incorporation into RNA fractions after the oral co-administration of [6-$^3$H]5-FU plus tuna oil (0.2 mL/mouse) was 3.60, 3.53, 3.96, 5.86, and 3.22 ($\times 10^3$) dpm/g tumor tissue at 15 min, 30 min, 1 h, 2 h, and 4 h (Fig. 6). The apparent $T_{max}$, $C_{max}$, and AUC (0–4 h) for the 5-FU plus tuna oil co-administration, were 2 h, 5.86 ± 1.50 ($\times 10^3$) dpm/g tumor tissue and 16.77 ($\times 10^3$) dpm·h/g tumor tissue, respectively. Thus, the apparent $T_{max}$ of 5-FU incorporation into RNA fractions was lengthened by the oral administration of

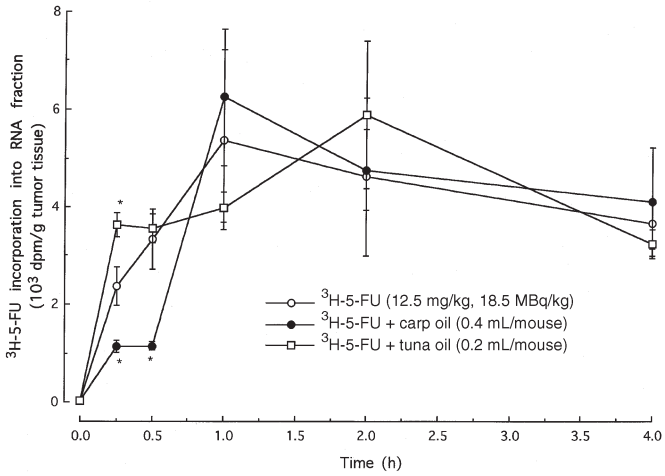



**FIG. 6.** [6-$^3$H]5-FU incorporation into RNA fractions of tumor after oral co-administration of [6-$^3$H]5-FU plus carp oil or [6-$^3$H]5-FU plus tuna oil. Results are expressed as mean ± SE of five mice in each group. *Significantly different from the administration of 5-FU alone, $P < 0.05$. See Figure 2 for abbreviation.

5-FU plus tuna oil, On the other hand, the apparent $T_{max}$ of 5-FU incorporation into RNA fractions was not affected by the oral administration of 5-FU plus carp oil. However, the apparent $C_{max}$ and AUC (0–4 h) were not shown to be different between the 5-FU-treated group and 5-FU plus carp oil group or 5-FU plus tuna oil group.

## DISCUSSION

In the present study, no antitumor activity was shown when carp oil (0.2 and 0.4 mL/mouse) or tuna oil (0.2 and 0.4 mL/mouse) was administered to sarcoma 180-bearing mice for 14 consecutive days. On the other hand, when 12.5 mg/kg/d of 5-FU was administered to sarcoma 180-bearing mice for 14 consecutive days, antitumor activities (T/C) were 40.9, 69.0, 61.4, 67.6, and 58.4%, respectively, on days 5, 7, 10, 12, and 14. The final T/C of tumor weight for 5-FU (12.5 mg/kg) was 64.5%. In addition, side effects such as myelotoxicity, immunocompetent organ toxicity, and the reduction of body weight did not occur. Therefore, to determine the enhancing effects of the two fish oils on the 5-FU-induced antitumor activity, we used the dose of 12.5 mg/kg/d of 5-FU, a dose which produced about 35–60% antitumor activity and did not cause side effects. The present study indicated that carp oil (0.4 mL/mouse) or tuna oil (0.2 or 0.4 mL/mouse) prevented tumor growth when combined with 5-FU (12.5 mg/kg/d) without side effects such as myelotoxicity (reduction of number of platelets, red cells, and leukocytes) or immunocompetent organ toxicity (reduction of spleen and thymus weights). Carp oil did not affect body weight, whereas tuna oil (0.2 mL/mouse) slightly reduced the body weight as compared to the effects of 5-FU alone. It was found that carp oil (0.4 mL/mouse) and tuna oil (0.2 and 0.4 mL/mouse) with 5-FU showed the 2.58-fold, 2.77-fold, and 3.61-fold antitumor activity, respectively, as compared with 5-FU alone. Thus, the present study showed that two fish oils containing unsaturated fatty acids enhanced the inhibition of tumor growth in combination with 5-FU *in vivo*. To study the mechanism of the antitumor activity of the combination of 5-FU plus the two fish oils, we designed an experiment in which the blood 5-FU level and 5-FU incorporation into tumor RNA fractions after the oral co-administration of 5-FU plus carp oil or 5-FU plus tuna oil to sarcoma 180-bearing mice were determined. From pharmacokinetic analysis after the oral co-administration of 5-FU plus carp oil or 5-FU plus tuna oil, apparent $C_{max}$ and AUC (0–120 min) in the blood 5-FU levels after oral co-administration of 5-FU plus carp oil were reduced more than that after 5-FU administration alone. However, AUC (0-4 h) of [6-$^3$H]5-FU incorporation into RNA fractions for the co-administration of [6-$^3$H]5-FU plus carp oil was similar to that of [6-$^3$H]5-FU administration. Thus, 5-FU incorporation into RNA fractions of tumor was similar to that of 5-FU alone, although the blood 5-FU level after co-administration of 5-FU plus carp oil (0.4 mL/mouse) was maintained at a low concentration. Therefore, these results suggest that the co-administration of 5-FU plus carp oil enhanced the 5-FU-induced antitumor activity without side effects. On the other hand, from the phamarcokinetic observation of 5-FU plus tuna oil (0.2 mL), 5-FU was rapidly absorbed following oral co-administration of 5-FU plus tuna oil. Furthermore, apparent $C_{max}$ of the blood 5-FU level was increased by the oral administration of 5-FU plus tuna oil, as compared with 5-FU alone. The AUC (0-4 h) of 5-FU incorporation into RNA fraction of tumor after the co-administration of 5-FU plus tuna oil was similar to that of 5-FU alone. These results suggest that the absorption of 5-FU is accelerated by the administration of tuna oil. Therefore, it seems likely that 5-FU-induced antitumor activity and side effects such as reduction in body weight may be caused by the decrease in $T_{max}$ and the increase in $C_{max}$ of the blood 5-FU levels after the co-administration of 5-FU plus tuna oil. Carp oil contained palmitic acid (22.8%), oleic acid (36.6%), linoleic acid (18.2%), and small amounts of EPA (0.9%) and DHA (2.3%). On the other hand, tuna oil contained palmitic acid (17.9%), oleic acid (17.5%), DHA (26.1%), and small amounts of linoleic acid (3.7%) and EPA (7.7%). Zhu *et al.* (21) reported that oleic acid and linoleic acid significantly prolonged the life spans of Ehrlich ascites carcinoma-bearing mice and inhibited the growth of Ehrlich solid carcinoma in mice compared with the findings in untreated control mice. And they suggested that the intraperitoneal administration of oleic acid and linoleic acid increased the contents of oleic acid and linoleic acid in phospholipids and triglycerides of tumor cells and consequently, that free fatty acids, especially unsaturated fatty acids, selectively inhibit the growth of tumor cells. There have been a number of reports that EPA and DHA have antitumor activity in tumor-bearing animals (3–6) and inhibit tumor growth (8). Yang *et al.* (8) reported that EPA inhibited vascular endothelial growth factor-induced angiogenesis in bovine carotid artery endothelial cells and suggested that the mechanism of the antitumor action of EPA may be related to its antiangiogenic action. It has been reported that DHA increases tumor permeability and uptake of cancer chemotherapy drugs such as adriamycin and mitoxanthrone and consequently enhances the sensitivity of tumor cells to the cytotoxicity of chemotherapy drugs (22–26). Ikushima *et al.* (27) reported that DHA enhanced the cytotoxic effect of vincristine twofold. Madhavi and Das (4) also reported that EPA augmented the cyototoxicity of vincristine in both vincristine-sensitive and -resistant human cervical carcinoma cells.

Therefore, it seems possible that the unsaturated fatty acids of the two fish oils might alter albumin-bindingof 5-FU or exert their effects *via* their incorporation into triglycerides or phospholipids. The unsaturated fatty acids in the fish oils, by altering the lipid composition of tumors or through some other mechanism, may enhance the susceptibility of 5-FU. Further studies are needed to identify which of the unsaturated fatty acids enhances of 5-FU-induced antitumor activity as well as the mechanisms involved. We conclude that carp oil and tuna oil enhanced the antitumor activity induced by 5-FU without increasing the side effects.

## ACKNOWLEDGMENT

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# Oxidative Stability of Low Density Lipoproteins and Vitamin E Levels Increase in Maternal Blood During Normal Pregnancy

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**ABSTRACT:** In 24 healthy pregnant women, parameters related to the oxidative stability of low density lipoproteins (LDL) were determined at three times during pregnancy and shortly after delivery. The fatty acid composition of plasma phospholipids (PL) and the plasma concentrations of vitamin E, vitamin A, and β-carotene were assessed in the same samples. Total triglyceride (TG), total cholesterol, LDL-cholesterol, and high density lipoprotein (HDL)-cholesterol concentrations were also determined. The length of the lag phase of isolated LDL challenged with $Cu^{2+}$ ions significantly increased with the progression of pregnancy. The oxidation rate and the amount of conjugated dienes formed increased and reached a maximum at 29–37 wk of pregnancy. Total TG, cholesterol, and LDL-cholesterol reached a maximum in the third trimester of pregnancy. β-Carotene remained stable, vitamin A decreased, and vitamin E significantly increased throughout pregnancy. Vitamin E plasma concentration correlated positively with the length of the lag phase. The increased levels of vitamin E could contribute to the higher resistance of LDL toward oxidation with progressing gestation, measured by the prolonged lag phase. Furthermore, vitamin E plasma levels correlated positively with TG concentration but not with LDL-cholesterol. The level of polyunsaturated fatty acids in PL decreased with the progression of pregnancy. No correlation was found between the fatty acid composition of plasma PL, nor with the cholesterol concentration, and the parameters studied related to the oxidative stability of LDL. The major finding of this study is the increased oxidative resistance of LDL with progressing gestation.

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The third trimester of pregnancy is accompanied by hyperlipidemia. As pregnancy progressess, the maternal plasma triglyceride (TG) concentration, the low density lipoprotein (LDL) content, and total cholesterol concentration increase (1–6). Late pregnancy is also associated with the predominance of small and dense LDL-particles (3,7). These small and dense LDL-particles have been shown to be more susceptible to oxidation (8). Hyperlipidemia and the occurrence of small and dense LDL particles during late pregnancy might increase the oxidative damage and impair the outcome of pregnancy. Many studies have determined either the antioxidative defense systems or peroxidation products during pregnancy (9–12). Uncomplicated pregnancy, but especially preeclampsia and diabetic pregnancy, is associated with high serum levels of lipid peroxides (8–13). During normal pregnancy the higher levels of lipid peroxides are accompanied by higher maternal levels of vitamin E compared to nonpregnant women (9,10,12–14). However, the increase in vitamin E levels is more pronounced, i.e., the vitamin E/lipid peroxide ratio increases with progressing gestation .

The aim of this study was to determine whether the oxidative stability of LDL changes during the course of pregnancy and, if so, whether this change correlates with changes in vitamin E, vitamin A, and β-carotene levels or with changes in the fatty acid composition of plasma phospholipids (PL).

## SUBJECTS AND METHODS

Healthy pregnant women attending the Department of Gynecology were asked to cooperate in this study. All pregnant volunteers signed a written informed consent form, approved by the ethics committee of Ghent University Hospital. Only singleton pregnancies were included. Inclusion criteria were: first pregnancy, normotensive (diastolic blood pressure <90 mm Hg), not diabetic, no proteinuria, and not suffering from renal or cardiovascular disease. The study population consisted of 24 healthy pregnant women. None of the women used any medication. Maternal venous blood was collected in EDTA tubes thrice during the course of pregnancy: earlier than 18 wk of gestation (median 12 wk), between 20 and 26 wk (median 23 wk), between 29 and 37 wk (median 32 wk) and shortly after delivery (median 39 wk; range 36–41 wk). Blood samples were temporarily stored on ice, and plasma was isolated by centrifugation (600 × *g* during 5 min at 4°C) within 24 h of collection. Plasma was stored with sucrose (60%, 10 μL/mL) to prevent lipoprotein aggregation during deepfreezing (–80°C).

*Preparation and oxidation of LDL.* LDL were prepared by sequential ultracentrifugation at 4°C according to Esterbauer *et al.* (15) and isolated from the appropriate density fraction ($d$ = 1.019–1.063 g/mL). EDTA was present throughout all the steps of the isolation in a concentration of 1 mg/mL density solution. Immediately after isolation, the LDL samples

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Abbreviations: HDL, high density lipoprotein; HUFA, highly unsaturated fatty acids (fatty acids with ≥20 carbon atoms and ≥3 double bonds); LDL, low density lipoproteins; PL, phospholipids; PUFA, polyunsaturated fatty acids; TG, triglyceride; *T*lag, length of the lag phase; *T*max, length of time required to obtain maximum levels of conjugated dienes.

were dialyzed at 4°C for 24 h against four changes of buffer (0.01 M $Na_2HPO_4$, 0.0022 M $NaH_2PO_4$, 0.16 M NaCl, 10 μM EDTA, and 0.1 μg/mL chloramphenicol; pH 7.4) which was made oxygen-free by vacuum degassing and subsequently was continuously purged with nitrogen. The protein content of the LDL fraction was determined according to Lowry *et al.* (16) with fatty acid-free serum albumin as standard. The concentration was initially adjusted to 500 mg/L with dialysis buffer. This solution was further diluted 10-fold in a quartz cuvette with EDTA and chloramphenicol-free phosphate buffer at a final protein concentration of 50 μg/mL.

Oxidation was initiated by addition of $CuCl_2$ (10 μM, in cuvette). The formation of conjugated dienes was determined by monitoring the change of absorbance at 234 nm at 30°C. The optical density was recorded every 3 min during a 3-h period. From the absorbance curve the following parameters were derived: length of the lag phase (*T*lag); length of the time required to obtain maximum levels of conjugated dienes (*T*max); length of the propagation phase, oxidation rate, and maximal amount of conjugated dienes formed. *T*lag (expressed in minutes) or the length of the lag phase is defined as the time interval between the addition of $CuCl_2$ to initiate oxidation and the onset of rapid oxidation. *T*max (expressed in minutes) is the time at which the absorbance reaches a maximum. After reaching the maximum value, the conjugated dienes slowly decreased by decomposition. The length of the propagation phase (during which the absorbance rapidly increases to a maximum) is the difference between *T*max and *T*lag. The oxidation rate was calculated from the slope of the tangent to the curve during the propagation phase and is expressed as moles of dienes formed per minute per gram of LDL protein. The maximal amount of conjugated dienes formed (expressed as mol dienes/g LDL-protein) was calculated by means of the molar extinction coefficient for conjugated dienes ($\varepsilon_{234}$ = 29,500 L/mol/cm). An actual experimental curve is given in Figure 1.

*Plasma lipid analyses.* Lipids were assayed using enzymatic-colorimetric methods based upon the technique of Allain *et al.* (17) for free and total cholesterol and that of Bucolo and David (18) for TG. LDL-cholesterol was calculated by difference between total cholesterol and cholesterol in the supernatant after precipitation of LDL with dextrane sulfate (QuantolipR, Immuno AG, Wien, Austria). HDL-cholesterol was determined in the supernatant after precipitation of the other lipoproteins with different concentrations of polyethylene glycol (QuantolipR, Immuno AG).

For the determination of the fatty acid composition of plasma PL, the following method was used. Lipids were extracted from 1 mL plasma according to Folch *et al* (19). The PL were prepared by thin-layer chromatography on rhodamine-impregnated silica gel plates using petroleum ether (bp 60–80°C; Merck Belgolab, Overijse, Belgium)/acetone 85:15 as mobile phase (20). The PL band was scraped off, and the fatty acids were converted into methyl esters by transesterifications with 2 mL of a mixture of methanol/benzene/HCl (80:20:5) (21). Fatty acid methyl esters were extracted with petroleum ether (bp 40–60°C), evaporated to dryness under a nitrogen flow at a temperature not exceeding 40°C, and analyzed by temperature-programmed capillary gas chromatography (Varian Model 3500) on a 25-m × 250 μm × 0.2 μm film thickness Silar 10C column (L. Restek, Interscience, Belgium) (21). The injection and detection temperatures were set at 285°C. The starting temperature of the column was 150°C, which was increased to 240°C after 3 min at a rate of 2°C/min. The carrier gas was nitrogen with a flow of 25 cm/s. Peak identification was performed by spiking with authentic standards (Sigma-Aldrich, Bornem, Belgium). Peak integration and calculation of the percentage composition were performed electronically with a Varian Model 4290 integrator.

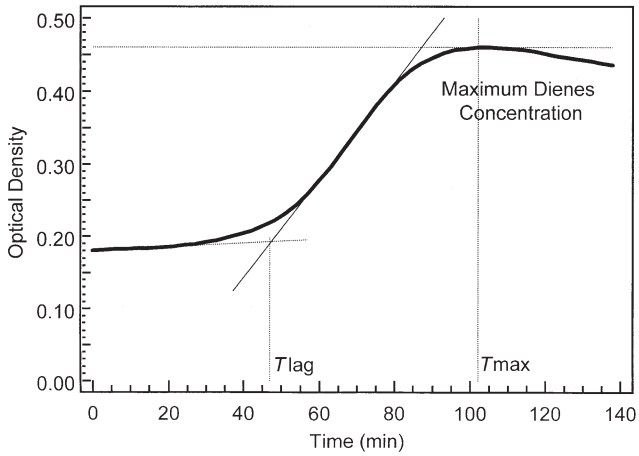


**FIG. 1.** Kinetics of density lipoprotein oxidation (actual experimental curve). *T*lag, length of the lag phase; *T*max, length of time required to obtain maximal levels of conjugated dienes.

*Plasma levels of lipid-soluble vitamins with antioxidant activity*. The concentrations of vitamin E, vitamin A, and β-carotene in plasma were measured by high-performance liquid chromatography as described by Catignani and Bieri (22). Peak identification was performed using the following standards: *d*-α-tocopherol, all-*trans*-retinol, and β-carotene (Sigma-Aldrich).

*Statistical analysis.* Normality of distribution was ascertained with the Kolmogorov-Smirnov test. Parameters related to LDL oxidative stability were log-transformed whereas the fatty acid fractions were arcsin transformed to reach normality of distribution. The vitamin levels (vitamin E, vitamin A, and β-carotene) had a normal distribution. Values are reported as mean (standard deviation). Differences in LDL oxidative stability-related parameters, in fatty acid composition of PL, and in vitamin levels between the first trimester and later stages in pregnancy or delivery were tested using the paired Student *t*-test. In order to avoid type 2 errors, due to multiple comparisons, a value of $P < 0.01$ was taken as the criterion of significance. Multiple regression and Spearman rank correlation coefficients were calculated to study the degree of association between LDL oxidative stability and antioxidant status-related parameters or plasma lipid concentrations. Trends during gestation were evaluated by computing Spearman's

rank correlation coefficients between gestational age and vitamin levels or plasma lipid concentrations. For the calculation of the correlation coefficients, samples from the same subject collected at different gestational ages were considered as independent samples. The data were analyzed using the MedCalc statistical program, version 6 (MedCalc Software, Mariakerke, Belgium) (23).

## RESULTS

*Clinical characteristics of the study population.* The mean age of the mothers at delivery was 30 yr (range 25–41 yr). The mean Body Mass Index of the women before pregnancy was 24.2 kg/m$^2$ (range 17.6–35.6 kg/m$^2$), and mean weight gain at delivery was 15 kg (range 7–23 kg). All pregnant women were nullipara, all pregnancies were uncomplicated, and the infants were born healthy with a mean birth weight of 3365 g (range 2590–4200 g) and a mean birth length of 51.2 cm (range 47–55 cm). The median Apgar score 1 min after birth was 8 (range 4–10) and 5 min after birth 9 (range 6–10). The sex ratio of the infants was 13 males and 11 females.

*Oxidative stability of LDL.* The oxidizability-related parameters of LDL throughout gestation are summarized in Table 1. *T*lag was significantly higher at later stages of pregnancy compared to the value earlier than 18 wk of gestation. *T*lag reached a maximum in the third trimester, namely 129% (SD 41.1%) of the value at the first antenatal visit. The length of the propagation phase did not change significantly during pregnancy. The oxidation rate increased to reach a maximum in the third trimester of pregnancy: 147% (SD 64.6%) of the oxidation rate at the first antenatal visit. A similar pattern was found for the maximal amount of conjugated dienes: the value in the third trimester was 142% (SD 52.4%) of the value at the first antenatal visit.

*Fatty acid composition of plasma PL.* Table 2 summarizes the fatty acid composition of the plasma PL. The sum of the highly unsaturated fatty acids (HUFA; fatty acids with 20 or more carbon atoms and with at least 3 double bonds) of the plasma PL steadily decreased with progressing gestation whereas the sum of the saturated fatty acids was slightly higher at delivery compared to the first trimester.

No correlation was found between the fatty acid fractions of plasma PL and any of the LDL oxidative stability-related parameters studied (data not shown).

*Plasma levels of cholesterol and TG (Table 3).* Cholesterol and TG concentrations could not be determined in all the plasma samples (aliquots were too small) obtained from this study population. Significance calculations were based on paired values. The values obtained for each women from the first trimester were compared with those obtained at later stages during pregnancy. The TG concentration reached a maximum in the third trimester of pregnancy. Both total cholesterol and LDL-cholesterol increased significantly during pregnancy and reached a maximum in the third trimester. HDL-cholesterol, on the other hand, reached a maximum in the second trimester and then leveled off again. To test whether the length of the lag phase depends on the plasma TG or LDL-cholesterol concentration, multiple regression analysis was performed. No significant correlations were found between any of the above-mentioned parameters.

*Plasma levels of lipid-soluble vitamins with antioxidant activity.* As was the case for the determination of the choles-

**TABLE 1**
**Oxidizability-Related Parameters of Low Density Lipoprotein (LDL)[a]**

| | <18 wk of gestation | 20–26 wk of gestation | 29–37 wk of gestation | Delivery |
|---|---|---|---|---|
| *T*lag (min) | 41.6 (16.23) | 49.2 (27.63) | 51.1 (19.93)* | 50.2 (28.88) |
| *T*max (min) | 102.5 (22.00) | 108.6 (32.28) | 111.12 (24.95)* | 113.75 (39.04) |
| Dienes (mol/g LDL-protein) | 138.0 (61.59) | 159.7 (54.99)* | 179.1 (64.54)** | 149.6 (63.54) |
| Oxidation rate (mol dienes/g LDL-protein/min) | 2.33 (1.16) | 2.72 (1.01)* | 3.04 (1.19)* | 2.48 (1.18) |

[a]Paired Student *t*-test after log transformation. Values significantly different from values obtained before 18 wk of gestation: *: $P < 0.01$; **: $P < 0.001$(mean with standard deviation in parentheses throughout gestation (n = 24).

**TABLE 2**
**Fatty Acid Composition (wt%) of Plasma Phospholipids[a]**

| | <18 wk of gestation | 20–26 wk of gestation | 29–37 wk of gestation | Delivery |
|---|---|---|---|---|
| SFA | 44.6 (1.43) | 45.6 (4.0) | 45.0 (0.9) | 46.5 (3.4)* |
| MUFA | 12.1 (1.6) | 11.7 (1.4) | 12.8 (1.3) | 12.7 (1.3) |
| PUFA | 39.7 (2.3) | 39.1 (3.8) | 38.9 (1.8) | 37.6 (3.6) * |
| HUFA | 19.4 (2.5) | 18.8 (2.8) | 17.9 (2.5)* | 17.8 (3.1)** |

[a]Mean with standard deviation in parentheses throughout gestation ($n = 24$). Paired Student *t*-test after arcsin transformation. Values significantly different from values obtained before 18 wk of gestation: *: $P < 0.01$; **: $P < 0.001$; ***: $P < 0.0001$. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids.

**TABLE 3**
**Plasma Levels of Cholesterol and Triglycerides (TG)[a]**

| | <18 wk of gestation ($n$ = 14) | 20–26 wk of gestation ($n$ = 15) | 29–37 wk of gestation ($n$ = 12) | Delivery ($n$ = 14) |
|---|---|---|---|---|
| TG | 110 (42.2) | 135 (48.7) | 186 (92.1)* | 161 (55.0) |
| Total cholesterol | 198.4 (30.8) | 238.1 (41.5)*** | 258.6 (42.6) | **212.6 (73.4) |
| LDL-cholesterol | 126.9 (29.9) | 155.2 (38.5)** | 171.6 (42.2)* | 134.6 (55.6) |
| HDL-cholesterol | 49.4 (13.5) | 55.8 (16.5)* | 49.7 (14.8) | 45.6 (19.5) |

[a] Expressed as (mg/dL) mean with standard deviation in parentheses throughout gestation. Paired Student *t*-test. Values significantly different from values obtained before 18 wk of gestation: *: $P < 0.01$; **: $P < 0.001$; ***: $P < 0.0001$. HDL, high density lipoprotein; for other abbreviation see Table 1.

**TABLE 4**
**Plasma Concentrations of Lipid-Soluble Vitamins with Antioxidant Activity[a]**

| | <18 wk of gestation ($n$ = 15) | 20–26 wk of gestation ($n$ = 17) | 29–37 wk of gestation ($n$ = 12) | Delivery ($n$ = 13) |
|---|---|---|---|---|
| Vitamin A | 1.40 (0.26) | 1.24 (0.37) | 1.03 (0.23)** | 0.87 (0.24)*** |
| Vitamin E | 21.54 (7.61) | 28.39 (13.13) | 31.16 (8.56)* | 30.19 (9.61)* |
| β-Carotene | 0.29 (0.22) | 0.26 (0.18) | 0.24 (0.12) | 0.23 (0.12) |

[a]Expressed as (mg/dL) mean with standard deviation in parentheses) throughout gestation. Paired Student *t*-test. Values significantly different from values obtained before 18 wk of gestation: *: $P < 0.01$; **: $P < 0.001$; ***: $P < 0.0001$.

terol concentration, vitamin plasma levels (μmol/L) could not be determined in all the plasma samples (aliquots were too small) obtained from this study population. Calculations of significance were based on paired values. The values obtained for each woman from the first trimester were compared with those obtained at later stages during pregnancy. The results are summarized in Table 4. Plasma levels of vitamin E significantly increased from the first trimester and reached a maximum in the third trimester, whereas β-carotene remained stable throughout pregnancy. Vitamin A in maternal plasma was significantly lower at delivery compared to the beginning of pregnancy. When the vitamin status of all the women over all the visits was plotted vs. gestational age, the plasma vitamin E levels were found to increase and the vitamin A levels to decrease during gestation (Fig. 2). To test whether the oxidative stability-related parameters correlated with the vitamin plasma levels, multiple regression analysis was performed.

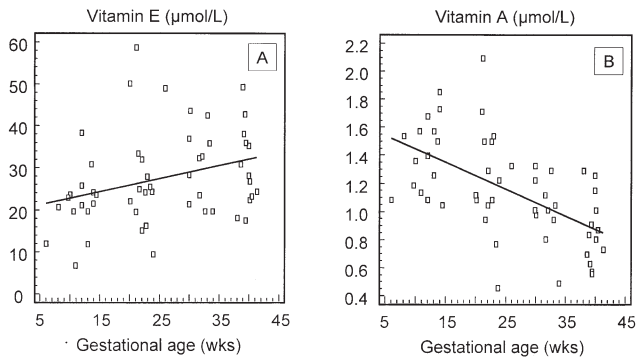


**FIG. 2.** Maternal plasma vitamin E (A) and vitamin A (B) levels (μmol/L) vs. gestational age. (Spearman's rank correlation coefficient, A: $r = 0.31$; $P = 0.025$; $n = 54$. B: $r = -0.62$; $P = 0.0001$; $n = 54$).

Only the vitamin E status correlated positively with *T*lag: $r = 0.54$; $P < 0.0001$ (Fig. 3) and with the amount of formed dienes: $r = 0.41$; $P < 0.01$. Neither vitamin A nor β-carotene correlated with the LDL oxidative stability-related parameters.

In our study population, no significant correlation was found between vitamin E plasma levels and total cholesterol or LDL-cholesterol concentration. On the contrary, vitamin E plasma levels correlated with the TG concentration during the course of pregnancy: $r = 0.41$; $P < 0.01$; $n = 49$.

## DISCUSSION

To our knowledge this is the first report on maternal plasma lipid-soluble antioxidant vitamin levels (vitamins E, A, and β-carotene) and *in vitro* formed peroxidation products (con-

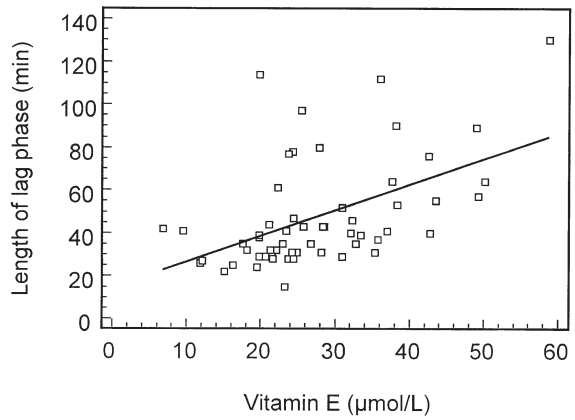


**FIG. 3.** Relation between the maternal plasma vitamin E concentration (μmol/L) and the length of the lag phase during *in vitro* oxidation of maternal low density lipoprotein (min). (Spearman's rank correlation coefficient, $r = 0.54$; $P = 0.0001$; $n = 57$).

jugated dienes of LDL), carried out in conjunction with determinations of the fatty acid compositions of maternal plasma PL at three times during normal uncomplicated pregnancy and at delivery.

Both the rate of formation and the amount of conjugated dienes formed reached a maximum in the third trimester. This could be due to a change in the composition of the LDL near the end of pregnancy. Indeed, structural changes in plasma lipoproteins during pregnancy have been described (1–7;24). An elevation of TG levels in all lipoprotein fractions during pregnancy compared to nonpregnant women was demonstrated previously (1,2,4,5). Longitudinal studies during various stages of pregnancy showed a steady rise of LDL-cholesterol and PL levels during pregnancy, which reached a maximum either at 36 wk of gestation (1) or at 2 wk postpartum (2). The observed increase in the concentration of plasma TG, cholesterol, and LDL-cholesterol in our study population is a well-known phenomenon during late pregnancy compared to early pregnancy or nonpregnant individuals (1,3–5). We observed an increase in HDL-cholesterol concentration that reached a maximum between 20 and 26 wk of gestation; others found a maximum of HDL-cholesterol at 28 wk of gestation (1).

The amount of conjugated dienes formed is a parameter for the concentration of substrate available for lipid peroxidation such as the amount of polyunsaturated fatty acids (PUFA) present in LDL. We did not determine the fatty acid composition of LDL, but it is conceivable that when the fatty acid composition of plasma PL changes there will be a related change in the LDL. In our study population the PUFA and HUFA status of plasma PL was significantly lower at delivery compared to the first trimester. No correlation was found between the fraction of PUFA or HUFA in PL and *T*lag or the amount of formed conjugated dienes.

It had been previously published that there is no correlation between the plasma α-tocopherol concentration and the α-tocopherol content of LDL (25). We found a positive correlation between *T*lag and plasma vitamin E concentrations during pregnancy. It is not clear how the higher plasma vitamin E concentrations can contribute to the higher resistance of LDL toward oxidation as measured by the prolonged lag phase. Vitamin E is tightly bound to LDL, and as LDL-cholesterol increases with progressing gestation it is expected that the vitamin E content will increase also. Our finding that vitamin E levels significantly rise during pregnancy is in line with reports by others (9,10,12–14). The observed increase in vitamin E levels during the course of pregnancy is probably not due to changes in dietary intake. Indeed, analysis of food frequency questionnaires surveyed at the beginning of pregnancy and in the third trimester of this study population revealed no significant differences in the vitamin E content of the diet, nor of the other vitamins, during the course of pregnancy (DeVriese, S.R., Matthys, C., De Henauw, S., Christophe, A.B., and Dhont, M., unpublished results). Our results suggest that when the amount of substrate available for lipid peroxidation in LDL increases (i.e., suggested by increased levels of conjugated dienes formed), the concentration of vitamin E increases also. Indeed, Esterbauer *et al*. (26) showed that the vitamin E content of LDL increases with the PUFA content of LDL. Vitamin A levels decreased with progressing gestation, as reported by others (14). We found no significant changes in maternal plasma β-carotene concentrations whereas others reported a decline in maternal β-carotene levels (14) or observed significantly higher β-carotene levels in pregnant women compared to nonpregnant women (27).

In conclusion, we report an increase in maternal plasma vitamin E levels, a decrease in vitamin A levels, and unchanged β-carotene levels during pregnancy. Furthermore, this study showed an increase in the oxidative stability of LDL with progressing gestation as measured by a prolonged lag phase after *in vitro* oxidation of isolated LDL with $Cu^{2+}$.

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# Oxidative Catabolism of α-Tocopherol in Rat Liver Microsomes

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**ABSTRACT:** The goal of this study was to clarify the mechanism responsible for the catabolism of α-tocopherol. The vitamin, bound to albumin, was incubated with rat liver microsomes and appeared to be broken down. Optimal production of the metabolite was obtained when 1 mg of microsomal protein was incubated with 36 μM of α-tocopherol in the presence of 1.5 mM of NADPH. Chromatographic and mass spectrometric analyses of the metabolite led to the conclusion that it consists of an ω-acid with an opened chroman ring, although we could not perform nuclear magnetic resonance analysis to confirm this. Our data show that α-tocopherol is ω-oxidized to a carboxylic acid and that this process can occur in rat liver microsomes in the presence of NADPH and $O_2$. The oxidation to the quinone structure appears to be a subsequent event that may be artifactual and/or catalyzed by a microsomal enzyme(s).

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Owing to its antioxidant properties (1,2), α-tocopherol, the biologically most active form of vitamin E, may act to prevent the initiation or progression of spontaneous atherosclerosis (3) and to decrease the risk of certain types of cancer (4,5) and of cataracts (5). Since the idea arose of possible beneficial effects of α-tocopherol on these degenerative diseases, an increasing number of people supplement their diet with high amounts of vitamin E. Despite the absence of overt toxicity when these excessive doses are taken, the possibility of unexpected toxic effects after long-term treatment should not be overlooked. In this regard, it is important to obtain more information on the physiological breakdown and/or excretion of α-tocopherol.

Apparently, excess absorbed tocopherols are taken up in several tissues but mainly in liver, and are readily excreted in bile (6). Furthermore, metabolites of α-tocopherol were found in the urine of rats (6), rabbits (7,8), and humans (9–11) when high doses of the compound were given. Analysis of these metabolites suggested that α-tocopherol is degraded, possibly *via* ω-oxidation of the phytyl side chain followed presumably by peroxisomal β-oxidation. An alternative possibility would be a one- step scission of most of the hydrocarbon tail, but this does not seem to occur (12). To investigate whether the isoprenoid side chain of α-tocopherol is indeed first ω-oxidized to a carboxylic acid, we incubated rat liver microsomes with α-tocopherol in the presence of NADPH and $O_2$ and analyzed the oxidation products.

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Abbreviations: APCI, atmospheric pressure chemical ionization; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; α-CEHC, 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman; DMF, dimethylformamide; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry.

## MATERIALS AND METHODS

*Materials.* Ready Safe Cocktail was from Beckman Instruments (Fullerton, CA). [3,4-$^{14}$C]All-*rac* α-tocopherol (specific activity: 53.75 Ci/mol) was a generous gift from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). The following products were obtained from Sigma (St Louis, MO): α-tocopherol, NADPH (tetrasodium salt), and butylated hydroxytoluene (BHT). Fatty acid-free bovine serum albumin (BSA) was from Boehringer Mannheim (Mannheim, Germany). Glutathione was obtained from Acros Organics (Pittsburgh, PA). α-Tocopheryl quinone was from ICN Biomedicals (Aurora, OH). The boron trifluoride/methanol reagent was obtained from Alltech (Deerfield, IL). Solvents were of analytical or high-performance liquid chromatography (HPLC) grade.

*Animals.* Male Wistar rats of approximately 150–200 g were used in all experiments; they were supplied and kept by the university facility for experimental animals. The rats were fed a standard laboratory diet, with unlimited access to water, and were fasted the night before the experiment. They were killed by cervical dislocation. After flushing with buffer A (see below) the liver was excised and homogenized as described below. The experimental protocol was approved by the university ethics committee.

*Tissue preparation.* Rat liver homogenates were prepared by adding three parts of cold buffer A (0.25 M sucrose/50 mM phosphate buffer pH 7.4/2.0 mM glutathione) to one part of liver (wet weight). The liver was homogenized in a Dounce hand homogenizer (8 up and down strokes with both pestle A and pestle B). After centrifugation of the homogenate at 400 × *g* for 5 min at 4°C, the resulting supernatant was centrifuged at 15,000 × *g* for 15 min at 4°C to remove most of the mitochondria, lysosomes, and peroxisomes. Microsomes were obtained by centrifuging the 15,000 × *g* supernatant at 105,000 × *g* for 60 min at 4°C (13). The resulting microsomal pellet was homogenized in buffer B [20% (vol/vol) glycerol/100 mM phosphate buffer pH 7.4/2.0 mM glutathione]. The microsomes were used on the day of preparation and were kept on ice before incubation. Protein determination was done

with the Bio-Rad protein assay (Richmond, CA) using BSA as standard (14). Although the purity of the microsomes was not routinely checked, the fractionation procedure has been in use in our laboratory for many years and yields a preparation that contains approximately 60, 20, 10, and 25% of, respectively, endoplasmic reticulum, peroxisomes/lysosomes, mitochondria, and protein of the liver (15).

*Preparation of solutions of α-tocopherol or α-tocopheryl quinone.* [3,4-$^{14}$C]α-Tocopherol, unlabeled α-tocopherol, or α-tocopheryl quinone was dissolved in an appropriate amount of dimethylformamide (DMF) and then mixed with a solution of BSA in a 66 mM Tris-HCl buffer pH 7.4 (molar ratio of α-tocopherol or α-tocopheryl quinone/BSA, 1:3). The suspension was vortexed immediately and diluted 1:2 with the same buffer. The final DMF concentration did not exceed 1.5% (vol/vol). Without inclusion of BSA we did not obtain a stable solution of α-tocopherol or α-tocopheryl quinone. Since α-tocopherol is sensitive to light and oxygen, solutions of α-tocopherol were always kept in the dark and sealed. The three compounds, dissolved in acetonitrile, were analyzed by HPLC (see below) to assess their purity. The cold and radiolabeleled α-tocopherol were both found to be approximately 97% pure, the remainder being mainly α-tocopheryl quinone; α-tocopheryl quinone appeared to be approximately 99% pure and contained virtually no α-tocopherol (data not shown).

*Incubation with microsomes.* Standard microsomal incubation medium consisted of 1.5 mM NADPH and radiolabeled α-tocopherol (specific activity 10 Ci/mol, at different concentrations; see further), or cold α-tocopherol (at a final concentration of 36 μM), or cold α-tocopheryl quinone (at a final concentration of 36 μM) in 66 mM Tris-HCl buffer pH 7.4. The incubations were performed under an atmosphere of 95% $O_2$ and 5% $CO_2$ in a gyratory shaker at 37°C for 15 to 120 min. The reaction was initiated by the addition of microsomes (0.1 mg to 5 mg of protein per vial), and the final volume was 1 mL. The following controls were included: incubation with buffer B instead of microsomes; and addition of HCl to stop the reaction (see below), before addition of microsomes.

*Analysis and purification of ω-oxidation products of α-tocopherol and [$^{14}$C]α-tocopherol.* The incubation of microsomes was stopped by the addition of 250 μL HCl 1 M. After vortexing, an aliquot (1 mL) was transferred to a screw-cap tube containing 3.75 mL of a chloroform/methanol (1:2, vol/vol) mixture, with 0.01% (wt/vol) BHT as antioxidant. After vigorous shaking, chloroform and water were added to a final ratio of chloroform/methanol/water of 2:2:1, resulting in a two-phase system. In the first experiments, which were done only with radiolabeled α-tocopherol, part of the chloroform layer was dried under nitrogen. The residue was then dissolved in methanol (70 μL) and subjected to HPLC using an Alltech (Deerfield, IL) Adsorbosphere C18 reversed phase column (4 μm; 150 × 3.9 mm). The eluant was methanol/water (96.25:3.75, vol/vol) at a flow rate of 1 mL/min, and the absorbance of the eluate was monitored at 280 nm. The radioactivity in the eluate was determined by collecting 0.5-mL fractions and counting in a Beckman LS-1701 liquid scintillation counter after addition of 5 mL Ready Safe cocktail.

In later experiments, the chloroform layer was evaporated to dryness under $N_2$. The lipid residue was dissolved in chloroform (500 μL) and applied to a Bond Elut $NH_2$ cartridge (Varian, Harbor City, CA). After washing with chloroform and chloroform/isopropanol (2:1, vol/vol), the ω-oxidation products were eluted with a solution of 3% acetic acid in diethyl ether. After evaporation of the diethyl ether, the residue was dissolved in a small volume (60 μL) of acetonitrile and analyzed by HPLC using an Alltech Adsorbosphere C8 reversed phase column (5 μm; 150 × 4.6 mm). Elution was performed isocratically with glycine-HCl buffer (30 mM, pH 2.6)/acetonitrile (60:40, vol/vol) for the first 5 min, followed by a linear gradient up to 100% acetonitrile over the next 10 min, and finally the column was eluted for 5 min with 100% acetonitrile. The eluate was monitored at 280 nm; and in some experiments with radiolabeled α-tocopherol, the radioactivity in the eluate was collected and counted as described above. The eluate of the other experiments, with labeled as well as with unlabeled α-tocopherol, was collected in 0.5-mL fractions, and those containing the ω-oxidation products were pooled and stored at –20°C until further analysis.

*Methylation of the ω-oxidation products.* The pooled fractions were evaporated to dryness under $N_2$, the residue was taken up in chloroform, and glycine was removed by washing with water. The chloroform layer was evaporated to dryness, and 200 μL of a boron trifluoride/methanol (14%, wt/vol) solution was added. The mixture was heated at 80°C for 4 min, cooled, and extracted with 1 mL of chloroform and 0.5 mL of water. The chloroform layer was removed and dried under $N_2$. The methylated products were purified on HPLC using an Alltech Adsorbosphere C8 reversed-phase column (5 μm; 150 × 4.6 mm). Elution was performed isocratically with water/acetonitrile (60:40, vol/vol) for the first 5 min, followed by a linear gradient up to 100% acetonitrile over the next 10 min; and finally the column was eluted for 5 min with 100% acetonitrile. The flow rate was maintained at 1 mL/min. In the experiments with radiolabeled α-tocopherol, the eluate was collected and counted for radioactivity as described above. In the experiments with unlabeled α-tocopherol the eluate was collected in 0.5-mL fractions. The fractions from different experiments, containing the methylated oxidation products, were pooled and used for analysis with HPLC coupled to a Finnigan Mat TSQ 70 triple stage quadrupole mass spectrometer, using atmospheric pressure chemical ionization (APCI) as ionization technique. HPLC was carried out with an Alltech Adsorbosphere C8 reversed phase column (5 μm; 150 × 2.1 mm). Elution was performed isocratically with water [containing 1% (vol/vol) acetic acid)/acetonitrile (60:40, vol/vol] for the first 5 min and then the same elution pattern was used as described above. The flow rate was maintained at 0.2 mL/min.

## RESULTS

*Optimization of incubation conditions.* The discovery of urinary metabolites of α-tocopherol in rabbits and humans given large doses of α-tocopherol suggests that the terminal methyl

group in the side chain may be ω-oxidized, followed by β-oxidation of the resulting carboxylic acid (7–10). With the aim of assessing whether this degradation mechanism is correct, we incubated microsomes with [3,4-$^{14}$C]α-tocopherol and investigated the breakdown products.

Since we had little radiolabeled α-tocopherol at our disposal, preliminary experiments related to the incubation conditions were not repeated or were done only twice. When the chloroform extracts, obtained after incubation of [3,4-$^{14}$C]α-tocopherol under standard conditions, were subjected to reversed-phase C18 HPLC analysis, using the isocratic system (see the Materials and Methods section), a radioactive peak was seen at the start of the chromatogram. The substance was not α-tocopherol (retention time of 12.5 min) or α-tocopheryl quinone (retention time of 7 min). Since it eluted at the solvent front, it could be a (mixture of) polar compound(s), which we refer to as "metabolite" in the following. No metabolite was formed in the absence of NADPH or at zero time of incubation; substituting buffer B for microsomes also abolished its formation. Studies on the concentration of α-tocopherol, on the amount of microsomal protein, and on the incubation time revealed that maximal production of metabolite was obtained when 1 mg of microsomal protein (Fig. 1A) was incubated with 36 μM of α-tocopherol (Fig. 1B) for 120 min (Fig. 1C) in the presence of 1.5 mM NADPH. There was a significant variability in the total amount of oxidation product formed between experiments. However, the effects of time, protein concentration, and substrate concentration were consistent. Over the 120-min incubation time, up to 2% of the added α-tocopherol was oxidized to the polar metabolite; of the remaining α-tocopherol, up to 5% was converted to α-tocopherylquinone (not shown). In order to exclude that the metabolite would have arisen from enzymatic degradation of α-tocopheryl quinone, present as a contaminant or generated *in situ*, microsomes were incubated for 120 min with α-tocopheryl quinone, as described in the Materials and Methods section. After purification, methylation, and mass spectrometry (MS; see below) we could not detect the presence of the metabolite in these incubation mixtures (results not shown), although it was present in mixtures incubated with α-tocopherol in parallel.

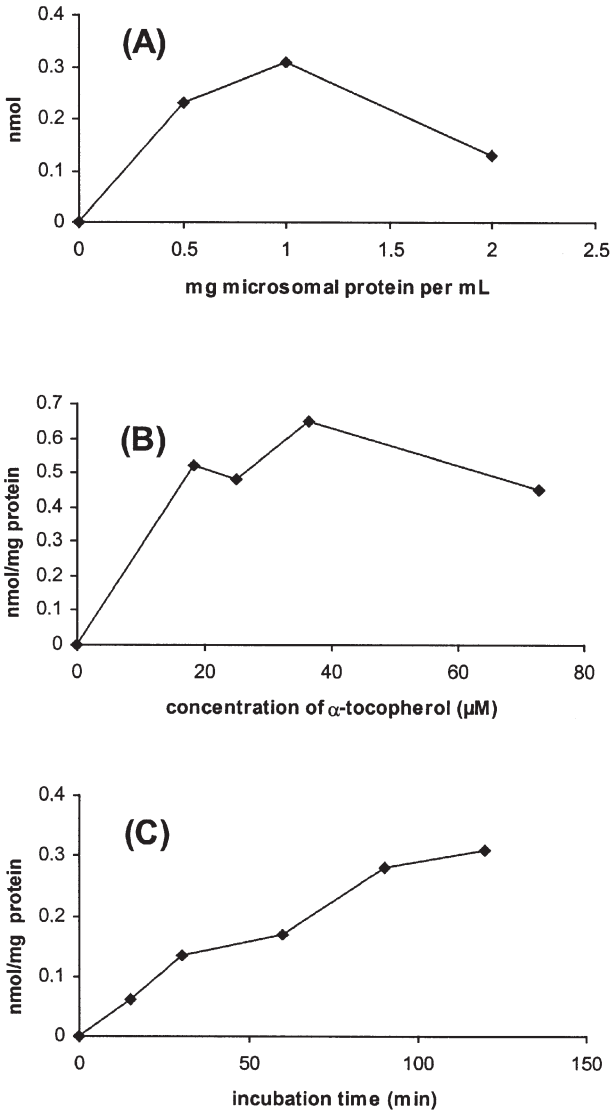


**FIG. 1.** α-Tocopherol catabolism as a function of (A) protein concentration, (B) concentration of α-tocopherol, (C) incubation time. The amount of metabolite formed was measured after incubation at 37°C in a reaction mixture containing 1.5 mM NADPH and, unless indicated otherwise in the abscissa, 36 μM of α-tocopherol and 1 mg of microsomal protein. Incubation time was 120 min except for condition (C) in which it was varied as indicated. The results are from one experiment.

Taken together, the data described above suggest that ω-oxidation did take part in the formation of the metabolite of α-tocopherol. The identification of this metabolite was the goal of the following experiments.

*Purification of the α-tocopherol metabolite.* To perform MS analysis, we needed at least 1 μg of pure unlabeled methylated product. A chloroform extract of liver microsomes contains a lot of organic material such as fatty acids, phospholipids and neutral lipids. In a first purification step, the extract was applied onto a weak anion exchange column. After elution, more than 90% of the radiolabeled metabolite was recovered in the acidic diethyl ether eluate. Analysis of this eluate on reversed-phase HPLC, using a C8 column and a glycine-HCl buffer pH 2.6/acetonitrile gradient (see the Materials and Methods section), revealed one major peak of radioactive material at 12.5 min with a shoulder of ultraviolet-absorbing material (Figs. 2A,B). When the glycine-HCl buffer was replaced by purified (grade I) water, the radiolabeled metabolite again eluted at the solvent front position. In the glycine-HCl buffer pH 2.6/acetonitrile mixture, the metabolite is probably fully converted to the acid form, whereas in the water/acetonitrile mixture the metabolite is probably mainly present in the ionic form. Thereafter, the radiolabeled metabolite was methylated and subjected again to HPLC using a C8 column and a water/acetonitrile gradient. In this system, we saw only one radioactive peak with a retention time of about 14 min, and hardly any signal at 280 nm

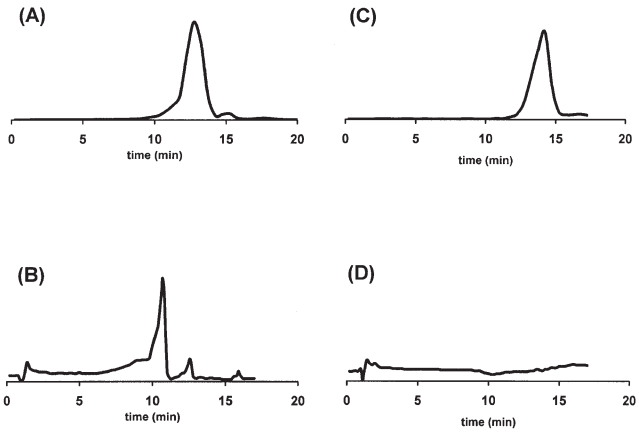


**FIG. 2.** Chromatograms of the α-tocopherol metabolite after purification on an $NH_2$ column (A and B) and of the methylated α-tocopherol metabolite (C and D). A and C show the radioactive signal and B and D show the absorbance at 280 nm. See the Materials and Methods section for experimental details. The results are from one representative experiment.

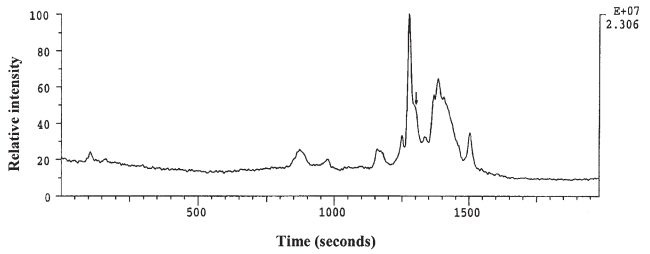


**FIG. 3.** Liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry chromatogram of the methylated metabolite of α-tocopherol. The mass spectrometer was set to scan the *m/z* range 50 to 800. The arrow indicates the position of the peak at *m/z* 491.2 (retention time: 21.7 min; see text).

(Figs. 2C, 2D). After pooling the methylated metabolite from several experiments, performed with unlabeled α-tocopherol, we finally obtained about 5 µg of material. This quantity was sufficient for MS but did not allow nuclear magnetic resonance analysis.

*Characterization of unlabeled methyl ester by liquid chromatography (LC)–MS.* The following elements led us to conclude that the metabolite obtained (i) is an ω-acid derived from α-tocopherol, and (ii) has an opened chroman ring (quinone structure).

The purification by HPLC was done under a normal atmosphere. Different groups have demonstrated that α-tocopherol can autoxidize to α-tocopheryl quinone under aerobic conditions (16–18). The ω-oxidation product of α-tocopherol also appears to be even more sensitive to this conversion than the parent compound.

Reversed-phase LC–MS analysis of the methylated metabolite was done as described in the Materials and Methods section. The mass spectrometer was set to scan the *m/z* range 50 to 800. The total ion chromatogram showed several peaks (Fig. 3), indicating the presence of a number of impurities that had not been detected at 280 nm. The calculated molecular weight of the methylated acid with the quinone structure is 490, whereas the methylated acid with the intact chroman ring has a molecular weight of 474. Since APCI is a "soft" ionization technique, the initial ions produced are mainly of the type $(M + H)^+$ from which the molecular weight can be deduced easily. Therefore, the peaks with a retention time between 15 and 25 min were monitored for *m/z* 491 and *m/z* 475. No significant peak was seen at *m/z* 475, but the ion with *m/z* 491.2 was present in a peak with a retention time of 21.7 min, although it was not separated from a much larger peak (Fig. 3). The mass spectrum of the peak associated with *m/z* 491.2 contained ions with *m/z* 345.0, 319.0, 244.8 and 374.0 (Fig. 4). Selected ion monitoring (not shown) of the chromatogram, for each of these fragments, and MS–MS of *m/z* 491.2 (Fig. 5) clearly showed that these fragments could not be derived from *m/z* 491.2. The fragment ions with *m/z* 263.0 and 294.9 showed approximately the same chromatographic pattern as the ion with *m/z* 491.2, but they were not present in the MS–MS spectrum of *m/z* 491.2. The ions with *m/z* 473, 459.2, 431.1, and 413.1 (Fig. 4), on the other hand, are fragments of *m*/z 491.2. The one with *m/z* 473 $(M + H - 18)^+$ might be formed by the loss of water, which normally indicates the presence of a hydroxyl group. The fragment ion observed at *m/z* 459.2 $(MH - 32)^+$ probably resulted from the loss of $CH_3OH$. The ion peak at *m/z* 431.1 $(MH - 60)^+$ might be explained by the loss of $HCOOCH_3$, indicating the presence of a methylated carboxyl group. The ion peak at *m/z* 413.1 might originate from the combined loss of $HCOOCH_3$ and $H_2O$ from the $(M + H)^+$ ion. We concluded that the mass spectrum indicates the presence of a molecule with a molecular weight of 490 containing a hydroxyl function and a methylated carboxyl group. As mentioned before, the methylated carboxylic acid derived from α-tocopheryl quinone by ω-oxidation has a molecular weight of 490 and has a free hydroxyl group. Therefore, our structural proposal for the metabolite is the ω-acid of the α-tocopheryl quinone. We believe that α-tocopherol is indeed ω-oxidized by microsomes in the presence of NADPH and $O_2$, apparently followed by oxidation to the quinone structure. The latter may occur during the incubation

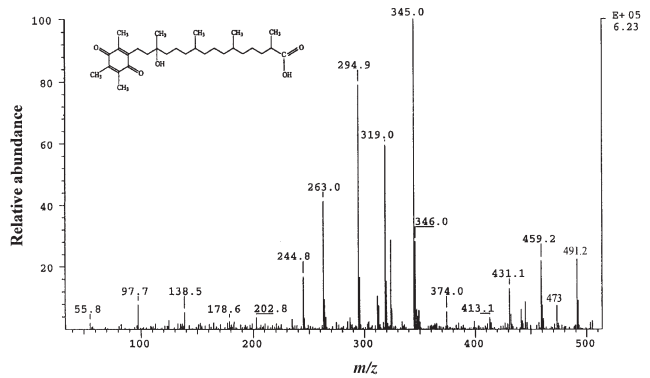


**FIG. 4.** Mass spectrum of the methylated metabolite, obtained at a retention time of 21.7 min (see text).

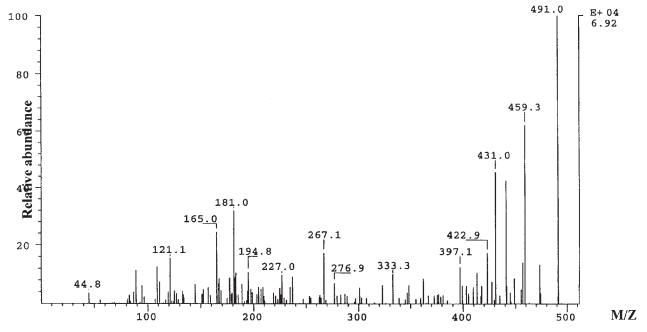


**FIG. 5.** Tandem mass spectroscopy spectrum of *m/z* 491.2, obtained at a retention time of 21.7 min (see text).

with the microsomes and/or during purification and analysis. Unfortunately the spectrum of the methylated metabolite contained ions that are not derived from this metabolite, but probably from impurities with more or less similar retention times. We have not identified these products.

## DISCUSSION

Both Simon *et al.* (8,10) and Schultz *et al.* (9) assumed that the metabolites of α-tocopherol found in the urine of rabbits and humans that had been given large doses of α-tocopherol were formed by ω-oxidation of the side chain followed by β-oxidation. According to Schultz *et al.* (9), the major urinary excretion product of vitamin E is the α-tocopherol-derived carboxylic acid 2,5,7,8-tetramethyl 2(2′-carboxyethyl)-6-hydroxychroman (α-CEHC). Other investigators (6,11) have confirmed this and found that α-CEHC is mainly derived from α-tocopherol stereoisomers that are present only in the synthetic vitamin. Wechter *et al.* (19) reported that γ-tocopherol is broken down in a similar fashion and that its metabolite is an endogenous natriuretic factor. The metabolites, tocopheronic acid [2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethyl benzoquinone] and its lactone (tocopheronolactone), found by Simon *et al.* (8,10), have a quinone structure, which means that the chroman ring was opened. Urinary metabolites of α-tocopherol are excreted as conjugates; before they can be analyzed chromatographically they must be hydrolyzed. Simon *et al.* used rather drastic procedures and did not avoid oxygenation during hydrolysis, whereas Schultz *et al.* (9) prepared their samples by enzymatic hydrolysis and excluded oxygen. The latter discovered that α-CECH was easily converted to α-tocopheronolactone by exposure to $O_2$, and they concluded that the Simon metabolites were in fact the result of oxidation of α-CEHC during sample preparation. On the basis of our results, we conclude that α-tocopherol can indeed be ω-oxidized to a carboxylic acid as hypothesized by Simon and Schultz, although we realize that the structural identification of the ω-acid of α-tocopherol is not definitive, since we could not obtain the product in pure form and in adequate quantities to perform further analysis (e.g., nuclear magnetic resonance). Furthermore, we have demonstrated that the conversion of α-tocopherol to the carboxylic acid can occur in liver microsomes. We did not obtain the ω-acid of α-tocopherol but of α-tocopheryl quinone, which seems to be formed by oxidation of the ω-acid of α-tocopherol, similar to the oxidation of α-CEHC to the Simon metabolites. Our control experiments, i.e., zero time incubations, incubations without microsomes, and incubations with α-tocopheryl quinone, strongly suggest that the proposed metabolite is not present as a contaminant in the substrate and is not formed by ω-oxidation of α-tocopheryl quinone. Thus, the microsomal ω-oxidation of α-tocopherol precedes the conversion to the quinone product that was finally obtained. Since we could only detect the latter, it appears that the conversion of the ω-acid of α-tocopherol to the corresponding quinone is rapid under our conditions, in contrast to the conversion of the parent compound (α-tocopherol) to α-tocopheryl quinone. This may be related to a differences in solubility, or the ω-acid may be a substrate for a microsomal enzyme catalyzing the opening of the chroman ring. The oxidation of α-tocopherol to a carboxylic acid presumably involves an ω-hydroxy intermediate, but we could not detect the latter in our system. When lauric acid is incubated with microsomes in the presence of NADPH and $O_2$, only hydroxylation occurs (20–22). However, literature data on the ω-oxidation of branched-chain fatty acids, incubated with washed microsomes in the presence of NADPH, indicate that the hydroxy compound is only a minor metabolite (23). Since α-tocopherol has a branched side chain, it is possible that the ω-hydroxy compound is not present or to such a low extent that we could not discern its presence. Microsomal alcohol and aldehyde dehydrogenases probably quickly converted the hydroxylated α-tocopherol to the ω-acid.

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# Lipid and Fatty Acid Profiles in Rats Consuming Different High-Fat Ketogenic Diets

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**ABSTRACT:** High-fat ketogenic diets are used to treat intractable seizures in children, but little is known of the mechanism by which these diets work or whether fats rich in n-3 polyunsaturates might be beneficial. Tissue lipid and fatty acid profiles were determined in rats consuming very high fat (80 weight%), low-carbohydrate ketogenic diets containing either medium-chain triglyceride, flaxseed oil, butter, or an equal combination of these three fat sources. Ketogenic diets containing butter markedly raised liver triglyceride but had no effect on plasma cholesterol. Unlike the other fats, flaxseed oil in the ketogenic diet did not raise brain cholesterol. Brain total and free fatty acid profiles remained similar in all groups, but there was an increase in the proportion of arachidonate in brain total lipids in the medium-chain triglyceride group, while the two groups consuming flaxseed oil had significantly lower arachidonate in brain, liver, and plasma. The very high dietary intake of α-linolenate in the flaxseed group did not change docosahexaenoate levels in the brain. Our previous report based on these diets showed that although ketosis is higher in rats consuming a ketogenic diet based on medium-chain triglyceride oil, seizure resistance in the pentylenetetrazol model is not clearly related to the degree of ketosis achieved. In combination with our present data from the same seizure study, it appears that ketogenic diets with widely differing effects on tissue lipids and fatty acid profiles can confer a similar amount of seizure protection.

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The high-fat ketogenic diet has regained popularity over the past 5 yr as a treatment of last resort for intractable epilepsy (1–3). Dairy fat is the predominant fat used in the classical ketogenic diet. This more common version of the diet is calculated to provide a fat to carbohydrate-plus-protein ratio of about 4:1. The diet has comparable anticonvulsant effects when prepared with other dietary oils such as medium-chain triglyceride (MCT) oil or corn oil (4,5), but MCT oil causes more gastrointestinal side effects (2) and corn oil is harder to incorporate into palatable foods. Animal studies modeling the protective effects of the ketogenic diet against seizures have used many different fat sources and have not shown consistent results between different seizure models (6–12). The mechanism by which the ketogenic diet suppresses or prevents seizures in humans is unknown, so we are attempting to reproduce its effects in animals in order to better understand how it works. We are not aware of any animal studies in which ketogenic diets containing different dietary fats have been compared.

The unusual amount and type of fat in the ketogenic diet is potentially problematic because this diet tends to be low in n-3 polyunsaturated fatty acids (PUFA), which are important for normal visual and neurological development (13,14). Interestingly, recent research implicates two of the n-3 PUFA, docosahexaenoate (DHA, 22:6n-3) and α-linolenate (ALA, 18:3n-3), as anticonvulsants (15,16). Owing to the potential role of n-3 PUFA as anticonvulsants and the substantial ketogenic potential of ALA (17), we recently evaluated the anticonvulsant efficacy of several high-fat ketogenic diets differing in fat composition. Ketogenic diets based on butter, ALA-rich flaxseed oil (FSO), or MCT all conferred moderate protection toward a low dose of pentylenetetrazol (PTZ) but not against electroshock-induced seizures (18). These beneficial effects were observed at blood ketone levels 30–80% lower than in humans on a diet of similar composition.

The present paper describes the changes in selected tissue lipids and fatty acid profiles from that study. We have emphasized the tissue levels of DHA and arachidonate (AA, 20:4n-6) because of the anticonvulsant effects of DHA (15) and the possible neuroexcitatory or proconvulsant effects of AA (19,20). A further aim was to determine whether the fatty acid profile of adipose tissue of rats on high-fat ketogenic diets tended to reflect the dietary fat profile as would be expected on diets containing 5–20 wt% fat.

## MATERIALS AND METHODS

*Animals.* All procedures involving animals received prior approval from the University of Toronto Animal Care Committee, the standards of which are set by the Canadian Council on Animal Care. Fourteen-day-old male Wistar rat pups arrived from the breeding facility (Charles River Canada, St. Constant, Québec) and were housed with their dams for 6 d before being weaned at 21 d of age and allocated to one of five dietary groups. To assist in adaptation to the ketogenic

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Abbreviations: AA, arachidonate; ALA, α-linolenate; ANOVA, analysis of variance; DHA, docosahexaenoate; EPA, eicosapentaenoate; FSO, flaxseed oil; LA, linoleic acid; MCT, medium-chain triglyceride; PTZ, pentylenetetrazol; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

**TABLE 1**
**Fatty Acid Composition (wt%) of the Fats Used to Formulate Different High-Fat Ketogenic Diets[a]**

| | CONTROL | MCT | FSO | BUTTER | MIXTURE |
|---|---|---|---|---|---|
| 8:0 | 0 | 57 | 0 | 2 | 32 |
| 10:0 | 0 | 33 | 0 | 4 | 20 |
| 12:0 | 0 | 1 | 0 | 4 | 1 |
| 14:0 | <1 | 0 | 0 | 13 | 4 |
| 16:0 | 12 | 1 | 6 | 32 | 10 |
| 18:0 | 5 | <1 | 3 | 10 | 3 |
| 18:1n-9 | 21 | 2 | 22 | 22 | 11 |
| 18:2n-6 | 53 | 4 | 21 | 7 | 7 |
| 18:3n-3 | 8 | <1 | 48 | 1 | 11 |

[a]All ketogenic diets contained 7 wt% soybean oil (see CONTROL group for fatty acid profile) and 65.2 wt% of the fat shown. CONTROL, 7 wt% soybean oil; MCT, medium-chain triglyceride oil; FSO, flaxseed oil; MIXTURE, ketogenic diet based on a 1:1:1 mixture by weight of MCT, FSO, and BUTTER.

diet, the amount of fat was gradually increased over the first 8 d by providing ratios of fat/protein + carbohydrate of 1:1 for 4 d, 2:1 for 4 d, and finally 3.5:1 for a further 40 d.

*Diets.* Control animals received the American Institute of Nutrition-93G (AIN-93G) diet (18). The experimental animals received ketogenic diets prepared in our laboratory. The ketogenic diets were prepared by mixing specific fats/oils with a custom-formulated powdered diet mixture (Dyets, Bethlehem, PA) of the following composition (g/kg): 198.6 cellulose, 0.27 AIN-93G Vitamin Mix (without sugar), 22.0 AIN-93G Mineral Mix (without sugar), 153.9 casein, 12.0 dextrose, 54.7 soybean oil, 524.6 fat/oil, 2.0 choline bitartrate, and 32.0 sodium cyclamate (Sucaryl$^{R}$). Sodium cyclamate is commonly used as a sweetening agent in foods given to children on the ketogenic diet. It was used to improve the flavor and consistency of the diet. Butter, FSO, MCT, or a mixture of equal parts of butter, FSO, and MCT oils were used to formulate each diet (see Tables 1 and 2). MCT was a gift of Mead Johnson Nutritionals (Evansville, IN). The other oils/fats were obtained commercially. Butter and MCT were used to mimic the classical and MCT versions of the ketogenic diet. FSO was chosen for this study because it is 55% by weight ALA. Diets and drinking water were available *ad libitum.*

*Blood and tissue analysis.* After 48 d on the diets and completion of the seizure tests (reported elsewhere; Ref. 18), each rat was anesthetized with pentobarbital (40 mg/kg). Blood was drawn by cardiac puncture, and the brain, liver, and perirenal adipose tissue were excised. Each organ was weighed and stored at −20°C for subsequent lipid extraction. Total lipid extracts were prepared using the extraction method of Folch *et al.* (21). Approximately 1 g of tissue (1 mL of plasma) was weighed into vials. An internal standard solution comprising heptadecanoate, L-α-phosphatidylcholine diheptadecanoyl, and triheptadecanoin (Sigma Chemical Co, St. Louis, MO) in chloroform was then added to each sample for the quantification of free fatty acids, phospholipids, triglycerides, and free cholesterol, respectively.

The organs were homogenized, methanol was added to give a 2:1 ratio to chloroform, and the lipid phase was separated after centrifugation. Lipid classes of the total lipid extract were fractionated by neutral thin-layer chromatography (TLC) using silica gel plates (20 × 20 cm Whatman LK6D plates precoated with 250 μm of silica gel 60Å). The plates were transferred to a covered TLC tank containing the mobile phase solvent system (petroleum ether/diethyl ether/acetic acid, 80:20:1, by vol) and allowed to develop for 30 min. The spots were visualized with ultraviolet light after spraying the plates with 2′,7′-dichlorofluorescein. The resulting bands were scraped off each plate and the phospholipid, cholesterol, and total lipid fractions were saponified in KOH-methanol for 60 min at 90°C. Triglyceride and free fatty acid bands were not saponified and were immediately methylated. After adding hexane and then acidifying, the fatty acid phase was removed and methylated in boron trifluoride/methanol. The fatty acid methyl esters were analyzed by capillary gas chromatography (30-m DB column; J&W Scientific, Folsom, CA) with automated sample injection and comparison of retention times against several standards of known composition.

Plasma total cholesterol was measured using a commercially available kit (Sigma Chemical Co.). Brain cholesterol was measured by capillary gas chromatography after preparation of *t*-butyldimethylsilyl derivatives of brain total sterols, with quantification against 5-α-cholestane as an internal standard.

*Data analysis.* Statistical analyses were performed using

**TABLE 2**
**Fatty Acid Composition (wt%) of Perirenal Adipose Tissue Total Lipids from Rats on Different Formulations of the High-Fat Ketogenic Diet[a]**

| | CONTROL | MCT | FSO | BUTTER | MIXTURE |
|---|---|---|---|---|---|
| 10:0 | 0.1 ± 0.02$^{a}$ | 14.4 ± 2.8$^{b}$ | 0.1 ± 0.01$^{a}$ | 1.3 ± 0.1$^{c}$ | 4.2 ± 0.4$^{d}$ |
| 12:0 | 0.2 ± 0.04$^{a}$ | 2.3 ± 0.3$^{b}$ | 0.1 ± 0.03$^{d}$ | 3.4 ± 0.2$^{c}$ | 1.6 ± 0.1$^{e}$ |
| 14:0 | 1.7 ± 0.1$^{a}$ | 2.8 ± 0.4$^{b}$ | 0.4 ± 0.1$^{d}$ | 11.1 ± 0.3$^{c}$ | 5.0 ± 0.2$^{e}$ |
| 16:0 | 24.7 ± 1.2$^{a}$ | 23.8 ± 4.5$^{a}$ | 7.9 ± 0.6$^{c}$ | 29.5 ± 0.4$^{b}$ | 18.0 ± 1.8$^{d}$ |
| 18:0 | 3.5 ± 0.5$^{a}$ | 4.5 ± 0.5$^{b}$ | 3.4 ± 0.3$^{a}$ | 7.0 ± 0.5$^{c}$ | 5.0 ± 0.5$^{d}$ |
| 18:1n-9 | 28.3 ± 1.3$^{a}$ | 17.3 ± 1.1$^{b}$ | 25.6 ± 0.4$^{d}$ | 30.8 ± 0.3$^{c}$ | 25.0 ± 0.4$^{d}$ |
| 18:2n-6 | 28.6 ± 2.4$^{a}$ | 25.9 ± 2.9$^{a}$ | 21.7 ± 0.6$^{c}$ | 10.6 ± 0.1$^{b}$ | 17.8 ± 0.9$^{d}$ |
| 20:4n-6 | 0.3 ± 0.1$^{a}$ | 1.1 ± 0.2$^{b}$ | 0.1 ± 0.02$^{c}$ | 0.3 ± 0.03$^{a}$ | 0.2 ± 0.02$^{d}$ |
| 18:3n-3 | 2.9 ± 0.3$^{a}$ | 3.2 ± 0.5$^{a}$ | 38.9 ± 0.5$^{c}$ | 1.7 ± 0.1$^{b}$ | 19.6 ± 1.3$^{d}$ |
| 22:6n-3 | 0.05 ± 0.06$^{a}$ | 0.2 ± 0.05$^{b}$ | 0.1 ± 0.06$^{a}$ | 0.02 ± 0.03$^{a}$ | 0.3 ± 0.04$^{b}$ |

[a]Values are mean ± SD, $n$ = 6 rats/group. In each row, values with different roman superscripts are significantly different, $P < 0.05$. For abbreviations see Table 1.

SigmaStat 2.03 software package (Jandel Scientific Software, San Rafael, CA). Differences between diet groups were analyzed using one-way analysis of variance (ANOVA) with the Tukey test for multiple comparisons. For data sets that were not normally distributed, a Kruskal-Wallis ANOVA on ranks, with Dunn's method for multiple comparison, was used.

## RESULTS

Final body weights of rats were (mean ± SD) CONTROL group, 351 ± 15 g; MIXTURE group, 333 ± 15 g; BUTTER group, 309 ± 19 g; FSO group, 290 ± 21 g; and MCT group, 205 ± 14 g. Body weights of all the ketogenic diet groups differed from each other at $P < 0.05$. Liver weights varied with body weight but did not differ as a proportion of body weight (4.0–4.3% of final body weight in all the groups (not significantly different). Brain weights (1.7–1.8 g) did not differ between groups.

Both the degree of ketosis and the response to seizures of the rats in this study have previously been reported (18). In brief, three ranges of plasma β-hydroxybutyrate were observed; the lowest values (0.09 ± 0.03 mM/L) were in CONTROLS, a middle range (0.7–0.9 mM/L) was seen in the BUTTER, FSO, and MIXTURE groups, and the highest values (5.2 mM/L) were in the MCT group. Seizures induced by PTZ were most severe in the CONTROL and the MIXTURE groups and were reduced by 33–50% in the BUTTER, MCT, and FSO groups ($P < 0.05$) (18).

In general, the proportions of fatty acids in adipose tissue broadly reflected their proportions in the diet (Tables 1 and 2). However, the MCT group had much higher linoleic acid (LA, 18:2n-6), ALA, and AA compared with either the MCT diet itself or the adipose tissue fatty acid profiles of other ketogenic diet groups ($P < 0.05$). The FSO and MIXTURE groups had 6–23-fold higher percentage of ALA and 4–10- fold higher percentage of DHA in adipose tissue than the other groups ($P < 0.05$).

The concentration of plasma phospholipids in the BUTTER group was 20–60% higher than in the other groups (Table 3). The MCT group had the lowest plasma triglyceride level, but this was significant only in comparison to the BUTTER group ($P < 0.05$). Free fatty acid concentrations were significantly higher than CONTROL in the FSO and MIXTURE groups, while the other groups were similar. No significant differences were found in plasma cholesterol between any of the groups (Table 3).

The concentration of liver phospholipids in the FSO group was significantly higher than both the CONTROL and MCT groups ($P < 0.05$). Liver triglycerides in the BUTTER and MIXTURE groups were about 10 times higher than in the other groups. Liver free fatty acids in the FSO and MIXTURE groups were nearly twofold higher than those in the other groups. No differences across groups were found in the concentration of brain total or free fatty acids. Except in the FSO group, brain cholesterol was 11–18% higher in all the ketogenic diet groups than in the CONTROL group ($P < 0.05$, Table 3).

The percentage composition of palmitate (16:0), stearate (18:0), and DHA in brain free fatty acids was similar in all the groups (Table 4). The proportion of LA in brain free fatty acids was significantly higher in the FSO group compared with all other diet groups, while the proportion of AA in the FSO group was significantly lower. ALA and EPA were not detected in the brain of the CONTROL, MCT, and BUTTER groups, but they were present at 0.2–0.3% of total brain fatty acids in the FSO and MIXTURE groups.

Both the total concentration and proportion of AA in liver free fatty acids of the MCT group were up to twofold higher than in the other groups (Table 5). The groups consuming FSO had the lowest proportion of free AA in liver and plasma. There were no significant differences between groups in the amount of free AA in the brain. There was significantly more free DHA in the liver in the FSO and MIXTURE groups, but there were no differences between groups in the concentration or proportion of free DHA in the brain (Table 5).

**TABLE 3**
**Lipid Concentrations in Plasma, Liver, and Brain of Rats on Different Formulations of the High-Fat Ketogenic Diet[a]**

| | CONTROL | MCT | FSO | BUTTER | MIXTURE |
|---|---|---|---|---|---|
| Plasma (mg/dL) | | | | | |
| Phospholipid | 7.9 ± 1.4[a] | 10.4 ± 2.1[a] | 7.4 ± 0.9[a] | 12.3 ± 1.0 [b] | 9.7 ± 1.3[a] |
| Triglyceride | 6.1 ± 1.5[a,b] | 2.5 ± 1.1[a] | 4.8 ± 4.3[a,b] | 8.7 ± 3.0 [b] | 4.8 ± 3.7[a,b] |
| Free fatty acid[b] | 53.8 ± 5.3[a] | 79.2 ± 19.7[a,b] | 91.8 ± 26.5[b] | 78.6 ± 20.5[a,b] | 89.9 ± 23.9[b] |
| Cholesterol | 96.3 ± 7.7 | 107.8 ± 15.4 | 96.3 ± 7.7 | 115.5 ± 15.4 | 103.9 ± 15.4 |
| Liver (mg/g) | | | | | |
| Phospholipid | 18.4 ± 1.0[a] | 28.9 ± 1.3[b] | 33.1 ± 3.9[c] | 31.9 ± 3.1[b,c] | 29.8 ± 1.4[b,c] |
| Triglyceride | 6.9 ± 3.7[a] | 7.3 ± 5.7[a] | 7.8 ± 2.4[a] | 73.2 ± 17.3 [b] | 68.8 ± 13.8[b] |
| Free fatty acid | 17.7 ± 5.4[a] | 22.3 ± 4.7[b] | 48.2 ± 10.9[c] | 28.6 ± 10.6[b] | 45.5 ± 13.6[c] |
| Brain (mg/g) | | | | | |
| Total lipid | 41.7 ± 0.6 | 40.9 ± 1.8 | 40.6 ± 0.8 | 40.1 ± 2.6 | 41.8 ± 1.6 |
| Free fatty acid | 2.0 ± 0.2 | 2.1 ± 0.2 | 2.1 ± 0.2 | 1.9 ± 0.6 | 2.4 ± 0.3 |
| Cholesterol | 17.5 ± 1.3[a] | 19.4 ± 0.7[b] | 17.5 ± 1.9[a] | 20.3 ± 3.1[b] | 20.7 ± 0.8[b] |

[a]Values are mean ± SD, $n = 6$ rats/group. In each row, values with different roman superscripts are significantly different, $P < 0.05$. For abbreviations see Table 1.
[b]Plasma free fatty acid units are μg/mL.

**TABLE 4**
**Fatty Acid Composition (wt%) of Brain Total Lipids in Rats on Different Formulations of the High-Fat Ketogenic Diet[a]**

| | CONTROL | MCT | FSO | BUTTER | MIXTURE |
|---|---|---|---|---|---|
| 14:0 | 0.2 ± 0.01[a] | 0.2 ± 0.01[a] | 0.2 ± 0.3[a] | 0.4 ± 0.04[b] | 0.3 ± 0.03[c] |
| 16:0 | 21.6 ± 0.3[a] | 22.0 ± 0.1[b] | 21.7 ± 0.2[a,b] | 21.8 ± 0.2[a,b] | 21.4 ± 0.4[a] |
| 18:0 | 12.4 ± 0.2 | 12.9 ± 1.0 | 12.3 ± 0.4 | 12.9 ± 1.4 | 12.3 ± 0.6 |
| 18:1n-9 | 21.2 ± 0.6[a] | 20.6 ± 0.4[b] | 21.8 ± 0.4[a,c] | 21.5 ± 0.2[a] | 22.2 ± 0.3[c] |
| 18:2n-6 | 1.5 ± 0.1[a] | 1.1 ± 0.1[b] | 2.9 ± 0.1[c] | 1.4 ± 0.2[a] | 2.1 ± 0.2[d] |
| 20:4n-6 | 13.4 ± 0.8[a] | 14.0 ± 0.6[b] | 11.8 ± 0.2[c] | 13.1 ± 0.7[a] | 12.3 ± 0.5[d] |
| 18:3n-3 | <0.1 | <0.1 | 0.3 ± 0.04[a] | <0.1 | 0.2 ± 0.01[b] |
| 20:5n-3 | <0.1 | <0.1 | 0.3 ± 0.04[a] | <0.1 | 0.2 ± 0.01[b] |
| 22:6n-3 | 16.4 ± 0.6 | 16.1 ± 0.8 | 16.0 ± 0.8 | 16.1 ± 0.8 | 16.4 ± 1.1 |

[a]Values are mean ± SD, $n = 6$ rats/group. In each row, values with different roman superscripts are significantly different, $P < 0.05$. For abbreviations see Table 1.

**TABLE 5**
**Free Fatty Acid Levels of Arachidonate and Docosahexaenoate in Plasma, Liver, and Brain of Rats on Different Formulations of the High-Fat Ketogenic Diet[a]**

| | | CONTROL | MCT | FSO | BUTTER | MIXTURE |
|---|---|---|---|---|---|---|
| Arachidonate | | | | | | |
| Liver | mg/g | 1.6 ± 0.4[a] | 2.4 ± 0.6[c] | 1.1 ± 0.2[b] | 1.7 ± 0.5[a] | 1.0 ± 0.5[b] |
| | % | 9.3 ± 0.7[a] | 11.6 ± 1.8[b] | 2.4 ± 0.4[c] | 6.5 ± 0.6[d] | 2.8 ± 0.6[c] |
| Plasma | g/mL | 2.6 ± 0.7[a,b] | 3.2 ± 0.9[a] | 2.1 ± 1.1[a,b] | 2.4 ± 0.5[a,b] | 1.8 ± 0.4[b] |
| | % | 5.4 ± 1.0[a] | 4.6 ± 0.8[a,c] | 1.9 ± 0.6[b] | 3.5 ± 0.5[c] | 2.2 ± 0.5[b] |
| Brain | g/g | 315 ± 33 | 340 ± 50 | 280 ± 48 | 303 ± 94 | 342 ± 30 |
| | % | 17.9 ± 0.7[a] | 18.5 ± 0.6[a] | 15.2 ± 1.1[b] | 18.1 ± 0.7[a] | 16.6 ± 0.7[c] |
| Docosahexaenoate | | | | | | |
| Liver | mg/g | 0.5 ± 1.0[a] | 0.8 ± 0.3[a] | 1.4 ± 0.3[b] | 0.8 ± 0.2[a] | 1.7 ± 0.4[b] |
| | % | 3.1 ± 0.7[a] | 3.8 ± 0.7[a] | 2.9 ± 0.6[a] | 3.1 ± 0.5[a] | 4.9 ± 0.3[b] |
| Plasma | g/mL | 0.4 ± 0.1[a,b] | 0.4 ± 0.1[a,b] | 0.8 ± 0.4[b] | 0.3 ± 0.1[a] | 0.9 ± 0.2[b] |
| | % | 0.5 ± 0.4[a] | 0.6 ± 0.3[a] | 0.7 ± 0.2[a,b] | 0.5 ± 0.1[a] | 1.1 ± 0.4[b] |
| Brain | g/g | 104 ± 20 | 115 ± 34 | 120 ± 29 | 107 ± 36 | 140 ± 26 |
| | % | 5.9 ± 0.9 | 6.2 ± 1.2 | 6.5 ± 0.9 | 6.4 ± 0.6 | 6.8 ± 1.2 |

[a]Values are mean ± SD, $n = 6$ rats/group. Values in each row with different roman letter superscripts are significantly different, $P < 0.05$. For abbreviations see Table 1.

## DISCUSSION

The higher ketosis in the MCT group (18) was probably due to the high proportion of medium-chain fatty acids (8:0, 10:0) in that diet. The higher level of plasma β-hydroxybutyrate in the MCT group was similar to that seen clinically but was no more protective against PTZ-induced seizures than the lower ketone levels seen in the FSO and BUTTER groups (18). Despite wide differences in the fatty acid profiles of the BUTTER, FSO, and MIXTURE diets, ketosis was similar in these groups, suggesting no clear role of individual dietary long-chain fatty acids in promoting ketosis. The lack of a clear relation between ketosis and seizure control in this model led us to seek other tissue lipid changes that might contribute to seizure protection.

We focused on possible changes in n-3 PUFA because of the anticonvulsant effects of both DHA and ALA in several different models (15,16,22). If levels of these n-3 PUFA in serum free fatty acids or in brain were elevated in the groups protected against the PTZ-induced seizures, we could conclude that at least part of the effect of ketosis could potentially be mediated by these fatty acid changes and not necessarily by ketosis itself. However, the present analysis does not clearly support a role for a specific fatty acid or dietary fat type in the mechanism by which ketogenic diets inhibit seizures because the MIXTURE and FSO groups consuming high amounts of ALA had higher n-3 PUFA in the tissues, but the MIXTURE group was not as well protected as the FSO group against the seizures induced by PTZ. Furthermore, the two most seizure-protected groups (BUTTER, FSO) had very few lipid or fatty acid changes in common that did not also occur in one or more other groups (Tables 2–5).

AA is an important fatty acid in membrane phospholipids, but excess free AA has a variety of excitatory and potentially neurotoxic effects (19,20) that could increase susceptibility to seizures. Our tissue fatty acid data do not support a proconvulsant effect of raised tissue levels of AA. This is because the MCT group, which had relatively high amounts of AA in adipose tissue, liver, and brain, had similar seizure incidence and scores to the rats on the other ketogenic diets. Hence, the proconvulsant effects of MCT-based ketogenic diets that are sometimes observed (9), especially when MCT oil is the predominant fat in the diet (23), do not appear to be directly related to increased tissue AA.

DHA protects against seizures (15), so we speculated that diets producing a high ratio of DHA to AA in blood or brain might be protective against seizures whereas a low DHA to AA ratio might promote seizures. Despite dramatic contrasts in ALA intake between the various groups in the present study, there was no significant difference in the percentage or concentration of DHA in brain total lipids or free fatty acids. At least in the free fatty acid fractions of plasma, liver, and brain, the DHA/AA ratio was not significantly correlated with seizure protection against PTZ (Table 5) (18). At the moment, therefore, any lipid- or fatty acid-related mechanism by which the ketogenic diet achieves seizure protection remains unclear. Our current lipid and fatty acid analyses of whole tissues are relatively crude and may need to become specific, focusing on particular brain regions or subcellular fractions.

The effect on adipose tissue composition of these ketogenic diets formulated with different fats was most interesting in the two groups given MCT oil (MCT and MIXTURE). In both these groups, the proportions of LA and ALA were much higher in adipose tissue than in the respective diets. This is unusual because proportions of LA and ALA in adipose tissue generally reflect dietary intake (24). However, on very high fat ketogenic diets containing MCT, it appears that the rat conserves LA and ALA or spares their release from adipose tissue more than usual. A possible explanation may be that the higher proportion of medium-chain fatty acids in the MCT diet effectively supports fat oxidation, thereby sparing PUFA such as LA and ALA that would normally be extensively β-oxidized (25). This apparent sparing of LA and ALA oxidation might help account for the higher adipose tissue AA and DHA in the MCT group.

Cholesterol levels in the rodent brain do not change even in the face of high saturated fat and cholesterol intake that would typically raise blood cholesterol by two- to threefold. Thus, as previously reported (26,27), exogenous cholesterol contributes little or nothing to brain cholesterol levels, probably because cholesterol crosses the blood-brain barrier poorly and because the brain can synthesize *de novo* all the cholesterol it requires (26). Interestingly, in the present study, the MCT, BUTTER, and MIXTURE groups had significantly higher brain cholesterol than that of the CONTROL and FSO groups ($P < 0.05$). These results illustrate that, although manipulation of dietary cholesterol itself does not affect brain cholesterol, a very high fat intake providing short- to medium-chain length fatty acids (6–14 carbons) can raise the level of cholesterol in the brain by 11–18%. This may be achieved by the long-term high level of blood ketones, which are important brain cholesterol precursors during early development (26).

One particular surprise in this study was the lack of significant change in plasma cholesterol, especially in the BUTTER group (Table 3). Weight gain was significantly reduced in all but one of the ketogenic diet groups, and this could potentially prevent an increase in plasma cholesterol. Our present data are consistent with the report that children with intractable epilepsy who are on the ketogenic diet have normal plasma cholesterol (28). However, adults on the ketogenic diet have the expected rise in plasma cholesterol associated with high saturated fat intake (3). The appropriateness of the rat as a model of human lipid metabolism while on a ketogenic diet therefore needs careful evaluation. We are aware of no reports involving humans on controlled experimental ketogenic diets, and there are few relevant reports describing lipid metabolism in animals on ketogenic diets. Rats are an important research tool for modeling seizure mechanisms and treatments, but it may be difficult to extrapolate between rats and humans. This caution applies not only to understanding the changes in cholesterol metabolism but also to the significant rise in liver triglycerides in the BUTTER and MIXTURE groups, changes that were not clearly obvious in plasma lipids (Table 3). If children on the ketogenic diet have such an elevation in liver triglycerides over 2–3 yr, compromised liver function would become a concern.

A ketogenic diet rich in n-3 PUFA may have indirect advantages for seizure control because, unlike dairy fat (29), n-3 PUFA do not raise serum lipids, even in a ketogenic diet (Table 3). In the present study, the FSO group had lower triglycerides in plasma and liver than those groups consuming butter (Table 3). The MCT group also had low plasma and liver triglycerides but, clinically, high intakes of MCT are commonly associated with gastrointestinal distress (2). FSO may therefore be an effective and acceptable alternative fat in the ketogenic diet. This has positive implications not only for children but also for adults who are looking to the ketogenic diet to control intractable seizures but who face serious dyslipidemia on the classical ketogenic diet (3).

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# Energy Value and Digestibility of Dietary Oil Containing Mainly 1,3-Diacylglycerol Are Similar to Those of Triacylglycerol

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**ABSTRACT:** Diacylglycerol (DAG) is a component of various vegetable oils. Approximately 70% of the DAG in edible oils are in the configuration of 1,3-DAG. We recently showed that long-term ingestion of dietary oil containing mainly 1,3-DAG reduces body fat accumulation in humans as compared to triacylglycerol (TAG) oil with a similar fatty acid composition. As the first step to elucidate the mechanism for this result, we examined the difference in the bioavailabilities of both oils by measuring food energy values and digestibilities in rats. Energy values of the DAG oil and the TAG oil, measured by bomb calorimeter, were 38.9 and 39.6 kJ/g, respectively. Apparent digestibility expressed according to the formula: (absorbed) × (ingested)$^{-1}$ × 100 = (ingested – excreted in feces) × (ingested)$^{-1}$ × 100 for the DAG oil and the TAG oil were 96.3 ± 0.4 and 96.3 ± 0.3% (mean ± SEM), respectively. The similarity in the bioavailabilities of both oils supports the hypothesis that the reduced fat accumulation by dietary DAG is caused by the different metabolic fates after the absorption into the gastrointestinal epithelial cells.

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Food energy values are calculated by application of Atwater conversion factors for carbohydrates, fats, and proteins or, alternatively, are determined by measurements using a bomb calorimeter (1). Fats and oils provide approximately 38 kJ/g of metabolizable energy compared to 17 kJ/g for proteins and carbohydrates (2,3). These energy availability coefficients have been approved and widely used to calculate the energy intake from various foodstuffs. Of course, these coefficients are generalized, and the precise energy of an individual food may vary depending on its composition and structure. These values are effective for most occasions except for specific experiments, therapeutics, and special diet programs where accurate caloric values are required. Research into the prevention of obesity would be a circumstances that would require precise determination of the food energy values.

Most edible oils contain diacylglycerol (DAG) as a minor constituent. Although the isoform of DAG that occurs in the process of triacylglycerol (TAG) digestion by lingual or pancreatic lipase is 1,2- or 2,3-DAG, a substantial fraction of DAG in edible oils has been converted to 1,3-DAG by acyl migration (4). We previously reported that the rate of lymphatic transport of TAG as chylomicron was significantly retarded in rats that had been intragastrically infused with DAG oil emulsion as compared to TAG oil emulsion (5). In the digestive tract, 1,3-DAG is hydrolyzed to glycerol and fatty acids (FA) through 1(3)-monoacylglycerol (MAG) (6) while TAG is hydrolyzed to 2-MAG and FA. Based on these results, we hypothesized that the limited availability of 2-MAG for re-esterification retards chylomicron-TAG transport in the rat infused with DAG oil emulsion. We recently reported that long-term ingestion of dietary DAG oil, in contrast to the TAG oil, reduces body fat accumulation in humans (7). We speculated that these effects are related to the different metabolic fates of the lipids caused by the structural differences of 1,3-DAG and TAG rather than a difference in food energy values between the oils. However, the difference in the energy value and digestibility between DAG and TAG has not been measured precisely.

Fats are digested in three different and coordinated processes: emulsification, hydrolysis of the substrate, and micellar solubilization of the hydrolysis products in the aqueous medium of the intestinal contents (8). Many determinants are therefore involved in these steps. It has been shown that intestinal fat absorption is influenced by TAG structures (9), FA chain length (10), the unsaturated/saturated FA ratio (10,11), the esterification form of FA (12), and the gastrointestinal flora (13–15).

The purpose of the present study was (i) to compare the energy values of the DAG and TAG oils by calculations and by bomb calorimetry, and (ii) to compare the absorption coefficients or digestibilities of the DAG and TAG oils to assess the contribution of the energy and digestibility differences to the functional differences observed in these dietary oils.

## EXPERIMENTAL PROCEDURES

*Fats.* The DAG oil was prepared by esterifying glycerol with FA from rapeseed oil by the method of Huge-Jensen *et al.* (16). The TAG oil was prepared by mixing rapeseed oil, soybean oil, and safflower oil so that the FA composition became similar to that of the DAG oil. All of these oils were obtained from Nissin Oil Mills Ltd. (Tokyo, Japan). Purified TAG and DAG were purchased from Sigma-Aldrich Japan (Tokyo, Japan). FA composition and acylglycerol composition were analyzed by gas chromatography. The measurements were performed by Japan Food Analysis Center (Tokyo, Japan).

*Calculation of available energy of dietary fats.* The FA composition and acylglycerol composition of each oil are shown in Table 1. The FA composition of the DAG oil was very similar to that of the blended TAG oil. The DAG concentration of the DAG oil was 87.0/100 g and the ratio of 1(3),2- to 1,3-DAG was 32:68. The amount of 1,3-isoform in equilibrium is intrinsic to the FA in the molecule (4). Using

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Abbreviations: DAG, diacylglycerol; FA, fatty acid; MAG, monoacylglycerol; TAG, triacylglycerol.

**TABLE 1**
**Fatty Acid and Acylglycerol Compositions of Diacylglycerol and Triacylglycerol Oil Used in the Study**

| Component | Diacylglycerol oil | Triacylglycerol oil |
|---|---|---|
| Fatty acids | g/100 g total fatty acids | |
| 16:0 | 2.4 | 6.0 |
| 18:0 | 0.7 | 2.2 |
| 18:1 | 28.0 | 29.1 |
| 18:2 | 60.3 | 57.8 |
| 18:3 | 5.6 | 2.5 |
| 20:0 | <0.05 | 0.4 |
| 20:1 | 0.2 | 0.6 |
| 22:0 | <0.05 | 0.3 |
| 22:1 | <0.05 | 0.2 |
| 24:1 | <0.05 | 0.2 |
| Others | 2.8 | 0.7 |
| Acylglycerols | g/100 g oil | |
| Triacylglycerol | 10.7 | 97.2 |
| Diacylglycerol | 87.0 | 1.1 |
| 1(3), 2-diacylglycerol | 27.8 | ND[a] |
| 1,3-diacylglycerol | 59.2 | ND |
| Monoacylglycerol | 0.82 | <0.05 |
| Free fatty acid | ND | ND |

[a]ND, not determined.

the approach outlined in the literature (17,18), we calculated the heat energy values of the DAG and the TAG oils.

The energy values for the FA were estimated by calculation using the equation

$$-\Delta H_c = 0.653n - 0.166d - 0.421 \quad [1]$$

where $-\Delta H_c$ is the heat of combustion in MJ/mol, $n$ is the number of carbon atoms/molecule of FA, and $d$ is the number of double bonds/FA, as described by Livesey (17). The energy value that was used for glycerol was 18.0 kJ/g (18). The heat of esterification was neglected because it amounts to only 3.8 kJ per mole of ester bond as determined with methyl stearate (19). In case of tristearin, this value corresponds to 13 J/g. A normalized percentage was used for the unknown components in the analysis of FA composition and acylglycerol composition.

*Energy measurements of the test oils.* The potential energies (combustion energies) of test oils of known acylglycerol and FA compositions were measured by a bomb calorimeter. Two measurements were carried out for each sample, and the averages were presented. The measurements were performed by Japan Food Analysis Center.

*Animals and experimental design.* Sixteen male Sprague-Dawley rats of 5 wk of age, obtained from CLEA Japan (Tokyo, Japan), were housed in metal cages and had free access to the TAG oil diet and drinking water. They were maintained in a temperature-controlled environment (22 ± 1°C) on a 12-h light/dark cycle. After an acclimatization period of 5 d, they were divided into two groups of eight, so that the body weight of each group became approximately equal, and transferred to the individual metabolic cages. Rats in one group were fed the DAG oil diet, and the others were fed the TAG oil diet (DAG group and TAG group). Food intake of all rats was recorded every day. Body weights of all rats were recorded every 3 or 4 d. Feces were collected and pooled from the last three study days, 13–15. All measurements and fecal collection were performed at the same time during the light cycle throughout the experiment. The digestibility coefficient is defined as the percentage of ingested fat that was not excreted in the feces (11,20,21). We calculated the value from the lipid analysis of the diet and 3-d fecal excretion. The study was approved by the Ethical Committee for the Experimental Animals of Kao Corporation.

*Diets.* The test diet contained 20 g/100 g of either DAG or TAG oil. The ingredients other than test oils were casein (20 g/100 g), cellulose (4 g/100 g), AIN-76 (22) mineral mixture (3.5 g/100 g), AIN-76 (22) vitamin mixture (1 g/100 g), and potato starch (51.5 g/100 g). Each diet was prepared in one batch for the entire experimental period and was stored at –22°C. The rats were provided with tap water *ad libitum*.

*Extraction of total fat from the diets and feces.* Collected feces were stored frozen and immediately freeze-dried as soon as the last specimen had been collected and frozen. The freeze-dried feces were ground with a mortar and pestle into a homogeneous mixture. Fecal lipids were extracted from the homogeneous mixture of freeze-dried feces using a modification of the method of Folch *et al*. (23). Dietary lipids were extracted by the same method as the feces extraction. The amount of extractable lipid was determined gravimetrically.

*Analysis of lipids by gas chromatography.* The lipid contents of the extracted total lipid were analyzed by gas chromatography. The lipid was silylated with trimethylsilylimidazole (GL Science, Tokyo, Japan) by the method of Sahasrabudhe and Legari (24). The trimethylsilyl ethers dissolved in chloroform were separated on a GC-18A gas chromatograph (Shimadzu, Kyoto, Japan) connected to a flame-ionization detector and fitted with a DB-1 capillary column (15 m × 0.25 mm × 0.1 μm; J&W Scientific, Folsom, CA). Operating conditions were: initial column temperature, 80°C; initial time, 3 min; rate of temperature increase, 10°C/min; final temperature, 335°C (held for 44.5 min); injector and detector temperature, 350°C; carrier gas, helium at 1.78 mL/min. Data interpretation was carried out by GC work station CLASS-GC 10 (Shimadzu, Kyoto, Japan) programmed for peak identification. The instrument was calibrated with a standard mixture of free FA, MAG, DAG, and TAG.

*Statistical analysis.* Data were expressed as means ± SEM. Statistical significance of the difference ($P < 0.05$) between the groups were determined by Student's *t*-test (two-tailed). The statistical calculations were performed with Stat View for Windows version 4.58 (Abacus Concepts. Inc., Berkley, CA).

## RESULTS AND DISCUSSION

*Heat energy values of test oils.* Since the FA compositions of the test oils were very similar (Table 1), the average molecular weights of the esterified FA in both oils were the same: 280.4 as free acids. The average molecular weights of TAG, DAG, and MAG in both oils were 879.2, 616.8, and 354.5, respectively. The theoretical energy values were calculated from the amount of FA and glycerol released from 1 g of test

oil and from the energy value for each FA (Table 2). The calculated energy values and those determined by bomb calorimeter for the DAG oil and the TAG oil are shown in Table 2. The energy values of distearin, tristearin, dicaprin, and tricaprin were also calculated and determined by bomb calorimeter as control fats. The energy values of distearin and tristearin determined by bomb calorimeter were 39.0 and 40.1 kJ/g, respectively, and the calculated values were 38.9 and 40.0 kJ/g, respectively. The energy values of dicaprin and tricaprin determined by bomb calorimeter were 34.7 and 35.8 kJ/g, respectively, and the calculated values were 34.7 and 36.0 kJ/g, respectively. The combustion energies measured by bomb calorimeter were in good agreement with the calculated values. The energy value of the DAG oil was approximately 98% of the TAG oil. Since the animal diet used in the present study contained 38.6 energy% fat, this caloric difference (2%) between the oils will produce a difference of only 0.8% in the energy value of the diet.

*Food consumption and body weight.* No significant difference between the rats fed different diets with respect to food consumption was seen. The body weight at the start of the experiment (day 1) did not differ statistically between the groups: 173.9 ± 2.1 and 174.5 ± 2.2 g (±SEM) for the TAG group and the DAG group, respectively. At the end of the experiment (day 15), the body weights did not differ significantly between the groups: 289.1 ± 4.4 and 283.8 ± 3.9 g (±SEM) for the TAG group and the DAG group, respectively.

The effect of different energy consumption on the growth or body weight has been studied quantitatively in the growth method for estimating the caloric availabilities of fats in the rat (25). This method compares the weight gain of rapidly growing rats receiving the test compound to the weight gain of animals receiving a substance of known caloric availability. The minimum energy added to create a standard curve was 209 kJ/100 g diet in this experiment. The 0.8% difference of the caloric value between the DAG diet and the TAG diet corresponds to 15 kJ/100 g. This difference is far less than the calories required to detect any differences in the growth of the rat at the maximum sensitivity. In the human trial (7), the contribution of the caloric difference to the results was much less (<0.1%) since the amount of the test oil substituted was only 10 g out of 42 g of total fat consumed daily. Our results in conjunction with the previous study suggest that the caloric difference between DAG and TAG is negligible.

Since the animal experiment in the present study was focused on the determination of the fat absorption coefficient, we adopted a typical experimental design in which rats were fed test foods for 2 wk. Although it was difficult to detect a difference in body fat accumulation or body weight gain between the treatment groups within 2 wk, the effect of substituting DAG for TAG on body fat accumulation in rats became significant by expanding the treatment period to 3 to 4 wk (6). Furthermore, Murase *et al*. (26) recently reported that substituting DAG for TAG prevented the increase in body fat associated with a high-fat and sucrose diet in obesity- and diabetes-prone C57BL/6J mice after 5 mon of *ad libitum* feeding. These results confirm a differential effect of DAG and TAG on body fat accumulation in rodents and thus replicate the data in humans.

*Fecal analysis.* The overall movements of fats in the rat during the last 3 d of the experiment are shown in Table 3. No significant differences between the diet groups were seen for all variables: food intake, fat intake, dried feces mass, fat excretion, fat content of dry feces, and fat absorption coefficients. We next examined the composition of the fecal lipids, which may be influenced by the composition of the diet, digestibility, and the intestinal microflora. To see the effect of

**TABLE 2**
**Composition of Experimental Oils, Energy Values of Each Component, and Comparison of the Theoretical and Experimental Energy Value of Each Oil**

| | | Diacylglycerol oil | | Triacylglycerol oil | |
|---|---|---|---|---|---|
| Fatty acid | Gross energy (kJ/g ) | Weight fraction (g/g oil) | Contribution to energy value (kJ/g oil) | Weight fraction (g/g oil) | Contribution to energy value (kJ/g oil) |
| 16:0 | 39.1 | 0.023 | 0.9 | 0.058 | 2.3 |
| 18:0 | 39.9 | 0.007 | 0.3 | 0.021 | 0.8 |
| 18:1 | 39.5 | 0.263 | 10.4 | 0.280 | 11.1 |
| 18:2 | 39.3 | 0.567 | 22.2 | 0.557 | 21.8 |
| 18:3 | 38.9 | 0.053 | 2.0 | 0.024 | 0.9 |
| 20:0 | 40.5 | —[a] | 0.0 | 0.004 | 0.2 |
| 20:1 | 40.2 | 0.002 | 0.1 | 0.006 | 0.2 |
| 22:0 | 41.0 | — | 0.0 | 0.003 | 0.1 |
| 22:1 | 40.7 | — | 0.0 | 0.002 | 0.1 |
| 24:1 | 40.9 | — | 0.0 | 0.002 | 0.1 |
| Fatty acids | | 0.913 | 35.9 | 0.956 | 37.6 |
| Glycerol | 18.0 | 0.145 | 2.6 | 0.105 | 1.9 |
| Total | | 1.000 | 38.5 | 1.000 | 39.5 |
| Experimental | | | 38.9 | | 39.6 |

[a]Below detection limit (<0.0005), regarded as zero.

**TABLE 3**
**Fat Intake and Excretion in Rats Fed Either Diacylglycerol or Triacylglycerol Diet for 3 d**

| Parameters | Diacylglycerol group | Triacylglycerol group |
|---|---|---|
| | g/3 d/rat | |
| Diet intake | 16.67 ± 0.23 | 16.71 ± 0.19 |
| Fat intake | 9.67 ± 0.24 | 9.67 ± 0.33 |
| Dried feces mass | 3.15 ± 0.17 | 3.35 ± 0.14 |
| Fat excretion | 0.354 ± 0.043 | 0.352 ± 0.022 |
| | g/100 g | |
| Fat contents of dry feces | 10.49 ± 0.38 | 11.18 ± 1.03 |
| | % | |
| Fat absorption coefficient | 96.3 ± 0.42 | 96.3 ± 0.26 |

dietary DAG on the lipid composition in the feces, we analyzed the fecal lipid profile by gas chromatography. No significant differences between the diet groups were observed in the pattern of the fecal lipids (data not shown).

The rat is the animal model most frequently used to predict digestibility of various food components. Bach Knudsen *et al*. (27) demonstrated that the digestibility values for protein, energy, and fat were very similar in rats and humans. Wisker *et al*. (28) also reported that digestibility of energy, protein, fat, and nonstarch polysaccharides in a low-fiber diet and in diets containing coarse or fine whole-meal rye are comparable in rats and humans. It should therefore be reasonable to predict that the digestibility of the DAG oil is similar to that of the TAG oil in humans from the results obtained in the present study. In light of these findings, the physiological differences between DAG and TAG observed in rats and humans are caused by the different metabolic fates after the absorption into the gastrointestinal epithelial cells.

These results encourage further studies on the mechanism of the function of dietary DAG in the reduction of body fat accumulation in humans.

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# Nonalcoholic Components in Wine Reduce Low Density Lipoprotein Cholesterol in Normocholesterolemic Rats

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**ABSTRACT:** Using an experimental model that enables the effects of alcohol to be distinguished from the effects of the nonalcoholic components present in wine, we determined whether wine has effects other than those of alcohol on the metabolism of cholesterol. Male rats were fed a standard diet and had free access to water and either wine or an equivalent alcohol solution for 45 d or 6 mon. Alcohol intake was similar in the two groups of animals. Consumption of the alcohol solution or wine did not influence plasma cholesterol or high density lipoprotein-cholesterol. At 45 d, the consumption both of wine and of alcohol solution reduced low density lipoprotein (LDL)-cholesterol and very low density lipoprotein cholesterol. At 6 mon, only the rats that consumed wine had reduced LDL-cholesterol. After 45 d of consuming alcohol solution, total cholesterol in the aorta was significantly increased mainly as a result of the rise in free cholesterol. In the aorta, the effect of wine consumption was similar to the effect of alcohol solution consumption, although it was less intense. The only clear effect that could be ascribed to the nonalcoholic components in wine was that the LDL-cholesterol was reduced in the long term, although aortic cholesterol was not.

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Considerable evidence suggests that small amounts of alcoholic beverages reduce the risk of vascular disease by about a third and reduce total mortality in middle and old age (1,2). But whether all alcoholic beverages protect to the same degree is not so clear because, as well as alcohol, they contain other characteristic compounds. The French Paradox (3) suggests that wine is more protective than other alcoholic beverages, but there is no agreement as to why this is so. Some authors consider that the benefit is directly due to the alcohol content and the extra benefit attributed to wine is due to drinking and diet patterns (1,4,5); others consider that the phenolic compounds in wine play a crucial role in cardioprotection (6, 7).

Phenolic compounds, particularly flavonoids, have been described as having considerable biological activity (8,9). Red wine is particularly rich in flavonoids and contains more than 100 different phenols, of which anthocyanins (0.2–0.8 g/L) and catechins as monomer and oligomer (1–3 g/L) are the two major classes (10). Oligomeric catechins from grape seed have an antiatherosclerotic effect in cholesterol-fed rabbits (11), and red wine reduces the progression of atherosclerosis in apolipoprotein E-deficient mice (12). This beneficial effect may be associated with the better resistance of low density lipoprotein (LDL) to oxidation and the changes in cholesterol metabolism. Although many studies show the antioxidant capacity of wine (13) *in vivo* in both humans and animals, there are fewer about the effects of wine on cholesterol metabolism, particularly in normocholesterolemic conditions. In using rats fed on a high-cholesterol diet, grape seed catechins were shown to have a pronounced antihypercholesterolemic effect because they enhance reverse cholesterol transport, reduce intestinal cholesterol absorption, and increase bile acid secretion (14,15). It has been suggested that free radicals are atherogenic not only because they modify LDL but also because they act on the cellular cholesterol metabolism (16,17). Flavonoids may affect cholesterol homeostasis because of their direct action on cellular cholesterol metabolism or because of their antioxidant power.

To determine whether wine really does have properties that are in addition to or different from those of alcohol, we developed an experimental model (18) that discriminates the effects of alcohol from the effects of the nonalcoholic components present in wine in healthy rats. Using this model, we determined whether wine has effects on cholesterol metabolism in normocholesterolemic rats that alcohol does not.

## MATERIALS AND METHODS

All experiments involving animals were approved by the ethics committee of the Universitat Rovirai Virgili. Male Wistar rats were purchased from IFFA-CREDO (Barcelona, Spain). They were bred at the Animal Service of the University under controlled conditions of light (12 h on/12 h off), humidity (70–80%), and temperature (20–22°C). The animals (weighing about 125 g) were individually housed in metabolic cages and were then divided into three groups of 14 animals each. One group of animals (Control Group = C) had free access to a standard diet (A04, Panlab S.L., Barcelona, Spain) and water. The remaining two groups had free access to a standard diet, water, and wine or an equivalent ethanol solution. Of these two groups, one consumed red table wine (Appellation Contrôlée Priorat, Spain) with an ethanol concentration of 13.5% and 2.64 g/L of overall phenolic compounds (Red Wine group = RW). The second group con-

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Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

sumed an aqueous ethanol solution (13.5% ethanol solution) equivalent to the red wine (Alcohol Solution group = AS). Both the wine and alcoholic solution were given to the rats in a glass feeding bottle (especially designed to avoid wine losses) that was separate from the water bottle, and they were allowed to consume as much as they desired (18). Spillage was also discounted as a possible source of wine loss because wine stains are easily observable and no such evidence was found when the cages were cleaned. Feces of 24 h were collected at day 1, day 44, and day 179 of treatment and stored at −80ºC until cholesterol and bile acids were determined. Seven nonfasted animals from each group were beheaded after 45 d and the other seven after 6 mon. Blood, with EDTA as anticoagulant, was centrifuged at 1500 × *g* for 5 min to obtain plasma. The liver, heart, and aorta were removed immediately, frozen with liquid $N_2$, and stored at −80ºC until they were used.

Lipoprotein was separated from fresh plasma using KBr (19,20) in a Beckman L8-70M centrifuge. Each fraction was dialyzed with phosphate-buffered saline for 24 h immediately after it had been obtained. Triglycerides (21) and cholesterol (22) in plasma and lipoproteins were measured by spectrophotometric-enzymatic methods. Total lipid content in the liver was determined by the method of Folch *et al.* (23).

The feces, liver, heart, and aorta were homogenized first with 9% NaCl and then with chloroform/methanol (2:1, vol/vol) to determine total cholesterol, free cholesterol, and cholesteryl esters. The homogenates were shaken for 24 h at 4°C. The organic fraction was separated and divided into two fractions: total cholesterol was determined in one and free cholesterol in the other. The fractions were evaporated under a stream of $N_2$ gas. For total cholesterol to be determined, the samples were hydrolyzed with 33% KOH, and cholesterol was extracted with hexane and evaporated with $N_2$ gas. All samples were dissolved in isopropanol, and cholesterol levels were measured using cholesterol oxidase. Cholesteryl ester values were calculated as total cholesterol minus free cholesterol from each animal. After they had been extracted with methanol from feces, bile acids were determined by the method described by de Wael *et al.* (24).

Analysis of variance was used to evaluate the effect of wine and alcohol. The statistical comparisons were made between the three groups (control, wine, and alcohol solution) of the same period (45 d or 6 mon). After a significant *F* test ($P < 0.05$), the Scheffé test was used to determine differences between group means. Although we studied consumption over two periods of time, each group was compared only with the other groups of the same period, because the aim of the analysis was to compare the effect of alcohol or wine consumption over short or long periods of time, not to compare the effect of age of the animals.

## RESULTS

Wine consumption was 1.9 ± 0.2 mL wine/d/rat. Alcoholic solution consumption was 1.7 ± 0.2 mL/d/rat, similar to that of the wine it was simulating. The daily alcohol consumption—as wine or alcohol solution—by rats was 0.2 mL alcohol/rat (0.6–0.4 mL alcohol/kg body weight). The percentage of daily energy supplied by alcohol was 1.5% in the two groups.

In the control animals, the values of the parameters studied were similar in the two periods studied, except for total cholesterol in the plasma and the aorta. The fact that total cholesterol increases in the plasma with age is in agreement with the results of other authors (25). The changes in total cholesterol in the aorta may be due to changes in the diameter of the artery and in the characteristics of the arterial wall during aging (26).

Figure 1 shows the levels of plasma cholesterol and lipoprotein cholesterol after 45 d and 6 mon of wine or alcohol consumption. There was no effect on plasma cholesterol and high density lipoprotein (HDL)-cholesterol. At 45 d, both wine and

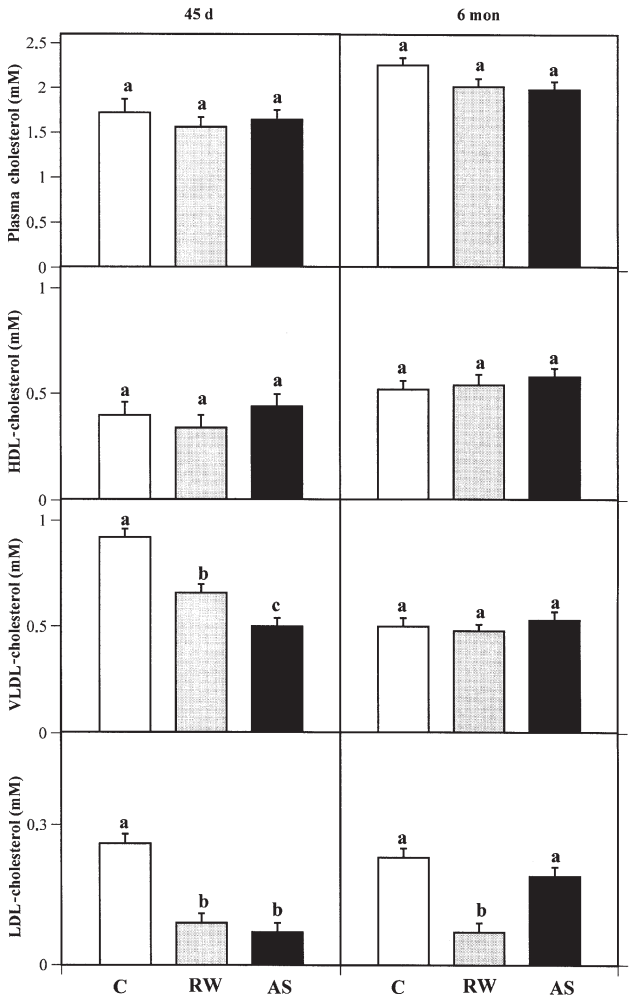


**FIG. 1.** Plasma cholesterol, HDL-cholesterol, VLDL-cholesterol and LDL-cholesterol in control rats (C) and in rats that consumed wine (RW) or an equivalent alcoholic solution (AS) for 45 d (left) or 6 mon (right). Each bar is the mean ± SEM of seven animals. Statistical analysis was performed by analysis of variance and the Scheffé test. The statistical comparisons were made between the three groups (control, wine, and alcohol solution) of the same period (45 d or 6 mon). Different superscripts indicate a significant difference between groups ($P < 0.05$). HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein.

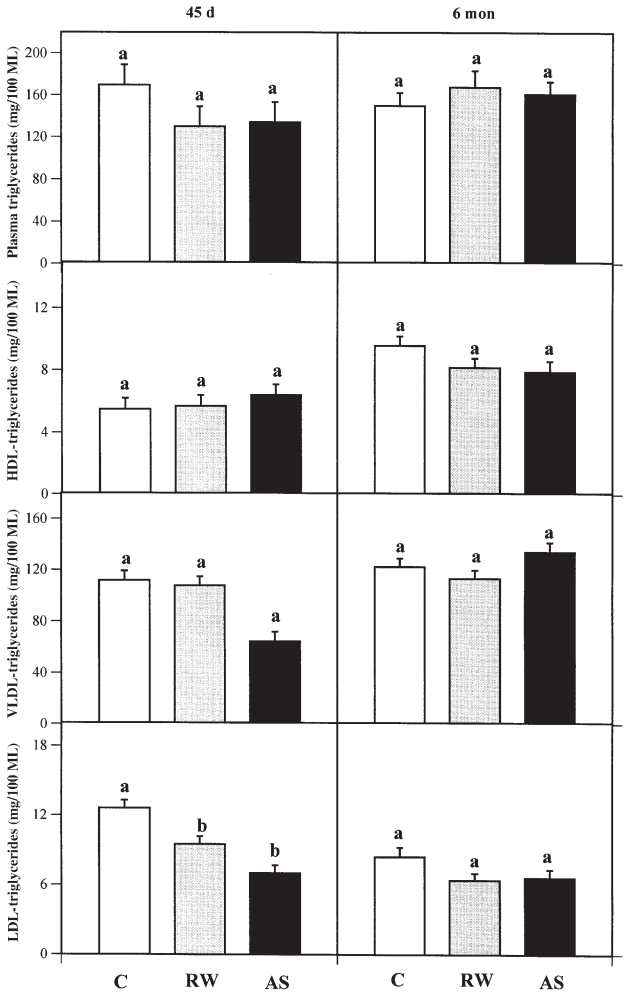


**FIG. 2.** Plasma triglycerides, HDL-triglycerides, VLDL-triglycerides and LDL-triglycerides in control rats (C) and in rats that consumed red wine (RW) or an equivalent alcoholic solution (AS) for 45 d (left) or 6 mon (right). Each bar is the mean ± SEM of 7 animals. Statistical analysis was performed by analysis of variance and the Scheffé test. The statistical comparisons were made between the three groups (control, wine, and alcohol solution) of the same period (45 d or 6 mon). Different superscripts indicate a significant difference between groups ($P < 0.05$). For abbreviations see Figure 1.

alcohol reduced LDL-cholesterol and very low density lipoprotein (VLDL)-cholesterol. At 6 mon, only the rats that consumed wine had reduced LDL-cholesterol.

Figure 2 shows the levels of plasma triglyceride and lipoprotein triglyceride of nonfasted animals. These levels were only slightly affected by wine and alcohol (Fig. 2) and only LDL-triglyceride was reduced in the rats that consumed wine or alcohol solution for 45 d.

The lipid content in the liver was similar in the controls and in the rats that consumed wine or alcohol (about 3 g lipids/100 g liver). Total cholesterol, free cholesterol, and cholesteryl esters in the liver (Table 1) were not affected by wine or alcohol consumption. In the control group, free cholesterol and cholesteryl esters were 70 and 30% of total liver cholesterol, respectively. This distribution was similar in the livers of the rats that consumed wine or alcohol (70–80% for free cholesterol and 20–30% for cholesteryl esters).

Table 2 shows the levels of total cholesterol, free cholesterol, and cholesteryl esters in the heart of control rats and rats that consumed wine or alcohol. Total cholesterol in the heart was not affected by wine or alcohol consumption, but cholesterol distribution—as free cholesterol and cholesteryl esters—was modified in rats that consumed wine. In the control group, free cholesterol and cholesteryl esters were 80 and 20% of the total cholesterol, respectively. At 45 d, however, free cholesterol was significantly reduced (60%) in the hearts of animals that consumed wine; at 6 mon, the distribution was similar to that of control animals. In the rats that consumed alcohol, free cholesterol and cholesteryl esters were 70 and 30% of total cholesterol, respectively, in the two periods studied.

Cholesterol content in the aorta was higher in rats that consumed alcohol (Table 3). After 45 d of alcohol consumption, total cholesterol in the aorta was significantly higher; alcohol also had this effect in the long term, but it was not significant. The effect of wine consumption was similar to the effect of alcohol consumption, although it was less intense.

Cholesterol excretion in the feces (Table 4) was not affected by alcohol or wine consumption. At 45 d, bile acid excretion in feces was lower in rats that consumed wine.

## DISCUSSION

Research has shown that the incidence of heart disease is lower in countries where wine is consumed on a moderate and regular basis than in countries where other types of alcohol are more commonly consumed (3). It has been suggested that moderate consumption of wine has positive effects because it

**TABLE 1**
**Total Cholesterol, Free Cholesterol, and Cholesteryl Esters in the Liver of Control Rats and Rats That Consumed Wine or an Equivalent Alcoholic Solution for 45 d or 6 mon[a]**

| | 45 d | | | 6 mon | | |
|---|---|---|---|---|---|---|
| | Total cholesterol (mg/g liver) | Free cholesterol (mg/g liver) | Cholesteryl esters (mg/g liver) | Total cholesterol (mg/g liver) | Free cholesterol (mg/g liver) | Cholesteryl esters (mg/g liver) |
| Control | 1.97 ± 0.17 | 1.27 ± 0.16 | 0.60 ± 0.13 | 2.22 ± 0.19 | 1.60 ± 0.18 | 0.62 ± 0.15 |
| Wine | 1.83 ± 0.14 | 1.49 ± 0.14 | 0.32 ± 0.11 | 2.20 ± 0.15 | 1.60 ± 0.15 | 0.63 ± 0.12 |
| Alcohol | 2.17 ± 0.15 | 1.40 ± 0.15 | 0.67 ± 0.12 | 1.82 ± 0.19 | 1.50 ± 0.18 | 0.33 ± 0.15 |

[a]Each value is the mean ± SEM of six or seven animals. Statistical analysis was performed using analysis of variance and the Scheffé test. The statistical comparisons were made between the three groups (control, wine, and alcohol solution) for the same period (45 d or 6 mon). There were no significant differences between groups.

**TABLE 2**
**Total Cholesterol, Free Cholesterol, and Cholesteryl Esters in the Hearts of Control Rats and Rats That Consumed Wine or an Equivalent Alcoholic Solution for 45 d or 6 mon[a]**

| | 45 d | | | 6 mon | | |
|---|---|---|---|---|---|---|
| | Total cholesterol (mg/g liver) | Free cholesterol (mg/g liver) | Cholesteryl esters (mg/g liver) | Total cholesterol (mg/g liver) | Free cholesterol (mg/g liver) | Cholesteryl esters (mg/g liver) |
| Control | 1.24 ± 0.05[a] | 0.98 ± 0.07[a] | 0.24 ± 0.06[a] | 1.10 ± 0.06[a] | 0.93 ± 0.09[a] | 0.16 ± 0.08[a] |
| Wine | 1.18 ± 0.04[a] | 0.71 ± 0.06[b] | 0.46 ± 0.05[a] | 1.03 ± 0.07[a] | 0.83 ± 0.10[a] | 0.17 ± 0.09[a] |
| Alcohol | 1.12 ± 0.06[a] | 0.78 ± 0.98[a,b] | 0.31 ± 0.07[a] | 1.06 ± 0.07[a] | 0.70 ± 0.10[a] | 0.36 ± 0.09[a] |

[a]Each value is the mean ± SEM of six or seven animals. Statistical analysis was performed using analysis of variance and the Scheffé test. The statistical comparisons were made between the three groups (control, wine, and alcohol solution) for the same period (45 d or 6 mon). Different superscripts indicate a significant difference between groups ($P < 0.05$).

**TABLE 3**
**Total Cholesterol, Free Cholesterol, and Cholesteryl Esters in the Aortas of Control Rats and Rats That Consumed Wine or an Equivalent Alcoholic Solution for 45 d or 6 mon[a]**

| | 45 d | | | 6 mon | | |
|---|---|---|---|---|---|---|
| | Total cholesterol (mg/g liver) | Free cholesterol (mg/g liver) | Cholesteryl esters (mg/g liver) | Total cholesterol (mg/g liver) | Free cholesterol (mg/g liver) | Cholesteryl esters (mg/g liver) |
| Control | 2.58 ± 0.45[a] | 1.70 ± 0.43[a] | 0.89 ± 0.36[a] | 1.11 ± 0.56[a] | 0.53 ± 0.05[a] | 0.59 ± 0.23[a] |
| Wine | 3.66 ± 0.39[a,b] | 2.80 ± 0.40[a] | 0.86 ± 0.31[a] | 2.05 ± 0.40[a] | 1.23 ± 0.38[a] | 0.82 ± 0.11[a] |
| Alcohol | 4.30 ± 0.32[b] | 2.84 ± 0.31[a] | 1.46 ± 0.25[a] | 2.36 ± 0.39[a] | 1.79 ± 0.38[a] | 0.57 ± 0.11[a] |

[a]Each value is the mean ± SEM of six or seven animals. Statistical analysis was performed using analysis of variance and the Scheffé test. The statistical comparisons were made between the three groups (control, wine, and alcohol solution) for the same period (45 d or 6 mon). Different superscripts indicate a significant difference between groups ($P < 0.05$).

contains not only alcohol but also phenolic compounds, in particular, flavonoids. To determine whether the nonalcoholic component of wine can modify cholesterol homeostasis, we used a healthy animal model that distinguishes the effects of alcohol from the effects of other components (18). There are differences in the lipoprotein metabolism among species; rats lack the cholesteryl ester transfer protein (25) and, unlike humans, are resistant to developing atherosclerosis due to high-cholesterol feeding, but it is not unusual to use the rat to study compounds, particularly flavonoids, that affect cholesterol metabolism (26,27).

The wine used in this study is from the Appellation Contrôlée Priorat (Spain). It is a young, tannic red wine with a relatively high alcohol content, which is commonly consumed with meals in our country and which, therefore, is representative of what could be called the Mediterranean consumption. The rats consumed wine regularly and in moderate quantities (1.9 ± 0.2 mL wine/d/rat) consumed. The amounts of alcohol solution drunk were similar to the amounts of wine (1.7 ± 0.2 mL alcohol solution/d/rat). The concentration of alcohol in the blood was below the limits of detection by the usual methods, so the experiment is not a study of alcohol abuse.

Lipoprotein metabolism plays a significant role in the pathogenesis of atherosclerosis and the risk of vascular disease: high levels of LDL and low levels of HDL are linked to the risk of cardiovascular disease. Although one of the effects of moderate drinking includes increased plasma HDL-cholesterol (28), these levels were not modified in the rats that consumed wine or alcohol solution. On the other hand, LDL-cholesterol was dramatically decreased by wine consumption. In the short term, it seems that the LDL-cholesterol levels are affected by the alcohol content of wine because after 45 d, the rats that consumed wine and the rats that consumed alcohol solution had lower LDL-cholesterol levels. This reduction in

**TABLE 4**
**Cholesterol and Bile Acids in the Feces of Control Rats and Rats Which Consumed Wine or an Equivalent Alcoholic Solution for 1 d, 45 d, or 6 mon[a]**

| | Cholesterol (mmol/d) | | | Bile acids (μmol/d) | | |
|---|---|---|---|---|---|---|
| | 1 d | 45 d | 6 mon | 1 d | 45 d | 6 mon |
| Control | 30.9 ± 3.4[a] | 51.9 ± 3.7[a] | 33.4 ± 4.3[a] | 27.0 ± 3.1[a] | 25.2 ± 3.6[a] | 22.4 ± 3.2[a] |
| Wine | 32.5 ± 3.2[a] | 44.2 ± 4.0[a] | 30.4 ± 4.0[a] | 21.2 ± 2.4[a] | 10.3 ± 2.9[b] | 22.0 ± 3.6[a] |
| Alcohol | 27.4 ± 3.1[a] | 50.5 ± 3.7[a] | 35.2 ± 3.7[a] | 20.4 ± 2.1[a] | 26.2 ± 2.9[a] | 19.8 ± 4.11[a] |

[a]Each value is the mean ± SEM of six or seven animals. Statistical analysis was performed by analysis of variance and the Scheffé test. The statistical comparisons were made between the three groups (control, wine, and alcohol solution) of the same period (1 d, 45 d, or 6 mon after treatment). Different superscripts indicate a significant difference between groups ($P < 0.05$).

LDL-cholesterol coincided with a reduction in VLDL-cholesterol, so it might be caused by a minor hepatic VLDL-cholesterol secretion induced by alcohol consumption. After 6 mon, only the rats that consumed wine had lower LDL-cholesterol levels, suggesting that it is the nonalcoholic components of wine that have this effect in the long term. This reduction was not the result of decreased VLDL levels, so it seems that the nonalcoholic components of wine increase the removal of LDL from blood. Rajendran *et al.* (29) showed that tincture of Crataegus, of which flavonoids are one of the major components, enhances the LDL-receptor activity in the liver of rats on an atherogenic diet, so the rats that consumed wine may have a greater influx of plasma cholesterol into the liver.

Despite this decrease in the LDL-cholesterol of rats that consumed wine, there was no reduction in the total cholesterol in the heart, and there was even a tendency for cholesterol to increase in the aorta. Arterial cholesterol also increased in the animals that consumed alcohol solution (significant at 45 d), suggesting that this effect was the result of the alcohol content of wine. However, the animals that consumed wine did not accumulate as much cholesterol in the aorta as the rats that consumed alcohol solution. This indicated that nonalcoholic components in wine counteract the accumulation of cholesterol in the aorta induced by alcohol. The accumulation of cholesterol in arteries is related to LDL-cholesterol, so we find our results difficult to explain. We found that cholesterol accumulation in the aorta of rats that had consumed alcohol solution was more significant at 45 d, when LDL-cholesterol was substantially reduced. In recent years, attention has been drawn to the relation between the qualitative features of plasma LDL particles and cardiovascular risk. The atherogenic potential of LDL particles has been related to their size (30), degree of oxidation (31), aggregation (32), and cholesteryl ester content (33). Carr *et al.* (33) showed that, in hamsters on a cholesterol diet, there is no linear relationship between plasma LDL-cholesterol concentration and aorta cholesterol, whereas cholesteryl ester content of LDL is directly associated with increased total aorta cholesterol. We can discount LDL oxidation because the lipid oxidation index in the plasma of rats that drink wine or alcohol solution is lower than in control animals (34).

Total cholesterol and free cholesterol in the liver and heart were not modified by either alcohol or wine consumption. However, after 45 d, cholesteryl ester concentrations were lower in the liver and higher in the heart in the wine group than in controls, although these differences were not significant. The concentrations of cholesteryl esters were not obtained by direct measurement but by the difference between total and free cholesterol values; therefore, small differences in the value of these two parameters lead to large differences in the cholesteryl ester values.

The effects of flavonoids on bile acid excretion are controversial: some authors describe an increase (14,15) while others describe a reduction (35). We observed a reduction in bile acid excretion after 45 d in rats that consumed wine, but this excretion was similar to that in control rats at 6 mon. It is possible that the specific type or class of flavonoids used and the concentration and time for which they are administered affect the excretion of cholesterol and bile acids.

Previous studies have stated that flavonoids are hypocholesterolemic (11,14,15,35) in animals on a hypercholesterolemic diet, but we have not detected this effect. In healthy animals, then, the nonalcoholic components of wine reduced LDL-cholesterol in the long term but did not reduce the cholesterol content of the aorta or the heart. Our animals did not have hypercholesterolemia, so it seems that wine or alcohol protection is only effective in hypercholesterolemic situations. Although human and rat lipoprotein metabolism are not directly comparable, our results agree with authors who suggest that drinking is protective in middle and old age (1), a period in life when metabolic disturbances are more frequent.

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# Wax Ester Biosynthesis in the Liver of Myctophid Fishes

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**ABSTRACT:** The biosynthetic properties of wax esters in the liver were compared between two types of myctophid fishes having different body lipid composition, i.e., three triglyceride-rich species (*Lampanyctus jordani*, *Diaphus theta,* and *Symbolophorus californiensis*) and three wax ester-rich species (*L. regalis, Stenobracius nannochir*, and *Stenobracius leucopsarus*). *n*-Heptadecanol (17:0-ALC) and/or 10-*cis*-heptadecenoic acid (17:1-ACID) was incubated with liver homogenate of the six myctophid fishes and with co-factors such as NADPH and ATP for 2 to 5 h. Considerable amounts of wax esters with odd-numbered fatty acids and/or alcohols were produced in the liver homogenate of the wax ester-rich species. *Stenobracius nannochir* and *L. regalis*, which exclusively contained wax esters as neutral lipids, showed the highest activity of wax ester synthesis, followed by *S. leucopsarus,* which contained triglyceride as the minor constituent. Only trace amounts at most of odd-numbered fatty acids and alcohols were incorporated into the wax esters after incubation with the liver homogenates of the triglyceride-rich fishes. Active interchange between the fatty acids and the alcohols occurred during wax ester biosynthesis in the wax ester-rich fishes. The chain elongation and shortening of acyl moieties were also observed during incubation. These results suggested that the deposition of lipids in myctophid fishes is mainly due to their biosynthetic activities.

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Myctophid fishes, which are abundant in the mesopelagic zone of the world's oceans, are regarded as one of the most promising unused marine biological resources (1–3).

We have examined nutritional components such as lipids (4,5) and amino acids (6) contained in the muscle of myctophid fishes caught in the subarctic and transitional waters of the Pacific ocean. We observed a fairly good correlation between the major tissue lipids and migration patterns of the myctophid fishes. Most myctophid fishes make diel vertical migrations to feed in the productive upper 100 m at night. Based on their migratory patterns, myctophid fishes in the subarctic Pacific are grouped into surface migrants, midwater migrants, semimigrants, and nonmigrants (7). Among the subarctic Pacific species, the nonmigratory or semimigratory species store large quantities of wax esters in their whole bodies (the wax ester-rich fishes), whereas the migratory ones contain triglycerides (the triglyceride-rich fishes) (4).

In this paper, we examined the capacity for wax ester biosynthesis in the liver of migratory, semimigratory, and nonmigratory myctophid fishes to elucidate the ecological role of wax esters in their lives.

## EXPERIMENTAL PROCEDURES

*Myctophid fishes.* The myctophid fishes were caught in the northwestern Pacific ocean by *Marusada-maru*, a commercial trawler, in August, 1996. Among the six species used in the experiments, three species (*Lampanyctus jordani, Diaphus theta,* and *Symbolophorus californiensis*) were rich in triglycerides (more than 90% of their total lipids) in whole body, whereas the others (*L. regalis, Stenobrachius nannochir,* and *Stenobrachius leucopsarus*) were rich in wax esters (more than 90% of their total lipids) in whole body (4). The fish were stored at 0°C for 10 h until the experiment.

*Incubation of liver homogenate with the marker fatty acid and alcohol to assess wax biosynthesis.* Crude enzyme was prepared by homogenizing 1.0 g of livers pooled from 5 to 10 individual myctophid fish in 10 mL of 0.05 M citrate phosphate buffer (pH 5.0) with a Teflon glass homogenizer. The enzymatic reaction was done principally according to the method of Mankura and Kayama (9). 10-*cis*-Heptadecenoic acid (17:1-ACID; Sigma, St. Louis, MO; 3.7 nmol), *n*-heptadecanol (17:0-ALC, Nu-Chek-Prep, Elysian, MN; 3.7 nmol), or a mixture of 17:1-ACID and 17:0-ALC [1:1 (mol/mol), total 3.7 nmol] was dissolved in 0.1 mL of 5% bovine albumin (inactivated by heating; Sigma) in 0.05 M citrate phosphate buffer (pH 5.0) with Triton X-100 (1.0 mg) and sodium deoxycholate (1.0 mg) by sonication for 5 min. The substrate solution was mixed with 0.9 mL of the crude enzyme solution (6.2 mg protein) added to CoA (0.15 μmol), ATP (5.0 μmol), $MgCl_2$ (4.0 μmol), NADPH (2.0 μmol), in the final volume of 1.0 mL. The reaction was allowed to proceed for 2 and 5 h at 30°C. Incubation was terminated by the addition of an equal volume of methanol. The amounts of the incorporated odd-numbered fatty acid and alcohol were calculated following Lepage and Roy (10). The wax ester content in liver was also measured before and after incubation.

The amount of incorporated odd-numbered fatty acids and/or alcohols equals the levels of odd-numbered fatty acids

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Abbreviations: 17:0-ALC, *n*-heptadecanol; 17:1-ACID, 10-*cis*-heptadecenoic acid; FID, flame-ionization detector; GC, gas chromatography; TLC, thin-layer chromatography.

and/or alcohols in liver homogenate with 17:1-ACID and/or 17:0-ALC) minus (the levels of odd-numbered fatty acids and/or alcohols in the liver homogenate without 17:1-ACID and/or 17:0-ALC).

Data for enzymatic reactions were averaged for three determinations per sample.

*Lipid analyses*. Lipids were extracted from the reaction mixture by the method of Bligh and Dyer (11) and analyzed qualitatively by thin-layer chromatography (TLC) using Kieselgel 60 plates (Merck, Darmstadt, Germany) with a mixture of *n*-hexane/diethyl ether (7:3, vol/vol) as a mobile phase. The lipids were detected by spraying the plates with 50% $H_2SO_4$ and heating. The lipid classes were, however, quantitatively determined by TLC with a hydrogen flame-ionization detector (FID) using an Iatroscan TH-10 (Iatron Laboratory, Tokyo) with silica gel rods (S-III) and *n*-hexane/diethyl ether/formic acid (80:20:1, by vol) as a mobile phase. All TLC data presented are from the averages of five measurements for each individual sample.

For the analyses of fatty acids and alcohols in the wax esters, the wax esters were isolated by TLC and saponified (4). The compositions of fatty acids and alcohols before incubation with odd-numbered alcohols and/or fatty acids were analyzed as described previously (4). After incubation, the fatty acids were isolated, hydrogenated (12), and then methylated (13) prior to gas chromatography (GC) analysis using a GC-380 gas chromatograph (GL Sciences, Tokyo, Japan) equipped with an FID. Hydrogenation was essential to simplify the chromatograms and to clearly identify the peaks of odd-numbered fatty acids on the chromatograms. The column was a CP-SIL 88 capillary column (0.25 mm × 50 m; Chrompack, Middelburg, the Netherlands). The FID and injection temperatures were 250°C and the column temperature was programmed from 170 to 225°C at 3°C/min. Fatty acids were identified by comparing retention times with those of authentic standards and by comparing chromatograms of standards subjected previously to GC–mass spectrometry.

Fatty alcohols obtained by the saponification of the wax esters were analyzed by GC after hydrogenation and acetylation. Acetylation improved the recovery and resolution of alcohols in GC analysis. Analytical conditions were the same as in the case of the wax ester except for the column temperature, which was programmed from 170 to 225°C at 4°C/min.

## RESULTS

*Lipid classes and wax ester compostition in the liver.* The lipid compositions in the liver of six species of myctophid fishes were examined (Table 1). They were somewhat different from the composition of the whole body reported previously (4), although the level of wax esters in the liver was higher in the wax ester-rich fish than in the triglyceride-rich fish. In the whole body of the wax ester-rich fish, such as *S. leucopsarus, S. nannochir* and *L. regalis*, the wax esters occupied more than 90% of the total lipids in the whole body. However, triglycerides (37.4–136 mg/g tissue) more than wax esters (30.3–78.5 mg/g tissue) were contained in the liver lipids of the wax ester-rich fish, as shown in Table 1. Small amounts of wax esters (0–7.4 mg/g tissue) were found in the liver lipids of three species among the triglyceride-rich fishes, although the major liver lipid classes were triglycerides.

The alcohol and fatty acid compositions in wax esters in liver of wax ester-rich species are shown in Table 2. The patterns of both alcohols and fatty acids were similar to wax esters extracted from the whole body reported previously (4), but the percentages of major alcohols and fatty acids were somewhat different.

*Synthesis of wax esters from 17:0-ALC and 17:1-ACID.* Figure 1 shows the increments of wax esters in the liver homogenate of four myctophid species during incubation with 17:1-ACID and/or 17:0-ALC. Large amounts of wax ester (372–865 μg/g tissue) were produced in the wax ester-rich fishes such as *S. nannochir* and *L. regalis* during incubation. The level of wax ester was lower (65–99 μg/g tissue) in *S. leucopsarus*. Only trace amounts (less than 2 μg/g tissue) of wax esters were detected in the triglyceride-rich fish. This result showed that the wax ester-rich myctophid fishes had a very high capability for biosynthesizing of wax ester. In the

**TABLE 1**
**Lipid Composition in Myctophid Fish Liver[a]**

| | Lipid class (mg/g tissue) | | | | |
|---|---|---|---|---|---|
| | Wax ester | Triglyceride | Free fatty acid | Sterol | Polar lipid |
| *Stenobracius leucopsarus* | 78.5 ± 2.0 (66.2) | 37.4 ± 2.3 (31.5) | 1.4 ± 0.1 (1.2) | 0.5 ± 0.1 (0.4) | 0.7 ± 0.3 (0.6) |
| *Stenobracius nannochir* | 31.5 ± 1.7 (22.2) | 102.6 ± 1.5 (73.3) | 1.5 ± 0.3 (1.1) | 0.7 ± 0.1 (0.5) | 3.5 ± 0.1 (2.5) |
| *Lampanyctus regalis* | 30.3 ± 1.9 (17.8) | 136.0 ± 2.4 (80) | 0.7 ± 0.3 (0.4) | 0.3 ± 0.1 (0.1) | 2.5 ± 0.8 (1.5) |
| *Diaphus theta* | 7.4 ± 1.2 (8.1) | 74.5 ± 1.9 (81.6) | 6.8 ± 0.9 (7.4) | 1.1 ± 0.3 (1.3) | 1.5 ± 0.6 (1.6) |
| *Symbolophorus californiensis* | 5.8 ± 0.8 (2.8) | 193.2 ± 2.4 (92.8) | 2.7 ± 0.3 (1.3) | 0.9 ± 0.2 (0.4) | 5.4 ± 0.5 (2.5) |
| *L. jordani* | 0 | 65.7 ± 0.3 (98.5) | 0.2 ± 0.1 (0.3) | 0.1 ± 0.1 (0.1) | 0.7 ± 0.2 (1.0) |

[a]Values presented as means ± SD ($n$ = 5). Values in parentheses are percentages of the total lipids.

**TABLE 2**
**Alcohol and Fatty Compositions (%) of Wax Esters in Liver of Myctophid Fishes[a]**

| | *S. leucopsarus* | *S. nannochir* | *L. regalis* |
|---|---|---|---|
| Alcohols | | | |
| 14:0 | 4.9 | 27.8 | 0.6 |
| 14:1 | 1.5 | 3.2 | 0.3 |
| 16:0 | 40.6 | 35.6 | 14.0 |
| 16:1 | 5.1 | 7.9 | 0.1 |
| 18:0 | 3.6 | 3.3 | 1.8 |
| 18:1 | 15.6 | 4.6 | 3.5 |
| 20:0 | 0.3 | 1.3 | 0.1 |
| 20:1 | 11.5 | 7.9 | 26.4 |
| 22:0 | 1.6 | 1.1 | 0.9 |
| 22:1 | 13.5 | 5.1 | 49.4 |
| 24:1 | 0.5 | 0.2 | 2.0 |
| Fatty acids | | | |
| 14:0 | 1.3 | 3.7 | 2.3 |
| 15:0 | 0.4 | 0.5 | 0.8 |
| 16:0 | 8.0 | 6.7 | 5.3 |
| 16:1n-7 | 11.2 | 14.3 | 5.0 |
| 18:0 | 7.8 | 2.2 | 2.4 |
| 18:1n-9 | 45.8 | 29.4 | 33.1 |
| 18:1n-7 | 0.1 | 2.5 | 1.1 |
| 18:3n-3 | 0.3 | 0.2 | 0.2 |
| 18:4n-3 | 1.5 | 1.7 | 0.7 |
| 20:0 | 1.9 | 2.1 | 1.3 |
| 20:1n-11 | 5.9 | 14.2 | 11.5 |
| 20:1n-9 | 1.2 | 1.1 | 7.6 |
| 20:4n-6 | 0.3 | 0.2 | 0.3 |
| 20:4n-3 | 0.3 | 0.8 | 0.5 |
| 20:5n-3 | 3.7 | 3.6 | 3.1 |
| 21:5n-3 | 0.2 | 0.3 | 0.1 |
| 22:0 | 0.1 | 0.4 | 0.3 |
| 22:1n-11 | 5.1 | 11.1 | 14.9 |
| 22:1n-9 | 0.2 | 0.6 | 1.3 |
| 22:5n-3 | 1.1 | 0.6 | 3.1 |
| 22:6n-3 | 2.9 | 3.0 | 4.1 |
| 24:1n-9 | 0.4 | 0.3 | 0.2 |

[a]For genus names see Table 1.

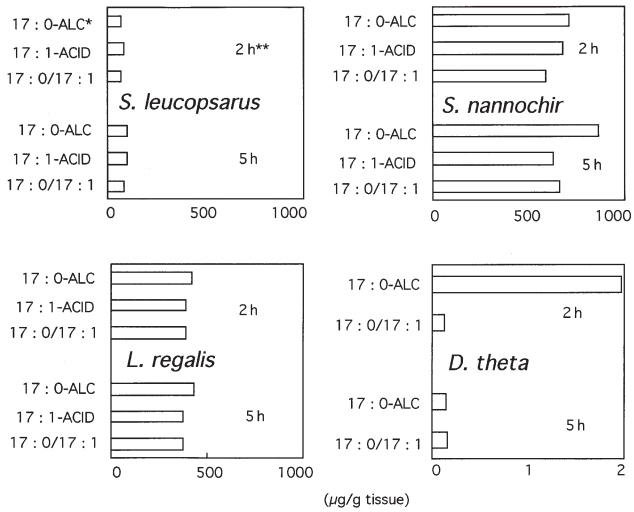


**FIG. 1.** Increment of wax esters in the liver of myctophid fishes during incubation with *n*-heptadecanol (17:0-ALC) and/or 10-*cis*-heptadecenoic acid (17:1-ACID). *17:0-ALC, incubated with 17:0-ALC; 17:1-ACID, incubated with 17:1-ACID; 17:0/17:1, incubated with 17:0-ALC and 17:1-ACID. ** Incubation time. Fish: *Stenobracius leucopsarus; S. nannochir; Lampanyctus regalis; Diaphus theta.*

wax-rich fish, the synthesis of wax ester reached a maximum at 2 h and no or very little increase was observed during 2 to 5 h (Fig. 1). The amounts of odd-numbered alcohols and fatty acids in wax esters after incubation of the liver homogenate of three wax-rich myctophid fishes with 17:0-ALC and/or 17:1-ACID are shown in Figure 2.

*Stenobracius leucopsarus*. When 17:0-ALC and a mixture of 17:0-ALC and 17:1-ACID were added to the liver homogenate, the total amount of odd-numbered alcohols was 32–95 nmol/g tissue. The level of odd-numbered alcohols was much higher (207–218 nmol/g tissue) when 17:1-ACID was added. In particular, greater amounts of odd-numbered alcohols with a carbon chain length other than 17 were observed. These observations showed that the added 17:1-ACID was reduced to alcohol after chain elongation and β-oxidation and then incorporated into wax ester in the liver.

When 17:1-ACID and a mixture of 17:0-ALC and 17:1-ACID were added to a liver homogenate of *S. leucopsarus*, the level of odd-numbered fatty acids was 25–68 nmol/g tissue. The level of odd-numbered fatty acids was higher (75–148 nmol/g tissue) when 17:0-ALC was added. Considerable amounts of odd-numbered fatty acids with a carbon chain length other than 17 were also found in the liver homogenate after incubation with 17:0-ALC. More than half of the added 17:0-ALC was transformed into fatty acids and then incorporated into wax ester in the liver.

From analyses of fatty acids and alcohols in wax esters of the liver of *S. leucopsarus*, one can see that most of the added fatty acids and alcohols were not directly incorporated into the wax esters of the liver but rather were transformed into the corresponding alcohols and fatty acids before incorporation into the wax esters.

*Stenobracius nannochir*. High levels of odd-numbered alcohols were observed in the wax esters after the incubation of liver homogenates with 17:0-ALC (533–974 nmol/g tissue) and 17:1-ACID (793–1123 nmol/g tissue). These levels were about five times higher than observed in *S. leucopsarus*.

The level of odd-numbered fatty acids in wax esters was higher in the liver homogenate with added 17:0-ALC (1600–2271 nmol/g tissue) and 17:1-ACID (977–1516 nmol/g tissue). This result was also different from that in *S. leucopsarus*. Added 17:1-ACID was incorporated into the wax esters as fatty acids, although the level of 17:1-ACID incorporated into the wax esters was less than that of 17:0-ALC. The level of 17:1-ACID incorporated into the wax esters as fatty acids in the liver of *S. nannochir* was 16 times higher than in *S. leucopsarus*. These observations suggested the occurrence of much higher wax ester biosynthetic activity in *S. nannochir* under the laboratory conditions used.

*Lampanyctus regalis.* Considerable amounts of odd-numbered alcohols were observed in the wax esters of liver homogenate incubated with 17:1-ACID (975–1049 nmol/g tis-

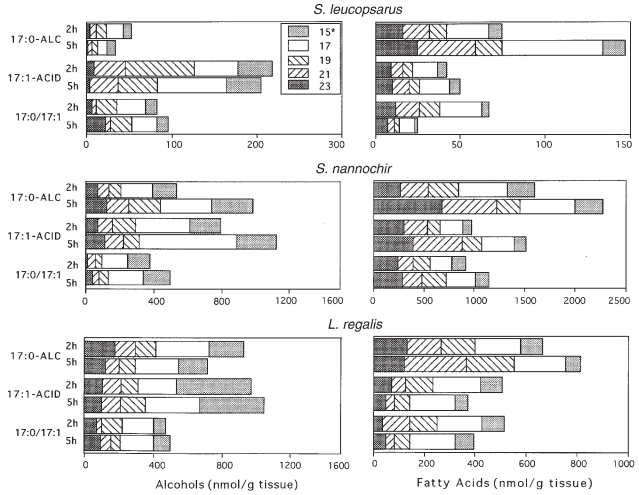


**FIG. 2.** Incorporated odd-numbered fatty alcohols (left panels) and fatty acids (right panels) in wax esters in the liver of wax ester-rich myctophid fishes after incubation with *n*-heptadecanol (17:0-ALC) and/or 10-*cis*-heptadecenoic acid (17:1-ACID). Upper: *S. leucopsarus;* middle: *S. nannochir;* lower: *L. regalis.* *Carbon chain length of odd-numbered alcohols and fatty acids in wax esters. See Figure 1 for abbreviations.

sue) and 17:0-ALC (721–930 nmol/g tissue). These results were similar to those observed in *S. nannochir*. However, the levels of incorporated fatty acids in *L. regalis* were lower than those in *S. nannochir*. The 17:0-ALC was incorporated into wax esters as fatty acids as well as alcohols, but 17:1-ACID was preferentially incorporated as alcohols rather than fatty acids.

*Triglyceride-rich species*. Among the three triglyceride-rich fishes, neither odd-numbered fatty acids nor alcohols were incorporated into the wax esters in *L. jordani* and *S. californiensis* after incubation with 17:0-ALC and/or 17:1-ACID. As no measurable amount of wax ester was synthesized after incubation of the liver homogenates of these two species with 17:0-ALC and 17:1-ACID, we suggested that wax ester is not biosynthesized in the liver of these species at all under these experimental conditions. In the case of *D. theta*, small amounts of odd-numbered fatty acids were detected in the wax esters after incubation with 17:0-ALC (Fig. 3) whereas no odd-numbered alcohols were detected at all in the wax esters. These results, if taken together, indicate that the activity of wax ester synthesis in the liver of triglyceride-rich myctophid fishes was very low and in some cases negligible.

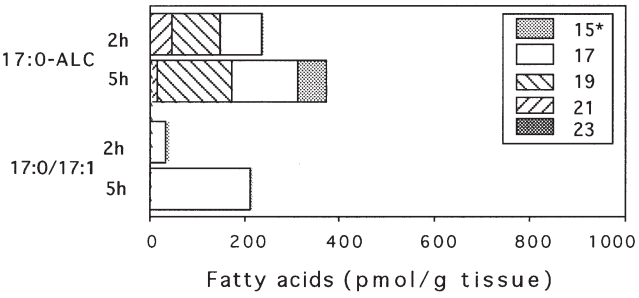


**FIG. 3.** Incorporation of odd-numbered alcohol (17:0-ALC) and fatty acid (17:1-ACID) in the fatty acids of wax esters in the liver of *D. theta* after incubation with 17:0-ALC or with 17:0-ALC plus 17:1-ACID. There was no incorporation detected of 17:0-ALC or 17:0-ALC/17:1-ACID into fatty alcohols of wax esters. For abbreviations see Figure 1.

## DISCUSSION

We have previously found that there were two types of myctophid fishes from the point of view of lipid properties (4). That is, one is a wax ester-rich fish, the other is a triglyceride-rich fish. We also analyzed the lipid composition of the stomach contents of myctophid fishes because body lipids might derive from prey organisms as reviewed by Benson and Lee (14). However, we found no significant differences in lipid composition in the stomach contents between the wax ester-rich and triglyceride-rich fishes. Thus, in this study we examined the capacity for biosynthesis of wax esters in the liver of the wax ester-rich and triglyceride-rich fishes to understand whether the wax ester synthesis in the liver may determine lipid compositions of the myctophid fishes.

The wax ester-rich fish had high contents of wax esters in the liver, which correlated with those in the whole body. We examined the synthesis of wax ester in their livers using 17:0-ALC and 17:1-ACID as substrates. In the liver, wax ester synthesis was much higher for the wax ester-rich fishes. In particular, activities in the nonmigrant species (*S. nannochir* and *L. regalis*) were greater than in the semimigrant species (*S. leucopsarus*). In the wax ester-rich fishes, most of the added 17:1-ACID and 17:0-ALC was transformed to alcohols and fatty acids, respectively, and then incorporated into the wax ester after chain elongation and β-oxidation. Both 17:1-ACID and 17:0-ALC were incorporated into the wax esters as fatty acids and alcohols without transformation. The 17:1-ACID, added to liver homogenates of the triglyceride-rich fishes, was incorporated in part into triglycerides, whereas most of it remained as free fatty acids (data not shown).

From these results, the wax content in the whole body of myctophid fishes (4) showed a good correlation with the wax ester biosynthetic activity in the liver rather than with foods consumed. The very active incorporation of $^{14}$C-labeled acetate into wax ester in the liver of orange roughy, which is known to accumulate wax ester in skin and swim bladder, was also reported (15). Moreover, we found that fatty acids and alcohols were mutually transformed during wax ester synthesis in the liver of the wax ester-rich myctophid fishes. Mankura and Kayama (9) reported the similar transformation from alcohols to fatty acids, but not from fatty acids to alcohols in the liver of carp.

Our results also showed that wax ester-rich myctophid species are restricted to nonmigratory and semimigratory species in the subarctic and transitional waters of the western Pacific. Nonmigrants (*S. nannochir* and *L. regalis*) especially showed high synthetic ability for wax ester. This supports the view that wax esters contribute to the static lift for neutral buoyancy.

## ACKNOWLEDGMENTS

This work was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan, No.06454096, and a grant from Marino Forum 21 Project "Technical Development of Utilization of Myctophid Fish Resources." We are indebted to Dr. Toshiyuki Tanabe of Tohoku National Fisheries Research Institute and Marino Forum 21 for providing the valuable myctophid samples analyzed.

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# Water-Soluble Organosulfur Compounds of Garlic Inhibit Fatty Acid and Triglyceride Syntheses in Cultured Rat Hepatocytes

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**ABSTRACT:** The putative hypolipidemic effect of garlic remains controversial. To gain further insight into the effect of garlic on lipid metabolism, the present study determined the inhibitory effects of water-soluble organosulfur compounds present in garlic on triglyceride (TG) and fatty acid synthesis in cultured rat hepatocytes. When incubated at 0.05 to 4.0 mmol/L with cultured hepatocytes, *S*-allyl cysteine (SAC) and *S*-propyl cysteine (SPC) decreased [2-$^{14}$C]acetate incorporation into triglyceride in a concentration-dependent fashion achieving a maximal inhibition at 4.0 mmol/L of 43 and 51%, respectively. The rate of [2-$^{14}$C]acetate incorporation into phosphlipids was depressed to a similar extent by SAC and SPC. SPC, SAC, *S*-ethyl cysteine (SEC), and γ-glutamyl-*S*-methyl cysteine decreased [2-$^{14}$C]acetate incorporation into fatty acid synthesis by 81, 59, 35, and 40%, respectively, at 2.0–4.0 mmol/L concentrations. Alliin, γ-glutamyl-*S*-allyl cysteine, γ-glutamyl-*S*-propyl cysteine *S*-allyl-*N*-acetyl cysteine, *S*-allylsulfonyl alanine, and *S*-methyl cysteine had no effect on fatty acid synthesis. The activities of lipogenic enzymes, fatty acid synthase (FAS), and glucose-6-phosphate dehydrogenase (G6PDH) were measured in cultured hepatocytes treated with the inhibitors. The activity of FAS in cells treated with 4.0 mmol/L SAC and SPC, respectively, was 32 and 27% lower than that of nontreated cells. Neither SAC nor SPC affected G6PDH activity. The results indicate that SAC, SEC, and SPC inhibit lipid biosynthesis in cultured rat hepatocytes, and further suggest that these *S*-alk(en)yl cysteines of garlic impair triglyceride synthesis in part due to decreased *de novo* fatty acid synthesis resulting from inhibition on FAS. Whether tissue concentrations of active garlic components can achieve levels required to inhibit TG synthesis *in vivo* warrants further investigation.

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The cardiovascular protective effects of garlic have been extensively investigated in the past decades. It has been reported that garlic and various garlic preparations (e.g., aged garlic extract) reduce platelet aggregation in humans (1,2) and production of thromboxane $B_2$ in rats (3). Aged garlic extract has also been shown to decrease development of fatty streak and fibro fatty plaques in rabbits (4). Similarly, garlic powder supplementation in healthy adults attenuated age-related increase in aortic stiffness by increasing aortic elasticity (5). A recent study with human volunteers has further demonstrated that aged garlic extract lowered the susceptibility of low density lipoprotein (LDL) to oxidation (6). The potential reduction of risk for atherosclerosis and cardiovascular diseases by garlic has been attributed primarily to its hypolipidemic property. However, the lipid-lowering effects of garlic in humans remain controversial. Although a number of studies reported that garlic lowered plasma total cholesterol, LDL-cholesterol, and triglyceride (TG) in animals (7–10) and in humans (11–14), several recent human intervention studies were unable to confirm the hypocholesterolemic effects of different garlic preparations (15–17). One of the possible explanations for the discrepancy may stem from the different ingredients of garlic or garlic preparations used in various studies (18). Moreover, the active components of garlic responsible for the putative lipid-lowering effects are ill defined (18). Raw garlic and garlic powder contain lipid-soluble thiosulfinates, thiosulfinate transformation products, and sulfides (19). Among these compounds, allicin and vinyl dithiins have been suggested as potential lipid-lowering agents (19). In addition, garlic in general, and aged garlic extract in particular, contains a significant amount of water-soluble organosulfur compounds including *S*-alk(en)yl cysteines and γ-glutamyl-*S*-alk(en)yl cysteines (19–21). We recently demonstrated that *S*-alk(en)yl cysteines and γ-glutamyl-*S*-alk(en)yl cysteines inhibited cholesterol biosynthesis in cultured rat hepatocytes (22). Among the compounds tested in cultured hepatocytes, three water-soluble *S*-alk(en)yl cysteines, i.e., *S*-allyl-cysteine (SAC), *S*-ethyl cysteine (SEC), and *S*-propyl cysteine (SPC), were the most potent inhibitors of cholesterol biosynthesis, achieving 42 to 55% maximal inhibition, albeit at high concentrations (22).

Despite extensive studies on understanding the effects of garlic on human lipid metabolism, little is known about the mechanisms underlying possible hypotriglyceridemic action of garlic or garlic preparations. A previous study from our laboratory demonstrated that garlic extracts inhibited [$^3$H]glycerol incorporation into TG in rat cultured hepatocyte in the presence of acetate (10). When oleate was incubated with [$^3$H]glycerol, such an inhibitory effect of the garlic extracts was no longer apparent (10). Consistent with these *in vitro* experiments, dietary supplementation of garlic decreased incorporation of [$^{14}$C]acetate into fatty acids in various tissues of the rabbit (23). It is therefore reasonable to speculate that the inhibition of TG synthesis by garlic may in part be attributed to an impairment of fatty acid synthesis. The reduction of hepatic synthesis of TG could suppress very low density lipoprotein (VLDL)

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Abbreviations: ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; FAS, fatty acid synthase; FBS, fetal bovine serum; G-6-P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GSAC, γ-glutamyl-*S*-allyl cysteine; GSMC, γ-glutamyl-*S*-methyl cysteine; GSPC, γ-glutamyl-*S*-propyl cysteine; $IC_{50}$, concentration required for 50% maximal inhibition; LDL, low density lipoprotein; PPB, potassium phosphate buffer; SAC, *S*-allyl cysteine; SAMC, *S*-allyl mercaptocysteine; SANC, *S*-allyl-*N*-acetyl cysteine; SASA, *S*-allylsulfonyl alanine; SEC, *S*-ethyl cysteine; SMC, *S*-methyl cysteine; SPC, *S*-propyl cysteine; TG, triglyceride; VLDL, very low density lipoprotein.

synthesis in the liver and hence decrease plasma TG level (24). Therefore, the present study was undertaken to determine the inhibitory potency of garlic-derived organosulfur compounds on TG and fatty acid synthesis *de novo* in cultured rat hepatocytes. The results indicated that SAC and SPC at high concentrations (2.0–4.0 mmol/L) were potent inhibitors of fatty acid and TG synthesis. In addition, SAC and SPC decreased the activity of fatty acid synthase (FAS) but had no effect on that of glucose-6-phosphate dehydrogenase (G6PDH).

## MATERIALS AND METHODS

*Animals*. Male Sprague-Dawley rats (200–300 g) were obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN) and fed a nonpurified diet (Purina Rat Chow; Ralston Purina, St. Louis, MO). The animals were housed individually in stainless steel cages at approximately 24°C and 50% relative humidity on a 12-h light/dark cycle (0600–1800). The animal protocol was approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

*Hepatocyte isolation and cell culture*. Hepatocytes were isolated from rats according to the method detailed previously (22). From each liver, 100–250 $\times$ $10^6$ cells were obtained with a viability of 92–94% judging by trypan blue exclusion. The cells were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units penicillin/mL and 100 μg streptomycin/mL) to obtain 0.5–0.8 $\times$ $10^6$ cells/mL of suspension. Aliquots (2 mL) of the suspension were plated in each well of a six-well culture plate (Becton Dickinson Labware, Franklin Lakes, NJ) and incubated at 37°C under an atmosphere of 95% air and 5% $CO_2$. After 4 h of incubation, nonadhering cells were removed and discarded. Hepatocytes that adhered to the culture plate were re-fed with DMEM and incubated overnight for 16 h.

*Metabolic study*. At the end of the overnight incubation, cells were washed three times with 2 mL of FBS-free DMEM, followed by incubation with 2 mL of the same medium containing sodium salt of [2-$^{14}$C]acetate (specific activity, 37 MBq/mmol) and 0.5 mmol/L nonlabeled sodium acetate in the presence or absence of organosulfur compounds. Four hours after incubation, the medium was discarded and cells were harvested with 1.3 mL of ice-cold water by scraping with a cell scraper.

*Lipid analysis*. Cell suspension was mixed with 20 mL of chloroform/methanol (2:1, vol/vol) to extract lipids according to the method of Folch *et al*. (25) with minor modifications. For measurement of [2-$^{14}$C]acetate incorporation into fatty acid, the lipid extract was saponified with 6 mL of 3.75% methanolic KOH in a sealed ampule at 90°C for 4 h (26). After removal of the nonsaponifiable fraction, the extracts were acidified with concentrated HCl, and the fatty acids were extracted with petroleum ether (b.p. 35–60°C). For quantification of [2-$^{14}$C]acetate incorporation into TG and phospholipid, the lipid extracts were separated by thin-layer chromatography (TLC) on Silica Gel H coated plates (Analtech, Inc., Newark, DE) using hexane/diethyl ether/acetic acid (80:20:1, by vol) as developing solvent (27). The silica gel bands corresponding to TG and phospholipid were scraped into scintillation counting vials. The radioactivity of $^{14}$C-labeled products was measured by liquid scintillation counting (Beckman Model LS 3801; Beckman Instruments, Fullerton, CA). The specific activity of [2-$^{14}$C]acetate incorporation into fatty acid, TG, and phospholipid was expressed as pmol acetate incorporated/4 h/mg cellular protein. Cellular protein was determined by the procedure of Lowry *et al*. (28). The relative rate of [2-$^{14}$C]acetate incorporation into fatty acid, TG, and phospholipid by cells treated with organosulfur compounds was expressed as percentage of control by calculating specific activity of treatment group/specific activity of control nontreatment group $\times$ 100. $IC_{50}$ (concentration required for 50% maximal inhibition) was calculated by regression and correlation analysis between substrate concentration and percentage inhibition.

*Determination of enzyme activities*. For measurement of enzyme activities, the cells were cultured in 60 mm-diameter culture dishes (Becton Dickinson Labware) plated with 3 $\times$ $10^6$ cells/dish. Cells were treated and incubated under the same conditions described above for metabolic study. After 4 h of treatment, the medium was removed, and the cells were washed three times with ice-cold phosphate-buffered saline. The dishes were placed on ice and immediately the following were added: 0.5 mL of potassium phosphate buffer (PPB, 100 mmol/L, pH 7.4) containing sucrose, 250 mmol/L; EDTA, 1 mmol/L; and dithiothreitol (DTT), 1 mmol/L (29,30). The cells harvested by scraping were subjected to freezing and thawing three times and centrifuged at 20,000 $\times$ *g* for 30 min at 4°C (29). The supernatant obtained was used to determine the enzyme activities and protein concentration. FAS activity was determined spectrophotometrically by the method of Nepokroeff *et al*. (31) in PPB (500 mmol/L, pH 7.0) containing malonyl CoA, 0.1 mmol/L; acetyl CoA, 0.05 mmol/L; NADPH, 0.1 mmol/L; EDTA, 1 mmol/L; and DTT, 5 mmol/L. The reaction was initiated by the addition of 0.1 mL malonyl CoA to a final volume of 1 mL, and the rate of oxidation of NADPH was monitored at 340 nm and 30°C with a Beckman DU®-50 LS 5801 spectrophotometer (Beckman Instruments). G6PDH was measured by the method of Deutsch (32). The reaction mixture contained Tris buffer (100 mmol/L), pH 7.8, KCl (100 mmol/L), NADP (0.4 mmol/L), $MgCl_2$ (5 mmol/L), and glucose-6-phosphate (G-6-P) (5 mmol/L). The reaction was initiated by the addition of 0.1 mL of G-6-P to a final volume of 1 mL. The reduction of NADP was monitored spectrophotometrically at 340 nm. The specific activity of enzyme was defined as nmol NADPH oxidized/mg protein/min for FAS and nmol NADP reduced/mg protein/min for G6PDH.

*Materials*. Culture media, FBS, penicillin, and streptomycin were purchased from GIBCO (Gaithersburg, MD). Collagenase D was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). All water-soluble organosulfur compounds of garlic: SAC, SEC, *S*-methyl cysteine (SMC), SPC, γ-glutamyl-*S*-allyl cysteine (GSAC), γ-glutamyl-*S*-methyl cysteine (GSMC), γ-glutamyl-*S*-propyl cysteine (GSPC), *S*-allyl cysteine sulfoxide (i.e., alliin), *S*-allyl-*N*-acetyl cysteine (SANC), *S*-allyl-mercaptocysteine (SAMC), and *S*-allylsulfonyl alanine (SASA) were pro-

vided by Wakunaga of America Co., Ltd. (Mission Viejo, CA). Sodium [2-$^{14}$C]acetate was obtained from Amersham Corp. (Arlington Heights, IL). All other chemicals of reagent grade were purchased from Sigma Chemical Co. (St. Louis, MO).

*Statistics*. Data are presented as means ± SEM (standard error of the mean). The comparisons of the test compounds were analyzed by analysis of variation (ANOVA) with the general linear model. When statistical significance was indicated by ANOVA, the Bunnett test was applied to identify the significant difference between the treatment and control at $P < 0.05$.

## RESULTS

Eleven water-soluble organosulfur compounds present in garlic or aged garlic preparations were tested for inhibition potency on fatty acid synthesis in cultured hepatocytes. The rate of [2-$^{14}$C]acetate incorporation into fatty acid in the untreated (control) group varied from 801 to 1127 pmol acetate/mg cellular protein/4 h among all experiments. The incorporation of [2-$^{14}$C]acetate into fatty acids was taken as a measure of fatty acid synthesis. The rate of fatty acid synthesis in the untreated cells for individual experiments was arbitrarily defined as 100%. Among *S*-alk(en)yl cysteines, SAC, SEC, and SPC, but not SMC, inhibited [2-$^{14}$C]acetate incorporation into fatty acid with maximal inhibition of 35–80% (Fig. 1). The inhibition was apparent at concentrations as low as 0.05 mmol/L for SAC and SEC, and 0.2 mmol/L for SPC (data not shown). The maximal inhibition was the highest by SPC, followed by SAC and SEC. Among γ-glutamyl *S*-alk(en)yl cysteines, GSMC inhibited the rate of [2-$^{14}$C]acetate incorporation into fatty acid to a maximum of 40%, while GSAC and GSPC did not alter the rate of the incorporation (Fig. 2). SAMC decreased the [2-$^{14}$C]acetate incorporation into fatty acid by 35% at 0.05 mmol/L (Fig. 2). Increasing concentration of SAMC to 2.0 and 4.0 mmol/L diminished fatty acid synthesis. Other water-soluble compounds (i.e., alliin, SANC, and SASA) had no effect on the rate of [2-$^{14}$C]acetate incorporation into fatty acid within the range of concentrations (0.05 to 4.0 mmol/L) tested (data not shown).

The concentration-dependent inhibition shown in Figures 1 and 2 permitted us to calculate maximal inhibition and $IC_{50}$ of water-soluble compounds on fatty acid synthesis. The maximal inhibition was the highest by SPC (81%) followed by SAC (59%), GSMC (40%), and SEC (35%), whereas the $IC_{50}$ (expressed as mmol/L) was the lowest with GSMC (0.72) followed by SEC (0.8), SPC (0.84), and SAC (0.91).

The effects of two potent inhibitors of fatty acid synthesis on the incorporation of [$^{14}$C]acetate into TG and phospholipid were determined. SPC and SAC inhibited the rate of [$^{14}$C]acetate incorporation into TG in a concentration-dependent fashion exhibiting 51 and 43% maximal inhibition, respectively, at 4.0 mmol/L (Fig. 3). A significant inhibition was observed at 0.05 mmol/L for SAC. In contrast, the inhibition by SPC was not apparent until the concentration was increased to 1.0 mmol/L. SAC and SPC inhibited [$^{14}$C]acetate incorporation into phospholipid as well, but the inhibitory potency was less than that on TG (Fig. 4). A significant inhibition of phospholipid production was not apparent until relatively high concentrations, i.e., 1.0 mmol/L for SAC and 2.0 mmol/L for SPC, were present in the incubation medium. The maximal inhibition was 43% for SAC, which was similar to that on TG (43%), and 30% for SPC, which was lower than that on TG (51%).

The marked inhibition on fatty acid biosynthesis led us to determine the effects of organosulfur compounds on the activities of lipogenic enzymes in cultured hepatocytes. The cells were incubated with SAC or SPC at the highest concentration (i.e., 4.0 mmol/L) that exhibited the maximal inhibition of fatty acid synthesis and at $IC_{50}$. The activity of FAS in

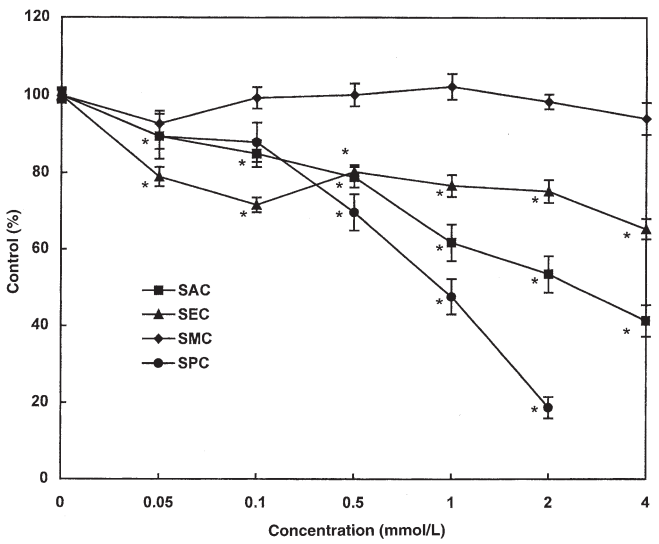


**FIG. 1.** Inhibition of [2-$^{14}$C]acetate incorporation into fatty acid by *S*-allyl cysteine (SAC), *S*-ethyl cysteine (SEC), *S*-methyl cysteine (SMC), and *S*-propyl cysteine (SPC) in primary rat hepatocyte culture. Data are expressed as a percentage of the control and represent means ± SEM of eight samples. *Statistically significant difference from controls at $P < 0.05$.

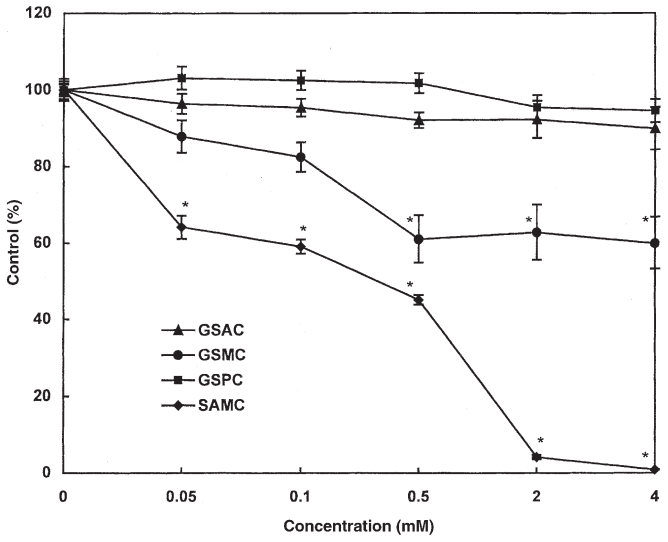


**FIG. 2.** Inhibition of [2-$^{14}$C]acetate incorporation into fatty acid by γ-glutamyl-*S*-allyl cysteine (GSAC), γ-glutamyl-*S*-methyl cysteine (GSMC), γ-glutamyl-*S*-propyl cysteine (GSPC), and *S*-allyl mercaptocysteine (SAMC) in primary rat hepatocyte culture. Data are expressed as a percentage of the control and represent means ± SEM of eight samples. *Statistically significant difference from controls at $P < 0.05$.

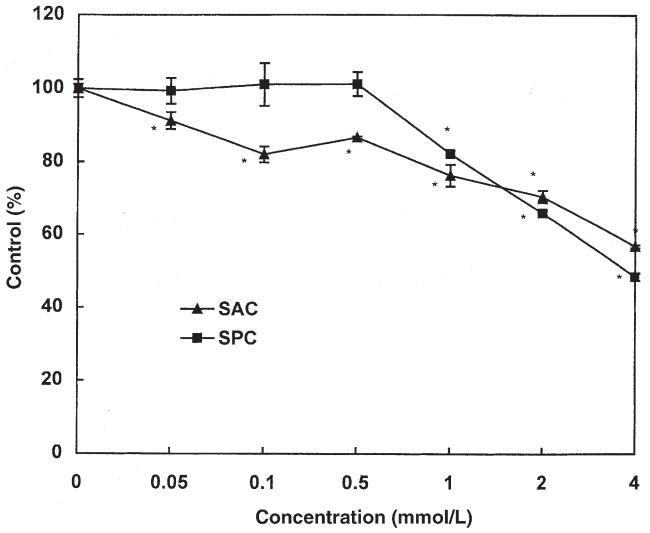


**FIG. 3.** Inhibition of [2-$^{14}$C]acetate incorporation into triglyceride by SAC and SPC in primary rat hepatocyte culture. Data are expressed as a percentage of the control and represent means ± SEM of eight samples. *Statistically significant difference from controls at $P < 0.05$. For abbreviations see Figure 1.

cells treated with 4.0 mmol/L SAC was 32% lower than that of nontreated cells (Table 1). SAC at $IC_{50}$ (i.e., 0.91 mmol/L) depressed the FAS activity by 19%. On the other hand, SPC depressed the activity of FAS only at 4.0 mmol/L but not at $IC_{50}$ (i.e., 0.84 mmol/L). Neither SAC nor SPC altered the activity of G6PDH at the concentrations tested (Table 1).

## DISCUSSION

Hypertriglyceridemia is a risk factor independent of plasma level of cholesterol for cardiovascular disease (33). Further, this risk factor is not necessarily associated with decreased plasma level of high density lipoprotein cholesterol, and hence a reduction of TG-rich lipoprotein (i.e., VLDL) may attenuate the progression of coronary artery disease (34).

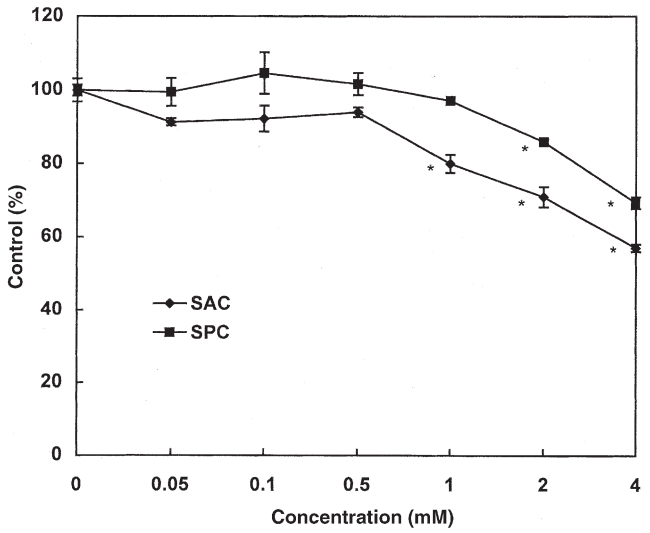


**FIG. 4.** Inhibition of [2-$^{14}$C]acetate incorporation into phospholipid by SAC and SPC in primary rat hepatocyte culture. Data are expressed as a percentage of the control and represent means ± SEM of eight samples. *Statistically significant difference from controls at $P < 0.05$. For abbreviations see Figure 1.

**TABLE 1**
**Activities of Fatty Acid Synthase (FAS) and Glucose-6-phosphate Dehydrogenase (G6PDH) in Hepatocytes Treated with or Without SAC and SPC[a]**

| Compounds | Concentration (mmol/L) | FAS[b] | G6PDH[b] |
|---|---|---|---|
| SAC | 0 | 3.71 ± 0.37 | 38.76 ± 3.43 |
| | 0.91 ($IC_{50}$) | 3.00 ± 0.19* | 37.12 ± 1.55 |
| | 4.0 | 2.53 ± 0.32* | 33.67 ± 1.81 |
| SPC | 0 | 4.06 ± 0.35 | 40.72 ± 1.96 |
| | 0.84 ($IC_{50}$) | 3.57 ± 0.18 | 40.29 ± 1.00 |
| | 4.0 | 2.94 ± 0.20* | 37.36 ± 2.65 |

[a] SAC, *S*-allyl cysteine; SPC, *S*-propyl cysteine.
[b]The specific activity of enzyme is defined as nmol NADPH oxidized/mg protein/min for FAS and nmol of NADP reduced/mg protein/min for G6PDH. Data represent means ± SEM of six samples. *Statistically significant difference from nontreated group, $P < 0.05$.

Evidence has suggested that garlic reduces the risk of cardiovascular disease by reducing the plasma level of cholesterol (4,12,13,23). Garlic may also decrease cardiovascular disease risk if it lowers plasma TG levels. In fact, some clinical studies have shown that garlic or garlic preparations did lower plasma TG levels (14,35). However, the mechanism underlying such a lowering action of garlic is unclear. Our previous study indicated that a TG-lowering effect of garlic extracts might stem in part from inhibition on hepatic TG synthesis (10). Furthermore, the reduction of TG synthesis was accompanied by depressed fatty acid synthesis in rat hepatocytes treated with various garlic extracts. Garlic is known to contain a large number of sulfur compounds (19). Although some of these compounds, such as SAC, have been shown to depress fatty acid synthesis (10), garlic constituents foremost responsible for inhibiting TG synthesis are yet to be determined. The present study revealed that sulfur-containing cysteine derivatives (i.e., SAC and SPC) were potent inhibitors of TG synthesis *in vitro*. Moreover, SAC and SPC were found to be the most potent inhibitors of fatty acid synthesis among *S*-alk(en)yl cysteines (e.g., SAC, SEC, SMC, and SPC) and γ-glutamyl-*S*-alk(en)yl cysteines (e.g., GSAC, GSMC, and GSPC) tested. These observations are consistent with our earlier study suggesting that garlic decreases hepatic TG production by inhibiting fatty acid synthesis (10). In addition, the current study further identified SAC and SPC as the most active garlic components for inhibition of fatty acid synthesis. The present data, however, do not exclude the possibility that garlic constituents affect the distal pathway of TG synthesis. For example, whether SAC, SPC, or other cysteine derivatives alter activities of glycerolipid-synthesizing enzymes such as glycerol-3-phosphate acyltransferase, monoacylglycerol acyltransferase, phosphatidate phosphohydrolase, and diacylglycerol acyltransferase is not known (36). It is worthwhile to note that the incorporation of [2-$^{14}$C]acetate into phospholipid was also inhibited in a similar manner as TG synthesis. Since phospholipid synthesis shares most acyl-

transferases and phosphatidate phosphohydrolase with TG synthesis, this finding tends to support the notion that the inhibition of TG synthesis by sulfur compounds may not involve esterification steps catalyzed by the acyltransferases.

Another important finding was that organosulfur compounds decreased fatty acid synthesis (Figs. 1 and 2), and the patterns of inhibition were similar to those on cholesterol synthesis as reported previously (22). Three *S*-alk(en)yl cysteines, SAC, SEC, and SPC, but not SMC, significantly decreased fatty acid synthesis with maximal inhibition of 59, 35, and 81%, respectively. Among γ-glutamyl *S*-alk(en)yl cysteines, GSMC reduced [2-$^{14}$C]acetate incorporation into fatty acid, while GSAC and GSPC did not affect fatty acid synthesis. Consistent with the unaltered rate of cholesterol synthesis, alliin, SANC, and SASA did not change [2-$^{14}$C]acetate incorporation into fatty acid, whereas SAMC containing two sulfur atoms markedly decreased fatty acid synthesis. However, unlike other water-soluble compounds, SAMC is highly cytotoxic, as reported earlier by this laboratory (22). Therefore, the reduction on [2-$^{14}$C]acetate incorporation into fatty acid by SAMC may result from cytotoxicity. The decrease of [2-$^{14}$C]acetate incorporation into fatty acid by other compounds, i.e., SAC, SEC, SPC, and GSMC, may result from impairment in the enzyme or enzymes regulating fatty acid synthetic pathways. In fact, the present study with cultured hepatocytes demonstrated that SAC and SPC incubated at the concentration (4.0 mmol/L) that caused maximal inhibition of fatty acid synthesis decreased the activity of FAS by 32 and 27%, respectively, when compared with nontreated cells. Neither SAC nor SPC exhibited an inhibitory effect on the activity of G6PDH. These data suggest that the sulfur-containing compounds depress fatty acid synthesis by inactivating FAS, the rate-limiting enzyme for fatty acid synthesis. Earlier animal studies by other investigators showed that garlic-supplemented diets decreased activities of not only lipogenic FAS but also G6PDH (7–9). The reason for the discrepancy in G6PDH activity observed between the present study and that of others (7–9) is not readily understood. However, it is important to point out that SAC and SPC isolated from garlic were used in the present *in vitro* experiment as compared with various garlic extracts fed to animals (7–9). Garlic extracts used in the studies contained not only SAC and SPC but also other water-soluble and fat-soluble sulfur compounds (37). Thus, animal studies are warranted to further delineate the differential effects of individually isolated compounds as compared with garlic extracts on lipogenic enzymes. Also, it should be stressed that whether SAC, SEC, SPC, and other sulfur-containing compounds interfere with fatty acid chain elongation by [2-$^{14}$C]acetate and hence play any role in the observed inhibition of TG synthesis remains to be ascertained.

Finally, the present study using cultured rat hepatocytes demonstrated that water-soluble organosulfur compounds of garlic, especially SAC and SPC, inhibited TG synthesis by depressing fatty acid synthesis *de novo*. The inhibition of fatty acid synthesis, on the other hand, was associated with decreased activity of FAS. Overproduction of TG-rich VLDL in liver is known to induce hypertriglyceridemia (38). It is therefore tempting to speculate that decreased hepatic TG synthesis by water-soluble organosulfur compounds may explain in part the hypotriglyceridemic effect of garlic reported previously (14,35). However, one must be cautious about the extrapolation of *in vitro* data to *in vivo* situations. It is important to stress that although maximal inhibition of fatty acid and TG synthesis was obtained at 4.0 mmol/L of alk(en)yl cysteines (i.e., SAC and SPC), significant inhibition of fatty acid synthesis was detected at a concentration as low as 0.05 mmol/L in the present *in vitro* study (Figs. 1,3). The inhibition of TG synthesis by SAC was also apparent in the same concentration range of 0.05 to 4.0 mmol/L (Fig. 3). It is essential to know whether these concentrations can be achieved under *in vivo* conditions. Unfortunately, there is no such information available for humans. Nonetheless, the present study *in vitro* clearly indicates that fatty acid and TG synthesis are inhibited by SAC and other alk(en)yl cysteines in a concentration-dependent manner. These findings further suggest that any effect of inhibition of the sulfur compounds on cholesterol synthesis and plasma cholesterol is likely dependent upon their tissue concentrations. Although garlic and garlic preparations have been shown to reduce (11–14, 39) or have no effect (15–17) on the plasma concentration of cholesterol and TG, the reported reduction of plasma concentration of cholesterol was mild with a mean of 9% according to the meta-analysis of Warshafsky *et al*. (39). Whether the low magnitude of the reduction in plasma cholesterol concentration is attributable to low tissue level of potential active components of garlic remains to be established. The reason for the contradictory observations of the effect of garlic on cholesterol is uncertain, but it may be explained in part by nonstandardized experimental designs. In addition, the present study further suggests the following parameters as important determinants of whether garlic affects plasma lipids: (i) the type of garlic preparations, e.g., garlic powder, garlic oil, or aged garlic extract, used in different studies; (ii) active components, e.g., lipid-soluble allicin and vinyl dithiin oils or water-soluble alk(en)yl cysteines, available in garlic preparations; and (iii) if the amount of active components is sufficient to achieve the plasma concentration required for inhibition of hepatic cholesterol and TG biosynthesis.

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# Regulation by Long-Chain Fatty Acids of the Expression of Cholesteryl Ester Transfer Protein in HepG2 Cells

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**ABSTRACT:** Cholesteryl ester transfer protein (CETP) is an important determinant of lipoprotein function, especially high density lipoprotein (HDL) metabolism, and contributes to the regulation of plasma HDL levels. Since saturated and polyunsaturated fatty acids (FA) appear to influence the CETP activity differently, we decided to investigate the effects of FA on the expression of CETP mRNA in HepG2 cells using an RNA blot hybridization analysis. Long-chain FA (>18 carbons) at a 0.5 mM concentration were added to the medium and incubated with cells for 48 h at 37°C under 5% $CO_2$. After treatment with 0.5 mM arachidonic (AA), eicosapentaenoic (EPA), and docosahexaenoic acid (DHA), the levels of CETP mRNA were less than 50% of the control levels (AA, $P = 0.0005$; EPA, $P < 0.01$; DHA, $P < 0.0001$), with a corresponding significant decrease in the CETP mass. These results suggest that FA regulate the gene expression of CETP in HepG2 and this effect is dependent upon the degree of unsaturation of the acyl carbon chain in FA.

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Cholesteryl ester transfer protein (CETP) is known to be a key protein in reverse cholesterol transport. By promoting the transfer and exchange of neutral lipids among plasma lipoproteins (1), it determines the plasma lipoprotein profile. A CETP deficiency markedly elevates plasma high density lipoprotein levels and decreases low density lipoprotein levels (2). The normal plasma CETP concentration of normolipidemic subjects ranges between 1.1 and 1.7 mg/L (3,4). CETP cDNA clones have been isolated from humans, cynomolgus monkeys, rabbits, and hamsters, with about an 80–95% sequence homology among the species (5–8). In humans, the organs with the most abundant expression of CETP mRNA are the liver, spleen, and adipose tissue, with lower expression levels in the small intestine, adrenal glands, kidney, and heart (5,6).

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Abbreviations: AA, arachidonic acid; aLA, α-linolenic acid; BSA, bovine serum albumin; C/EBP, CCAAT/enhancer binding protein; CETP, cholesteryl ester transfer protein; DgLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; FA, fatty acid; gLA, γ-linolenic acid; LA, linoleic acid; LCAT, lecithin-cholesterol acyltransferase; LPL, lipoprotein lipase; OA, oleic acid; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid; SA, stearic acid; SDS, sodium dodecyl sulfate; SREBP, sterol regulatory element-binding protein; SSPE, saline-sodium dihydrogenphosphate-ethylenediaminetetraacetate buffer.

The level of CETP mRNA is responsive to various environmental factors (9,10). High-fat and high-cholesterol diets (i.e., atherogenic diets) have been shown to raise both the mass and mRNA levels of CETP (6,8,11), whereas marine lipids, mainly n-3 polyunsaturated fatty acids (PUFA), have been shown to reduce plasma cholesterol and triglyceride levels (12) and to decrease the CETP mass level in hypercholesterolemic subjects (13). A recent study also reported that plasma CETP activity increased in a palmitic acid-rich diet in comparison to a stearic acid (SA)-rich diet (14) and that dietary *trans* fatty acids (FA) (e.g., elaidic acid) significantly increased CETP activity in comparison to diets rich in either SA, linoleic acid (LA) or palmitic acid (15,16).

*In vitro* studies with CaCo-2 cells (17) and HepG2 cells (18) have demonstrated that oleate or butyrate can up-regulate CETP secretion. Although the effects of various dietary FA on plasma CETP activity have been reported (19,20), whether various types of FA regulate the synthesis or expression of the CETP mass and/or the mRNA has yet to be explained.

To elucidate the action of dietary FA on CETP expression, we investigated the effects of long-chain FA on the mRNA and protein levels of CETP using HepG2 cells as an *in vitro* model.

## MATERIALS AND METHODS

*Cell culture*. The human hepatoma cell line HepG2 was purchased from the Riken gene bank (Wako, Japan), and the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 265 µg/mL thymidine (all from Life Technologies Ltd., Grand Island, NY) and 10% (vol/vol) fetal calf serum (Intergen Co., Purchase, NY) at 37°C under 5% $CO_2$. For the experiments described herein, the cells were seeded in 60-mm diameter collagen-coated culture dishes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at a density of $2 \times 10^5$/dish and then were cultured to approximately 90% confluency.

*FA treatments*. HepG2 cells were grown to 90% confluency in the same medium and then were incubated with 10% (vol/vol) fetal calf lipoprotein-deficient serum-DMEM supplemented or not with 0.5 mM of FA: SA (18:0), oleic (18:1, OA), LA (18:2), alpha-linolenic (18:3, aLA), gamma-linolenic (18:3, gLA), arachidonic (20:4, AA), eicosapentaenoic (20:5, EPA) and docosahexaenoic (22:6, DHA) acids, which were dissolved in 10% essential FA-free bovine serum albumin (BSA) (all from the Sigma Chemical Co., St. Louis, MO).

Following a 48-h incubation at 37°C, cell-conditioned media were collected to determine the CETP mass, and the cells were dissolved in a denaturing solution for RNA extraction.

*Northern blot analysis*. Total RNA was isolated using an acid guanidinium thiocyanate-phenol-chloroform extraction method (21). The yield of purified RNA samples was determined by absorbance at 260 nm. RNA samples (15 µg per lane) were separated by electrophoresis in 1% agarose/5.5% formaldehyde gel containing 0.84 µM ethidium bromide, followed by capillary transfer to nylon membranes (Nytran-N, Schleicher & Schuell GmbH, Dassel, Germany) that were then cross-linked by exposure to ultraviolet light. The membranes were prehybridized (4 to 5 h) at 42°C, and then were probed overnight at 42°C with a CETP cDNA fragment (365–624 bp) which had been labeled with [alpha-$^{32}$P]dCTP (NEN Life Science Products, Inc., Boston, MA) using a commercial kit (Random Primer DNA Labeling Kit; Takara Co., Otsu, Japan) in 5 × saline-sodium phosphate-ethyltetraacetate buffer (SSPE), 5 × Denhardt's, 10% dextran sulfate, 50% deionized formamide, 1% sodium dodecylsulfate (SDS), 0.2 mg/mL of heat-denatured salmon sperm DNA, and 1 mg/mL of BSA. The blot was washed twice at 42°C and twice further at 50°C for 30 min with 2 × saline-sodium citrate buffer (SSC), 0.2% SDS and then was exposed to an imaging plate (Fuji Film, Tokyo, Japan). The mRNA levels were quantitated by estimating the photostimulated luminescence per area of the corresponding band with an imaging analyzer (BAS 2000; Fuji Film). The data obtained were normalized for a glyceraldehyde phosphate dehydrogenase level. Northern hybridization detected CETP mRNA in HepG2 dose-dependently ($r = 0.977$, $P < 0.0001$) when the total RNA per lane varied in mass from 0 to 20 µg.

*Measurement of the CETP mass*. The CETP mass secreted by HepG 2 cells into 4 mL of culture medium/60-mm i.d. dish was measured using an enzyme-linked immunosorbent assay (ELISA) (22). One milliliter of a cultured medium was concentrated to about 100 µL using Microcon-10 (Amicon, Inc., Beverly, MA), and then the CETP mass of the concentrated medium was determined using the CETP (Chugai) ELISA kit (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan). The results obtained were then adjusted to the original volume.

*Analysis of the FA content in HepG 2 cells*. The cells were washed three times with PBS without $Ca^{2+}$/$Mg^{2+}$ before the cell lipids were extracted by the method of Folch *et al.* (23). The lipids were analyzed after methylation using HCl/methanol with margaric acid (17:0) as an internal standard as described in a previous paper (24). The FA content was then measured by gas–liquid chromatography (PerkinElmer Auto System GC, Palo Alto, CA) on an Rscot Sillier 5CP capillary column (0.25 mm i.d. × 50 m; Nihon Chromato Works Ltd., Tokyo, Japan). The protein concentration of the cells was determined with a Micro BCA Protein Assay Reagent kit (Pierce Laboratories Inc., Rockford, IL) using BSA as a standard.

*Statistical analysis*. The results are presented as the mean ± SE. Statistical comparisons of the experimental groups and a linear regression analysis were performed using one-way analysis of variance, and each group was compared with each other by Fisher's protected least significant difference test. The level of significance was set at $P < 0.05$. Analyses were performed using the StatView J4.51.1 software package (Abacus Concepts Inc., Berkeley, CA).

## RESULTS

*Expression of CETP by FA treatment.* To investigate the influence of FA on the CETP mRNA levels, HepG2 cells were treated with 0.5 mM of various kinds of FA. After 48 h of incubation, both the cell growth and morphology were normal in these treatments. Figures 1A and 1B show the effect of various FA on the levels of mRNA and protein of CETP in the HepG2 cells, respectively. After treatment with 0.5 mM of AA, EPA, and DHA, the expression of CETP mRNA was less than 50% of that of the control (Fig. 1A, $P < 0.0001$). De-

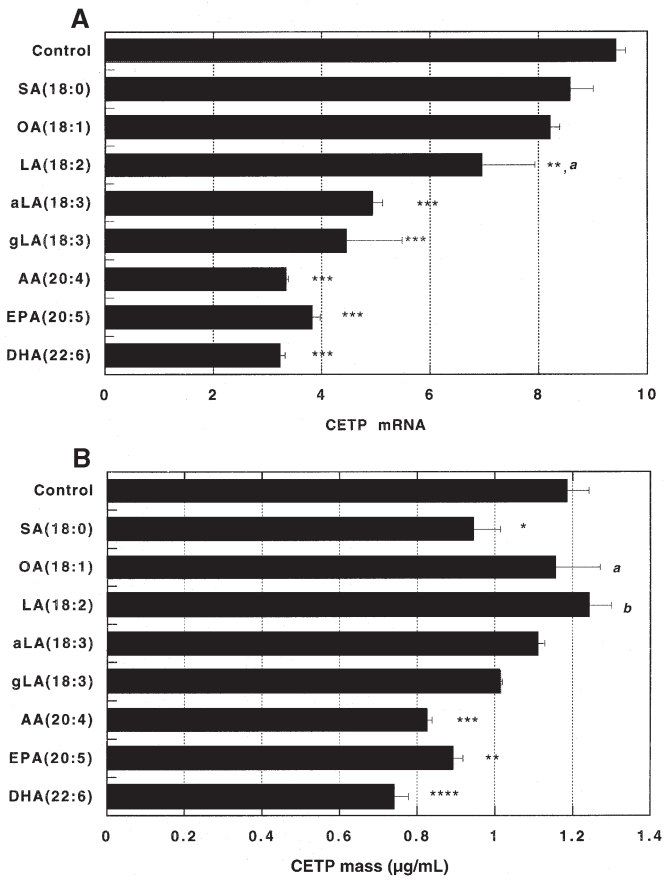


**FIG. 1.** Comparative effects of various saturated and unsaturated fatty acids (FA) on the expression of the cholesteryl ester transfer protein (CETP) mRNA (A) and mass (B). HepG2 cells were incubated with 0.5 mM of various FA or 1.25% bovine serum albumin (as a control) for 48 h in Dulbecco's modified Eagle's medium with 10% lipoprotein-deficient serum. The CETP mRNA and mass were measured as described in the text. Each data point represents the mean ± SE from three dishes. In A, statistical differences are shown as $^{**}P < 0.005$, $^{***}P < 0.0001$ relative to the control; and in B, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P = 0.0005$, $^{****}P < 0.0001$; and in both, $^{a}P < 0.05$, $^{b}P < 0.005$ relative to stearic acid (SA). OA, oleic acid; LA, linoleic acid; aLA, alpha-linoleic acid; gLA, gamma-linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

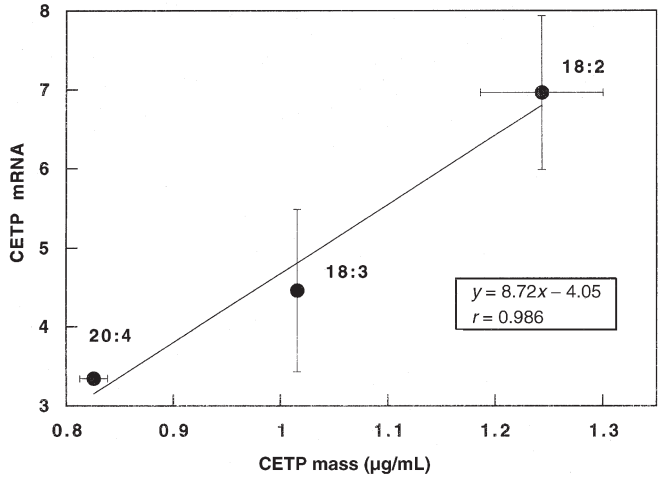


**FIG. 2.** Relationship between the CETP mRNA and mass secreted into the medium after the treatment of n-6 FA. A linear regression analysis demonstrates a positive correlation between CETP mRNA and mass ($r$ = 0.986). Each data point represents the mean ± SE from three dishes. For abbreviations see Figure 1.

creases in the CETP mRNA levels correlated with the increases in the degree of unsaturation of FA. As shown in Figure 1B, AA, EPA, and DHA also induced a significant decrease, relative to the control, in the CETP mass levels that correlated with the expression of CETP mRNA (AA, $P$ = 0.0005; EPA, $P < 0.01$; DHA, $P < 0.0001$). On the other hand, in the presence of 0.5 mM of SA, OA, and LA, each of which contains 18 acyl carbons, the induction of the CETP mass was facilitated as the degree of unsaturation increased (OA vs. SA, $P < 0.05$; LA vs. SA, $P < 0.005$), even though the mRNA level decreased in these cases.

*Correlation between the CETP protein mass and the mRNA level.* To analyze the correlation between the CETP mass and the mRNA level, we plotted the levels of the CETP mass and mRNA obtained after treatment with both the n-6 and n-3 series of FA, respectively (Figs. 2,3). These plots indicated that the n-6 and n-3 series each reduced both the CETP mass and mRNA level as the degree of unsaturation increased. The degree of unsaturation was strongly related to the expression of CETP if FA were classified into two groups consisting of the n-6 and n-3 series. Furthermore, the CETP mass and the mRNA levels both showed a positive correlation in this experiment (n-6, $r$ = 0.986; n-3, $r$ = 0.998).

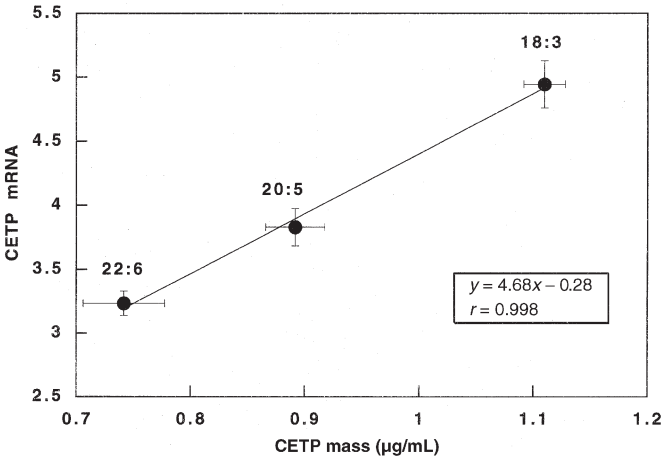


**FIG. 3.** Relationship between the CETP mRNA and mass secreted into the medium after the treatment of n-3 FA. A linear regression analysis demonstrates a positive correlation between CETP mRNA and mass ($r$ = 0.998). Each data point represents the mean ± SE from three dishes. For abbreviations see Figure 1.

*Incorporation and metabolism of FA.* As shown in Table 1, each FA added to the media increased the cellular FA concentration in comparison to the control, which was incubated without FA, thus indicating that FA were incorporated into HepG2 after 48 h of incubation. The metabolism of PUFA in humans and other animals proceeds by both chain elongation, such as biosynthesis of dihomo-γ-linolenic acid (DgLA, 20:3n-6) from gLA (18:3n-6), and desaturation, such as biosynthesis of gLA (18:3n-6) from LA (18:2n-6). For example, in the n-9 series, the concentration of OA (18:1n-9) and 20:3n-9, the metabolites of SA (18:0n-9), increased from 639.7 to 1424.9 nmol/mg protein and from undetectable levels to 78.2 nmol/mg protein after 48 h of incubation, respectively. In the n-6 series, DgLA and AA (20:4n-6), the metabolites of LA, were undetectable but rose to 172.1 nmol/mg protein and from 38.0 to 65.2 nmol/mg protein, respectively. Similarly in the n-3 series, 20:4n-3, the undetectable metabolite of aLA, increased to 203.8 nmol/mg protein. By the addition of LA, DgLA increased to 172.1 nmol/mg protein, while AA rose to a level that was 27.2 nmol/mg protein more than the control. Similarly, 20:4n-3 increased to 203.8 nmol/mg protein, while EPA (20:5n-3) was not detectable after treatment with aLA. These results supported the findings of previous reports in which the low activity of Δ5-desaturase in HepG2 resulted in the accumulation of DgLA or 20:4n-3 (24).

## DISCUSSION

These studies demonstrate that FA influence both the expression of CETP mRNA and protein in HepG2 cells. The human hepatoma-derived cell line HepG2 reveals many characteristic differences from normal differentiated hepatocytes, and very low levels of CETP gene expression and CETP activity in the medium have been reported (18,25,26). In the present study, using a northern blot analysis, we succeeded in detecting low amounts of radioactivity in bands corresponding to CETP mRNA.

FA of various chain lengths and degrees of unsaturation have been reported to affect the expression of enzymes and proteins involved in lipid metabolism (27–31). For example, previous studies reported that long-chain FA increase lipoprotein lipase (LPL) mRNA but reduce the degree of LPL activity, therefore presumably regulating the posttranslational processing of LPL (29). Skretting *et al.* (30) also found that lecithin-cholesterol acyltransferase (LCAT) activities and mRNA levels in HepG2 cells decreased due to a posttranscriptional mechanism following sodium butyrate treatment. Faust *et al.* (17) reported that CETP secretion measured by

**TABLE 1**
**Composition of Total Fatty Acid in HepG2 Cells[a]**

| Added PUFA | 18:0 | 18:1n-9 | 18:2n-6 | 18:3n-3 | 20:3n-9 | 20:3n-6 | 20:4n-6 | 20:4n-3 | 20:5n-3 | 22:4n-6 | 22:6n-3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 128.9 ± 15.5 | 639.7 ± 86.7 | 14.2 ± 1.9 | ND | ND | ND | 38.0 ± 4.9 | ND | ND | ND | ND |
| 18:0 | 540.4 ± 34.1[e] | 1424.9 ± 73.6[d] | 9.6 ± 4.9 | ND | 78.2 ± 4.3 | ND | 46.7 ± 2.5 | ND | ND | ND | ND |
| 18:1n-9 | 131.9 ± 23.0 | 1738.4 ± 281.6[e] | 9.85 ± .05 | ND | 88.8 ± 14.5 | ND | 34.9 ± 5.5 | ND | ND | ND | ND |
| 18:2n-6 | 216.4 ± 29.4[a] | 502.0 ± 68.4 | 1275.4 ± 154.2[e] | ND | ND | 172.1 ± 21.2 | 65.2 ± 9.4[b] | ND | ND | ND | ND |
| 18:3n-3 | 179.0 ± 7.6 | 446.7 ± 18.8 | 14.3 ± 0.8 | 1115.3 ± 50.5 | ND | ND | 34.7 ± 1.3 | 203.8 ± 6.5 | ND | ND | ND |
| 20:4n-6 | 153.1 ± 2.3 | 228.9 ± 5.9[a] | 7.1 ± 1.2 | ND | ND | ND | 633.1 ± 12.2[e] | ND | ND | 226.1 ± 9.8 | ND |
| 20:5n-3 | 234.9 ± 9.6[c] | 422.9 ± 19.0 | 15.0 ± 3.2 | ND | ND | ND | 42.8 ± 1.7 | ND | 619.7 ± 42.7 | ND | 513.7 ± 37.5 |
| 22:6n-3 | 148.4 ± 26.9 | 352.9 ± 63.6 | 11.5 ± 1.7 | ND | ND | ND | 28.9 ± 4.9 | ND | 9.4 ± 5.9 | ND | 911.0 ± 146.4 |

[a]Data are expressed as nmol/mg cell protein and are the means ± SE from three dishes. ND, not detectable. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.005$; [d]$P < 0.0005$; [e]$P < 0.0001$; significantly different from the control according to the analysis of variance and Fisher's protected least significant difference test.

the CETP activity was regulated in response to the OA concentration in CaCo-2 cells.

We therefore investigated whether various types of FA, particularly those with different degrees of unsaturation, influenced the expression of CETP. The results demonstrated that increasing degrees of unsaturation of FA reduced the CETP mRNA levels; for example, the expression of the CETP mRNA decreased to less than 50% of that of the control with 0.5 mM of AA, EPA, or DHA (Fig. 1A). The CETP mass levels also decreased significantly based on the levels of CETP mRNA expression. These results were also supported by the findings of previous studies, which suggested that the mRNA level is a major determinant of secreted CETP protein (11,26). The increasing FA concentration in HepG2 cells after FA treatments also indicated that the FA influenced the expression of CETP mRNA and protein (Table 1). Although the mechanism of regulation of CETP protein secretion by FA remains unknown, some interesting findings have appeared. Agellen *et al.* (26) indicated that CCAAT/enhancer binding protein (C/EBP)-α levels influence CETP mRNA expression, since C/EBP-α binds to the site of the CETP gene promoter and activates the CETP promoter activity. Raclot *et al*. (27) found that C/EBP-α mRNA level is influenced by PUFA in the following order: DHA-rich diets > EPA-rich diets > OA-rich diets. Our observations are in accord with this finding, which may, in part, suggest that the mechanisms for the modulation of the mRNA levels by PUFA occur indirectly through the expression levels of C/EBP because the transcriptional levels of CETP are partly dependent on C/EBP. Ritsch *et al.* (32), however, reported that C/EBP had no specific influence on the expression of CETP in HepG2. Another possible mechanism for the regulation of the CETP gene expression by FA may be the influence of apolipoprotein regulatory protein-1 (ARP-1) and/or v-erbA-related proteins-3 (Ear-3)/chicken ovalbumin upstream promoter transcription factor (COUP-TF) (33), which are orphan receptors, and sterol regulatory element-binding proteins (SREBP) (34,35). These transcriptional factors, especially SREBP-1 (34,35), play an important role in controlling CETP gene expression. Kim *et al.* (36) described how SREBP-1 expression in the liver nuclei of fish oil-fed mice decreased by 57% compared with safflower oil-fed mice. Yahagi *et al.* (37) reported that dietary PUFA drastically decreased the mature, cleaved form of SREBP-1 protein in the nucleus in the liver of wild-type mice, presumably due to the reduced cleavage of the SREBP-1 precursor protein. Therefore, the regulation of the CETP gene expression by FA may be mediated by the cooperative interaction with SREBP-1 and other transcriptional factors.

On the other hand, the concentration of FA in lipoprotein particles influences the CETP-mediated cholesteryl ester transfer activity *in vitro* (20,38,39); that is, a low concentration of FA stimulates and promotes CETP activity while increasing the FA concentrations above an optimal level, thus inhibiting the CETP activity in a dose-dependent manner. Both the length and degree of unsaturation of the FA acyl carbon chain also affect the CETP activity. The CETP activity is

thus likely to be influenced by double bonds in the *trans* instead of the natural *cis* configuration (15,16); however, we did not investigate the effects of *trans* fatty acids on the gene expression of CETP. Further studies are needed to reveal whether a different configuration (*cis*- or *trans*-) of FA possibly controls the expression of CETP mRNA.

Our present data indicate that FA regulate gene expression of CETP in HepG2, and this effect is dependent upon the degree of unsaturation of the acyl carbon chain in FA. FA may therefore be convenient modulators of both the CETP expression level and the cholesteryl ester transfer activity.

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# Modulation of Prostaglandin H Synthase Activity by Conjugated Linoleic Acid (CLA) and Specific CLA Isomers

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**ABSTRACT:** Conjugated linoleic acid (CLA) has been shown to inhibit tumorigenesis in animal models and is cytostatic to numerous cell lines *in vitro*. However, the mechanism of action is unknown. In the current study, we determined the effects of CLA and specific isomers of CLA on the rate of oxygenation of arachidonic acid by prostaglandin H synthase (PGHS) in ram seminal vesicle microsomes. The enzyme was incubated with 0.1 to 100 μM CLA or specific isomers of CLA for 2 min prior to the addition of 44 to 176 μM arachidonate. The isomers tested were 9(*E*),11(*E*) CLA; 9(*Z*),11(*E*) CLA; 9(*Z*),11(*Z*) CLA, and 10(*E*),12(*Z*) CLA. For a positive inhibitor control, flurbiprofen was used at 0.75 to 2.50 μM. Enzyme activity was assessed by measuring the rate of oxygen consumption. Inclusion of CLA or specific isomers of CLA in the incubation mixtures inhibits PGHS. The efficacy differs for each isomer, with the 9(*Z*),11(*E*) CLA isomer being the most effective and the 9(*Z*),11(*Z*) CLA isomer being the least effective inhibitor among the four CLA isomers tested. The $K_i$ values obtained by Dixon replots range from 18.7 μM for the most effective isomer, 9(*Z*),11(*E*) CLA, to 105.3 μM for the least effective isomer, 9(*Z*),11(*Z*) CLA. The $K_i$ value for flurbiprofen with ram seminal vesicle microsomes was 0.33 μM. As the concentration of arachidonate was increased, the CLA-dependent inhibition of PGHS decreased, suggesting competitive inhibition. The results of this study demonstrate the potential of CLA and specific isomers of CLA to modulate prostaglandin biosynthesis.

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Conjugated linoleic acid (CLA) is a term used to describe a mixture of positional and geometric isomers of linoleic acid (1,2). Dietary CLA has been shown to inhibit tumorigenesis in a number of animal models, which is in distinct contrast to the effects of the parent compound linoleic acid (3–8). More recently, the suppression of mammary tumor growth by CLA was reported to be lost when the content of CLA in the neutral lipid fraction of mammary gland tissue is depleted (9). Although the nutritional requirements for observing the tumor-inhibitory effects of CLA have been fairly extensively investigated, very little is known about the mechanism by which the compounds act.

In addition to the whole animal studies reported above, *in vitro* studies on several cell lines have shown CLA to be cytostatic when present in the culture medium at concentrations in the micromolar range (10). As in the tumor studies just mentioned, the precise mechanism by which CLA modulates biological activity is unknown although some studies have suggested the compound may have antioxidant activity, inhibit peroxidases, alter the response to estrogen, interact with peroxisome proliferator-activated receptors (PPAR), or perturb eicosanoid biosynthesis (5,6,11–15). CLA has also been shown to reduce prostaglandin $E_2$ ($PGE_2$) production in phorbol ester-induced events in murine keratinocytes and mouse epidermis (16–18). This leads to the important question of whether the mixture of CLA isomers, a specific isomer of CLA, or a metabolite of CLA functions as the active agent.

Three families of enzymes are responsible for the initial generation of bioactive lipid oxidation products from linoleic acid (LA) and arachidonic acid (AA). Cyclooxygenases, lipoxygenases, and cytochromes P-450 all produce relatively short-lived, pluripotent products that play a role in a myriad of processes at both the cellular and higher levels of organization (19–21). Numerous studies have shown that modulation of the activity of these oxidative enzymes has significant consequences for biochemical processes in both normal and pathologic situations (22–24).

Given the structural similarities between many of the bioactive lipid oxidation products and CLA, in particular the presence of a conjugated diene moiety, it is conceivable that CLA itself may interact with either the enzymes involved in the generation of these bioactive lipids or the ultimate cellular receptors of these bioactive compounds. Therefore, to investigate the former possibility, the present investigations were undertaken to determine if CLA and specific isomers of CLA are capable of altering the activity of a representative cyclooxygenase, prostaglandin H synthase-1 (PGHS).

## EXPERIMENTAL PROCEDURES

Ram seminal vesicle (RSV) microsomes were used as the source of PGHS and were prepared in our laboratory. CLA, a mixture of isomers, was obtained from Nu-Chek-Prep Inc. (Elysian, MN). According to the manufacturer, the isomer composition was 41% 9(*Z*),11(*E*) and 9(*E*),11(*Z*) CLA; 44% 10(*E*),12(*Z*) CLA; 10% 10(*Z*),12(*Z*); 5% 9(*Z*),11(*Z*), 9(*E*),11(*E*), and 10(*E*),12(*E*) CLA; and less than 1% 9(*Z*),12(*Z*) linoleate. The following specific isomers of CLA were obtained from Matreya (Pleasant Gap, PA), and their structures are shown in Scheme 1: 9(*Z*),11(*E*) CLA,

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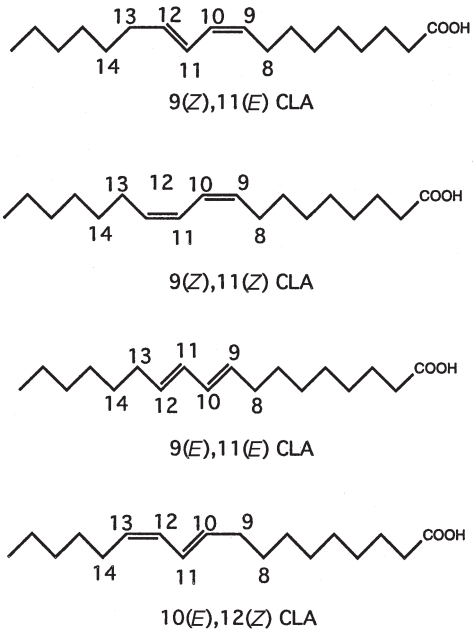
Abbreviations: AA, arachidonic acid; CLA, conjugated linoleic acid; DDC, diethyldithiocarbamate; LA, linoleic acid; $PGE_2$, prostaglandin $E_2$; PGHS, prostaglandin H synthase-1; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acids; RSV, ram seminal vesicle.




**SCHEME 1**

9(*Z*),11(*Z*) CLA, 9(*E*),11(*E*) CLA, and 10(*E*),12(*Z*) CLA. All other reagents were obtained from routine laboratory suppliers and were of the highest grade available.

*PGHS.* RSV, a tissue source high in PGHS, were used to prepare RSV microsomes for enzyme assays. The RSV were homogenized in 100 mM potassium phosphate pH 7.8, containing 300 μM diethyldithiocarbamate (DDC), 250 mM mannitol, and 10 mM EDTA using a Polytron tissue homogenizer. The RSV microsomes were purified through a series of three centrifugations. After retaining the supernatant for the first two steps and the pellet for the last, the homogenate was centrifuged at 650 × *g* for 15 min, 8700 × *g* for 10 min, and then ultracentrifuged at 100,000 × *g* for 80 min. The pellet was resuspended in 10 mM Tris pH 8.0, containing 0.5 mM EDTA, 1% Tween 20, and 300 μM DDC for storage at −80°C until use. The protein concentration was determined by the method of Bradford (25).

*Enzyme assays*. Enzyme activity was determined by measuring the rate of oxygen consumption using a Clark electrode obtained from Yellow Springs Instruments Inc. (Yellow Springs, OH). The electrode was connected to a strip chart recorder to monitor changes in oxygen concentration. Reactions were maintained at a constant temperature of 37°C using a Neslab Endocal circulating bath. For all experiments, incubations were performed in duplicate with control incubations at the beginning and end of a series of incubations to assess possible loss of enzyme activity over the course of the experimental period.

The rate of oxygen consumption by PGHS, using AA as substrate, was determined in 1.0-mL incubations containing the following components: 100 mM Tris buffer pH 7.8, 150 μg RSV microsomal protein, 0.5 mM phenol, 44–176 μM AA, and various concentrations of inhibitor. The compounds tested for inhibition were CLA, specific isomers of CLA, flurbiprofen, LA, and oleic acid. The term CLA is used to describe the commercially available mixture of isomers described above. Fatty acids were delivered as solutions in ethanol. The final ethanol concentration did not exceed 2.0%. The CLA, or specific isomers of CLA, were added to the reaction 2 min prior to the substrate, AA. Reactions were monitored for 3 min after substrate addition, then terminated by the addition of sodium dithionite. The initial concentration of oxygen was assumed to be 190 μM based on $O_2$ solubility at 37°C, and the concentration of oxygen after the addition of sodium dithionite was taken to be 0 μM (26). The rate of oxygen consumption reported was measured at maximal velocity, which typically occurred 0.3–1 min after substrate addition. The rate of nonenzymatic oxygenation of AA was monitored and subtracted from all incubations.

## RESULTS

The activity of PGHS, using AA as substrate, is readily determined by monitoring the uptake of molecular oxygen by means of a Clark electrode. In the following experiments, the effect of CLA, and specific isomers of CLA, on PGHS activity was determined. A representative experiment is shown in Figure 1. As shown, the addition of 9(*Z*),11(*E*) CLA inhibited the rate, and extent, of oxygen consumption catalyzed by RSV microsomal PGHS. Additional incubations were performed to determine whether CLA is a substrate for PGHS and whether the observed inhibition of PGHS is CLA-specific. When CLA, at concentrations up to 100 μM, was incubated with PGHS in the absence of AA, no oxygen uptake was detected. Thus, CLA does not serve as a substrate for the enzyme (data not shown).

It is conceivable that the observed inhibition of PGHS by CLA is not specific for CLA but is due to an alteration in substrate availability as a result of altered partitioning in the presence of an increased fatty acid concentration. To examine this possibility, 100 μM oleic acid was substituted for CLA in the presence of 44 μM AA. The observed reaction rates were 105 ± 11.3 μM $O_2$/min in the controls and 103 ± 17.0 μM $O_2$/min in the presence of oleic acid. Therefore, the observed effects on PGHS are CLA-specific.

On the basis of the preceding results, a series of experiments was performed to evaluate the concentration dependence for the substrate, AA, and the inhibitors CLA and specific isomers of CLA. The concentration of AA was varied from 44 to 176 μM, and the concentration of CLA, or the specific isomers of CLA, was varied from 0.1 to 100 μM. Lineweaver-Burk and Dixon plots were used to determine $K_m$, $V_{max}$, and $K_i$ for each inhibitor. In the absence of inhibitors, the RSV microsomal protein with AA as substrate yielded a $V_{max}$ of 1177 μM $O_2$/mg·min and a $K_m$ of 84.5 μM. The reported $K_m$ value for homogeneous PGHS is 10 μM (27). The higher $K_m$ with RSV microsomes is most likely due to the fact that the preparation of microsomes does not yield pure PGHS.

In Dixon analysis, the manner in which the lines intersect allows one to determine the type of inhibition(28). From the transformation of the data to 1/*V* vs. [*I*], shown in Figure 2, the commercial CLA mixture and the specific isomers of CLA

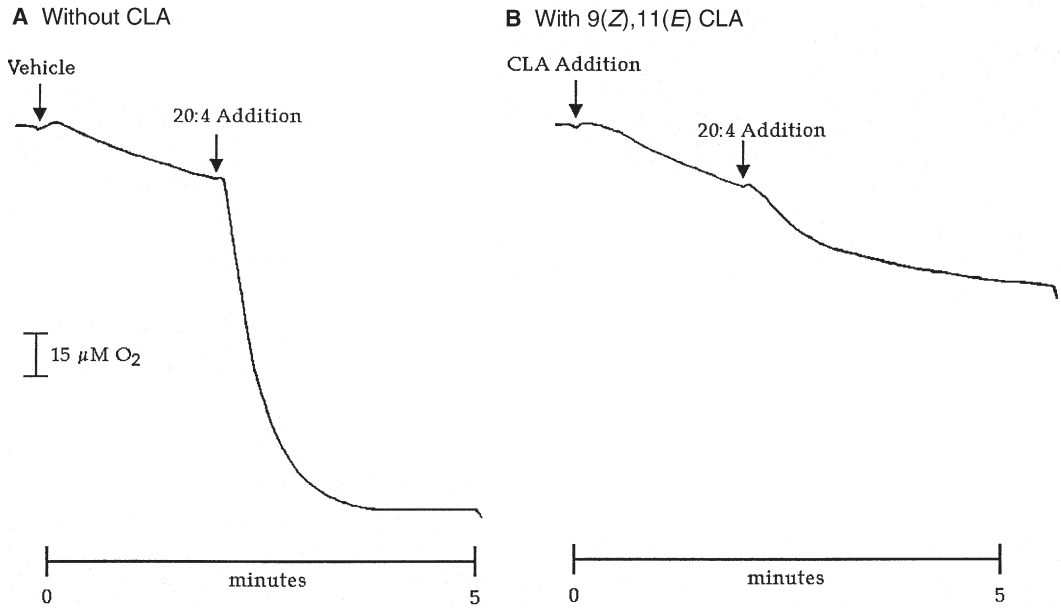



**FIG. 1.** Representative oxygraph recording of the effect of 9(*Z*),11(*E*) conjugated linoleic acid (CLA) on prostaglandin H synthase (PGHS). (A) PGHS in the absence of CLA. (B) PGHS in the presence of 100 μM 9(*Z*),11(*E*) CLA.

appear to exert competitive inhibition. The degree of inhibition of PGHS was concentration-dependent and isomer-specific. The 9(*Z*),11(*E*) CLA isomer was the most potent of the four isomers tested. The least effective was 9(*Z*),11(*Z*) CLA. Furthermore, whereas the degree of inhibition varied for each specific isomer, the extent of inhibition with the commercial mixture was not directly proportional to the isomer composition.

In a Dixon plot for the analysis of inhibition, the point where all lines intersect equals $-K_i$. Since this point is not always easy to identify, an additional method for determining $K_i$ values, the Dixon replot, was employed (28). This technique plots the slopes from the Dixon graph against 1/[*S*]. The resulting slope of the line in a Dixon plot is $K_m/V_{max}K_i$. Using this reciprocol replot and the previously determined $V_{max}$ and $K_m$ values, the $K_i$ values for CLA and the specific isomers of CLA are reported in Table 1. The $K_i$ values range from 18.7 to 105.3 μM with the 9(*Z*),11(*E*) CLA isomer having the lowest value and the 9(*Z*),11(*Z*) isomer having the highest.

To determine the potency of CLA as an inhibitor of PGHS, LA, a weak substrate of PGHS, and flurbiprofen, a known inhibitor of PGHS, were also tested. Addition of 100 μM LA reduced the rate of oxygen consumption in the presence of 44 μM AA. Unlike the results with CLA, the total extent of $O_2$ consumption by PGHS was not affected by LA. The observed reduction in reaction rate with 100 μM LA was equivalent to that of 100 μM 9(*Z*),11(*E*) CLA. The rate was reduced from 104.8 μM $O_2$/min to 22.9 ± 4.0 and 23.8 ± 1.6 μM $O_2$/min for LA and 9(*Z*),11(*E*) CLA, respectively. The effects of 0.75–2.50 μM flurbiprofen on RSV microsomes were also examined. The calculated $K_i$ for flurbiprofen in these experiments is 0.33 μM.

Finally, experiments were performed to investigate whether the inhibition of PGHS by CLA is time dependent. Either the commercial mixture or the 9(*Z*),11(*E*) isomer, 100 μM each, were incubated with PGHS for 2 and 30 min prior to the addition of AA. The results are shown in Table 2. The time of inhibitor preincubation did not affect the observed inhibition. For CLA, the percentage of control activity was 42.2 and 54.2% after 2- and 30-min preincubation, respectively. The more potent 9(*Z*),11(*E*) CLA gave 17.0% of control after 2 min and 16.3 % of control after 30 min of preincubation. A

**TABLE 1**
**$K_i$ Values for CLA, Specific CLA Isomers, and Flurbiprofen with RSV Prostaglandin H Synthase-1**

| Inhibitor | $K_i$ (μM)[a] | Normalized $K_i$[b] |
|---|---|---|
| CLA | 28.34 | 85.88 |
| 9(*Z*),11(*E*) CLA | 18.73 | 56.76 |
| 9(*Z*),11(*Z*) CLA | 105.32 | 319.15 |
| 9(*E*),11(*E*) CLA | 24.69 | 74.82 |
| 10(*E*),12(*Z*) CLA | 23.84 | 72.24 |
| Flurbiprofen | 0.33 | 1.00 |

[a]$K_i$ values were calculated using Dixon replot analysis (28).
[b]$K_i$ values normalized against the $K_i$ value for flurbiprofen reported by Rome and Lands (34). CLA, conjugated linoleic acid; RSV, ram seminal vesicle.

**TABLE 2**
**The Effect of Inhibitor Preincubation on Prostaglandin H Synthase-1**

| | Preincubation (2 min) | | Preincubation (30 min) | |
|---|---|---|---|---|
| | μM $O_2$/min[a] | % of control | μM $O_2$/min[a] | % of control |
| Control[b] | 147.5 ± 8.0 | | 133.0 ± 7.4 | |
| CLA[b] | 62.2 ± 6.1 | 42.2 | 72.1 ± 10.8 | 54.2 |
| 9(*Z*),11(*E*) CLA[b] | 25.1 ± 5.0 | 17.0 | 21.7 ± 3.4 | 16.3 |

[a]Rates are the average of duplicates. Errors are the standard deviation.
[b]Incubations were performed in 44 μM arachidonic acid in the presence of 100 μM inhibitor. Controls contained an equal volume of CLA solvent. For abbreviation see Table 1.

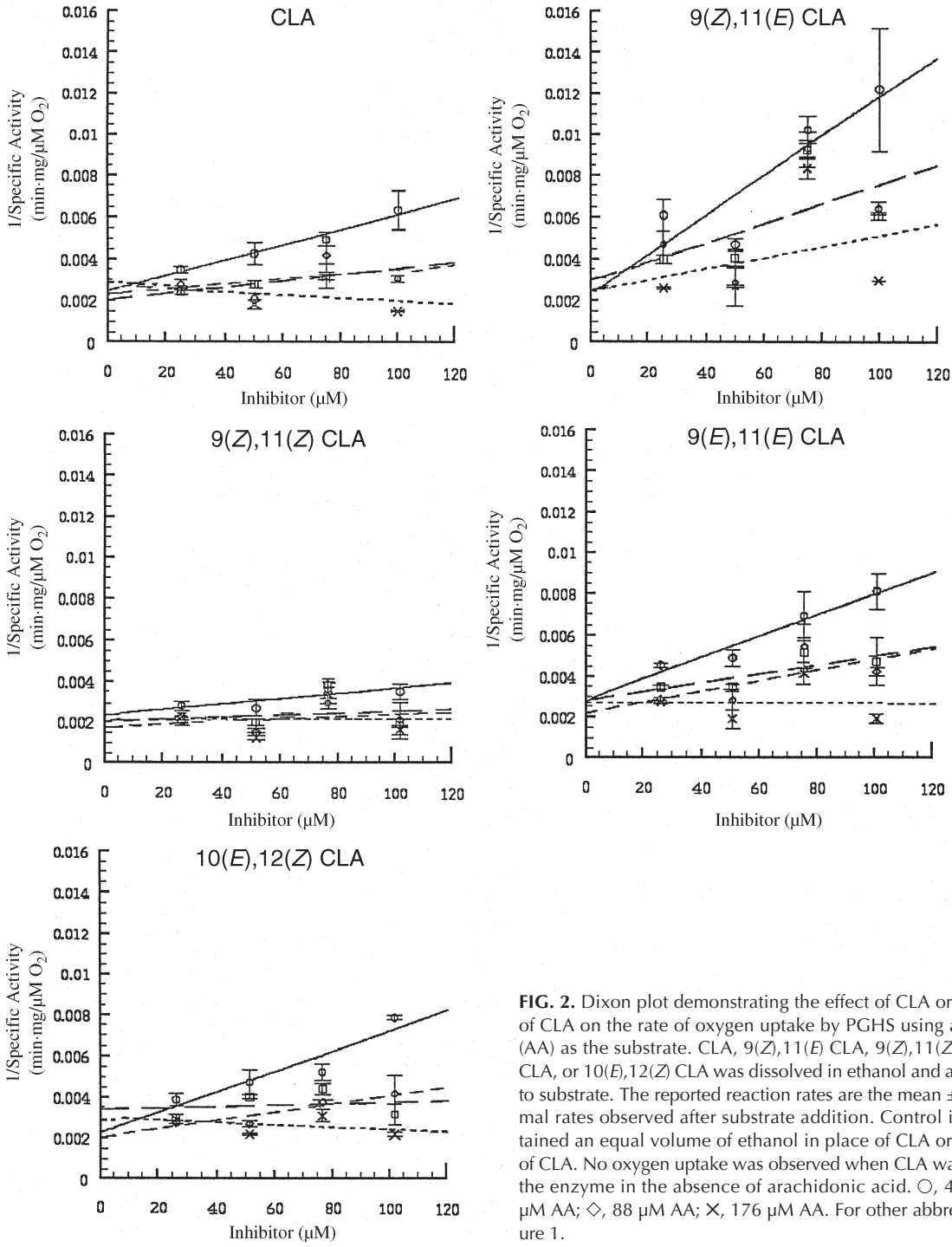



**FIG. 2.** Dixon plot demonstrating the effect of CLA or specific isomers of CLA on the rate of oxygen uptake by PGHS using arachidonic acid (AA) as the substrate. CLA, 9(*Z*),11(*E*) CLA, 9(*Z*),11(*Z*) CLA, 9(*E*),11(*E*) CLA, or 10(*E*),12(*Z*) CLA was dissolved in ethanol and added 2 min prior to substrate. The reported reaction rates are the mean ± SD of the maximal rates observed after substrate addition. Control incubations contained an equal volume of ethanol in place of CLA or specific isomers of CLA. No oxygen uptake was observed when CLA was incubated with the enzyme in the absence of arachidonic acid. ○, 44 μM AA; □, 66 μM AA; ◇, 88 μM AA; ×, 176 μM AA. For other abbreviations see Figure 1.

9.9% decrease in velocity was also observed in the controls between 2 and 30 min.

## DISCUSSION

The modulation of biological activities by CLA has not yet been fully characterized (7,11,12,29,30). A number of potential mechanisms have been suggested including antioxidant activity, inhibition of peroxidase activity, alteration of estrogen response, modulation of eicosanoid systems, and interaction with PPAR (5,6,11–15). Data in the present report provide support for one means by which CLA and specific isomers of CLA can modulate biological function. Specifically, these experiments demonstrate inhibition of PGHS by CLA and specific isomers of CLA. It is well known that prostaglandin biosynthesis is involved in tumor growth in numerous systems (22,31). For example, many colon tumors produce high levels of prostaglandins and other eicosanoids, and

inhibitors of prostaglandin biosynthesis inhibit colon tumorigenesis (22). Recently it was reported that in both cultured systems and whole animals, CLA treatment reduces $PGE_2$ production (16,18,32). The experiments in the present report are consistent with these observations; however, the concentrations of CLA required to inhibit PGHS activity may be beyond the physiologically significant range.

The mixture of fatty acids known as CLA contains compounds that have structural similarities to products derived from enzymatic polyunsaturated fatty acid (PUFA) oxygenation. In particular, the conjugated diene moiety of CLA is also present in hydroxyeicosatetraenoates and hydroxyoctadecadienoates. Owing to this conjugated diene moiety, CLA has the potential to function as a product analog, and such interactions could be one means by which CLA exerts biological activity. It is noteworthy that while CLA inhibits the oxygenation of AA by PGHS, CLA does not serve as a substrate for the enzyme.

From the data, we conclude that the efficacy with respect to the inhibition of PGHS differs for each isomer of CLA. The 9(*Z*),11(*E*) CLA isomer is the most effective inhibitor followed by the 9(*E*),11(*E*) CLA, 10(*E*),12(*Z*) CLA, the commercial mixture CLA, and finally the 9(*Z*),11(*Z*) CLA isomer. The fact the CLA mixture showed less inhibition than some of the individual isomers, and more than others, supports the evidence of differing isomer efficacy. Although the efficacy of the individual isomers is not consistent with the isomer composition of the commercial mixture, the most effective isomer is a major component in this mixture. It is interesting that the most potent isomer, 9(*Z*),11(*E*) CLA, is the primary isomer produced in the rumen by linoleate isomerase and is the isomer most preferentially incorporated into phospholipids (5,33).

To place the inhibition of PGHS by CLA in the proper context, we compared CLA and the specific isomers of CLA to LA, a weak substrate for PGHS, and flurbiprofen, a known inhibitor of PGHS. The rate of AA metabolism in the presence of 100 μM LA was equivalent to the rate observed in the presence of 100 μM 9(*Z*),11(*E*) CLA. As previously mentioned, CLA does not appear to be metabolized by PGHS, but it does compete for the active site as efficiently as the weak substrate LA. The known inhibitor of PGHS, flurbiprofen, was also compared to CLA. The $K_i$ obtained in the present experiments is 0.33 μM, which is somewhat smaller than the value of 1 μM reported by Rome and Lands (34), who also used crude PGHS. Thus, CLA is significantly less effective than flurbiprofen as an inhibitor. On the other hand, flurbiprofen is an especially potent PGHS inhibitor; therefore, comparisons to other inhibitors may be more enlightening. For example, if the $K_i$ value for flurbiprofen is adjusted to 1 μM and the values for CLA are normalized to this value, then CLA and the specific isomers of CLA appear to be as potent as indomethacin with respect to the inhibition of PGHS (34). Based on the Dixon analysis presented in Figure 2, the mode of inhibition of CLA and of the specific CLA isomers appears to be competitive. This would appear to be pure competitive inhibition as there was no evidence of time-dependent phenomena with CLA. The absence of time-dependent inhibition by CLA is in clear distinction to the effects of many PGHS inhibitors and may influence the *in vivo* efficacy of these fatty acids compared to xenobiotic inhibitors.

Of the four isomers tested, those containing *trans* geometry were the most potent inhibitors of PGHS, and the all-*cis* isomer, 9(*Z*),11(*Z*) CLA, was the weakest. Thus, the presence of a *trans* configuration, involving C-11 in conjugation with another double bond, may be important for inhibition of PGHS by CLA. Enzymatic oxidation of AA to $PGH_2$ involves an initial hydrogen abstraction at C-13 of AA. The C-11 of CLA is the same distance from the ω end of the molecule as the C-13 of AA. However, C-13 of AA is a doubly allylic carbon whereas C-11 of CLA is vinylic. This hydrogen abstraction of C-11 of CLA is relatively unfavorable, which may play an important role in the ability of CLA to block the active site of PGHS. Furthermore, it is unlikely the inhibition of PGHS by CLA involves covalent interactions as the inhibition is not time-dependent and CLA itself does not serve as a substrate for the enzyme.

In an effort to explain some of the biological activity of CLA, several recent reports have examined the possibility of antioxidant activity (5,6). However, other investigators have observed that CLA is a relatively weak antioxidant and produces oxidation products somewhat different from those usually observed with PUFA; thus this mechanism of inhibition is unlikely (35,36). The present report describes an alternative mechanism by which CLA can modulate biological activity, in particular the inhibition of fatty acid oxygenases. The inhibition of the cyclooxygenase activity of PGHS, reported in the present work, is in distinction to the previously reported inhibition of peroxidase-mediated activation of the food mutagen 2-amino-3-methylimidazo[4,5-f]quinoline by the peroxidase activity of PGHS (11). Therefore, modulation of both the cyclooxygenase and peroxidase activities of PGHS could contribute to the biological activity of CLA (37).

## ACKNOWLEDGMENTS

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# Hen Egg Yolk and White Contain High Amounts of Lysophosphatidic Acids, Growth Factor-Like Lipids: Distinct Molecular Species Compositions

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**ABSTRACT:** Hen egg yolk and white were found to contain high amounts of lysophosphatidic acid (acyl LPA) in addition to small amounts of lysoplasmanic acid (alkyl LPA). The levels of acyl LPA in hen egg yolk (44.23 nmol/g tissue) and white (8.81 nmol/g tissue) were on the same order as or higher than the levels of acyl LPA known to be required to elicit biological responses in various animal tissues. Noticeably, there is a marked difference between the fatty acid composition of egg yolk acyl LPA and of egg white acyl LPA; egg yolk acyl LPA predominantly contains saturated fatty acids as the acyl moiety, whereas egg white acyl LPA primarily contains polyunsaturated fatty acids. We found that the level of acyl LPA, especially polyunsaturated fatty acid-containing acyl LPA, in egg white was augmented markedly during the incubation at 37°C, while there was no change in egg yolk. We confirmed that egg white contains both the substrate, i.e., polyunsaturated fatty acid-containing lysophosphatidylcholine (LPC), and the enzyme activity catalyzing the hydrolysis of polyunsaturated fatty acid-containing LPC to the corresponding acyl LPA. Egg yolk LPA and egg white LPA may play separate physiological roles in the development, differentiation, and growth of embryos.

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Lysophosphatidic acid (1- or 2-acyl-*sn*-glycero-3-phosphate, acyl LPA) and lysoplasmanic acid (1-*O*-alkyl-*sn*-glycero-3-phosphate, alkyl LPA) are common precursor molecules of the *de novo* synthesis of a variety of glycerolipids in animal tissues. In addition to the role as a metabolic intermediate, it is becoming evident that LPA act as important intercellular mediators in various tissues and cells (1–6). LPA have been shown to exert diverse biological activities *in vitro* and *in vivo*. For example, LPA induce hypertension in rats (7), smooth muscle contraction (8), activation of human or feline platelets (9–11), cell proliferation (12), neurite retraction (13,14), neurotransmitter release (15), cell survival (16), apoptosis (17), tumor cell invasion (18), accelerated development of embryos (19), and stimulation of ovum transport in oviducts (20). It has long been assumed that these cellular responses are mediated through specific binding sites for LPA expressed on the cell surface (1–4), yet detailed characteristics of such binding sites remained obscure until recent cloning of the cDNA for LPA receptors (21–26). Although the exact physiological roles of LPA as messenger molecules are not yet fully elucidated, evidence is accumulating that LPA play some essential roles in embryonic development and differentiation (19,20,27,28) in addition to other pathophysiological processes such as wound healing (29).

What then are the actual tissue levels of LPA? Furthermore, what types of molecular species of LPA are present in animal tissues? Little information is available concerning these important issues except for a few cases such as rat brain (30,31), rat liver, heart, testis, kidney, and lung (31), rat plasma (32,33), human plasma (34–36), human serum (36), and human ascites fluids (37). The levels of LPA required to elicit biological responses differ considerably depending on the types of response as well as the tissues and cells involved (1–6), and the intrinsic biological activities differ markedly among various molecular species of LPA (7,8,11,13,38–45). Thus, detailed information concerning the tissue levels as well as the molecular species composition of LPA is essential for better understanding the physiological roles of LPA in animal tissues. Several years ago, Tigyi *et al.* (46) reported that an LPA-like molecule was present in human amniotic fluid, mouse ascites fluid, and human and rat cerebrospinal fluids, although neither the chemical structure nor the amounts of the LPA-like molecule were determined in their pioneering study. Also, even though information on levels as well as the molecular species composition of LPA in embryonic tissues is particularly important, little has appeared concerning LPA in embryonic tissues. Tokumura *et al.* (27,28) did demonstrate the occurrence and the production of LPA in human ovarian follicular fluids.

In the present study, we examined in detail whether LPA are present in hen eggs. Hen eggs were chosen for these reasons: (i) Hen eggs contain sufficient amounts of egg yolk and white for accurate lipid analyses. (ii) Hen eggs are often used in the field of developmental biology. We found that hen egg yolk and white contain high amounts of LPA. We also found that the molecular species composition of acyl LPA obtained

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Abbreviations: Acyl LPA, lysophosphatidic acid (1- or 2-acyl-*sn*-glycero-3-phosphate; alkyl LPA, lysoplasmanic acid (1-*O*-alkyl-*sn*-glycero-3-phosphate); GC, gas chromatography; LPA, monoradyl-*sn*-glycero-3-phosphate (lysophosphatidic acid, lysoplasmanic acid, and lysoplasmenic acid); LPC, lysophosphatidylcholine; MS, mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; TLC, thin-layer chromatography; TMS, trimethylsilyl.

from egg white is strikingly different from that of acyl LPA obtained from egg yolk.

## MATERIALS AND METHODS

*Chemicals.* [$^3$H]Arachidonic acid (100 Ci/mmol) and 1-[$^{14}$C]-palmitoyl-*sn*-glycero-3-phosphocholine [LPC (1-[$^{14}$C]16:0)] (56 mCi/mmol) were obtained from NEN (Boston, MA). Arachidonic acid, heptadecanoic acid, LPC (1-16:0), *sn*-glycero-3-phosphocholine cadmium chloride complex, and phospholipase $A_2$ *(Naja naja)* were obtained from Sigma (St. Louis, MO). Phospholipase D *(Streptomyces chromofuscus)* was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Dicyclohexylcarbodiimide was obtained from Wako Pure Chem. Ind. (Osaka, Japan). Dimethylaminopyridine was from Aldrich Chemical Co. (Milwaukee, WI). 1(2)-Heptadecanoyl-*sn*-glycero-3-phosphate [acyl LPA (17:0)] was prepared according to the method described previously (30). All other chemicals were reagent grade.

*Preparation of 1-[$^3$H]arachidonoyl*-sn-*glycero-3-phosphocholine and 2-[$^3$H]arachidonoyl*-sn-*glycero-3-phosphocholine.* 1-[$^3$H]Arachidonoyl-*sn*-glycero-3-phosphocholine [LPC (1-[$^3$H]20:4n-6)] was prepared as follows. *sn*-Glycero-3-phosphocholine and [$^3$H]20:4n-6 (5 mCi/mmol) were mixed in chloroform (ethanol-free) containing dicyclohexylcarbodiimide and dimethylaminopyridine. The mixture was stirred at room temperature for 24 h under nitrogen gas (47). The resultant radiolabeled phosphatidylcholine [PC (1,2-di[$^3$H]20:4n-6)] was purified by thin-layer chromatography (TLC) using chloroform/methanol/water (65:25:4, by vol). LPC (1-[$^3$H]20:4n-6) was prepared from PC (1,2-di[$^3$H]20:4n-6) by treatment with phospholipase $A_2$ and purified by TLC using chloroform/methanol/water (55:25:4, by vol) as the solvent system. LPC (2-[$^3$H]20:4n-6) was prepared as follows. Total lipids were extracted from rabbit heart by the method of Bligh and Dyer (48) and fractionated by TLC using chloroform/methanol/water (65:25:4, by vol) as the solvent system. Choline glycerophospholipids were extracted from the silica gel and then treated with 0.5 M methanolic sodium methoxide solution at room temperature for 20 min. The resultant lysoplasmenylcholine (with a small amount of lysoplasmanylcholine) was extracted and purified by TLC using chloroform/methanol/water (55:25:4, by vol) as the solvent system. To obtain 1-*O*-alkenyl-2-[$^3$H]20:4n-6-*sn*-glycero-3-phosphocholine, lysoplasmenylcholine and [$^3$H]20:4n-6 (5 mCi/mmol) were mixed in chloroform (ethanol-free) containing dicyclohexylcarbodiimide and dimethylaminopyridine at room temperature for 24 h (47). Plasmenylcholine (1-*O*-alkenyl-2-[$^3$H]20:4n-6) was purified by TLC using chloroform/methanol/water (65:25:4, by vol) as the solvent system. LPC (2-[$^3$H]20:4n-6) was prepared immediately before use from plasmenylcholine (1-*O*-alkenyl-2-[$^3$H]20:4n-6) by treatment with 10 mM $HgCl_2$ at 37°C for 40 min to cleave an alkenyl ether bond. LPC (2-[$^3$H]20:4n-6) was purified by TLC using chloroform/methanol/water (55:25:4, by vol) as the solvent system.

*Purification of LPA from hen egg yolk and white.* Fresh unfertilized hen eggs were obtained from a local poultry farm. Egg yolks and whites were carefully separated and homogenized in a mixture of chloroform/methanol/water using a Waring blender (the final volume of the mixture, 760 mL for egg yolk and 380 mL for egg white; the final ratio of chloroform/methanol/water in the mixture, 1:2:0.8, by vol). Butylated hydroxytoluene (final content 0.05%) was added to prevent lipid peroxidation. In experiments where fatty acid composition of acyl LPA was determined by gas chromatography (GC), acyl LPA (17:0) was added as an internal standard (40 nmol for egg yolk and 20 nmol for egg white). To make two phases, chloroform and an aqueous ammonia solution (0.07%) were added, as described previously (final ratio of chloroform/methanol/water; 2:2:1.8, by vol) (30). After vigorous shaking, the mixture was left to stand at 4°C for 12 h. The mixture was then centrifuged to allow phase separation. We confirmed that LPA were recovered exclusively from the upper phase (30); the upper phase was carefully transferred to glass tubes and washed with chloroform. Four hundred (for egg yolk) or 200 mL (for egg white) of chloroform and a 3.2 (for egg yolk) or 1.6 mL (for egg white) of 12 M HCl were then added to the upper phases. After vigorous shaking and centrifugation, the lower phase was carefully aspirated. The upper phase was washed twice with chloroform, and the lower phases were taken and combined. LPA were purified by TLC using chloroform/acetone/methanol/acetic acid/water (4.5:2:1:1.3:0.5, by vol) as the solvent system in a developing tank sealed with nitrogen gas. The area corresponding to LPA were scraped off the TLC plates into a glass tube. LPA were extracted from the silica gel by the modified method of Bligh and Dyer (48) where HCl was added (final concentration of HCl in the upper phase; 0.07 M) prior to the phase separation. LPA were further purified by TLC using chloroform/methanol/25% ammonia (65:35:5, by vol) as the solvent system and then by TLC using chloroform/acetone/methanol/acetic acid/water (4.5:2:1:1.3:0.5, by vol) as the solvent system (30).

*Mild alkaline hydrolysis of LPA.* LPA obtained from egg yolk and white were treated with 0.2 M NaOH in 90% methanol or 90% methanol alone (control) for 20 min, as described previously (30). The LPA remaining were then extracted by a modified method of Bligh and Dyer (48) where HCl was added to the extraction mixture prior to the phase separation to acidify the upper phase. The lower phase was transferred to another tube, and the upper phase was washed twice with chloroform. The combined lower phases were evaporated to dryness. The amount of LPA (alkali-stable or total) was determined by measuring lipid phosphorus (30).

*Analysis of the fatty acyl moiety of acyl LPA by GC.* The fatty acyl moiety of acyl LPA was converted to fatty acid methyl esters by treating purified acyl LPA with 0.5 M methanolic sodium methoxide. The resultant fatty acid methyl esters were extracted and analyzed in a gas chromatograph (GC8A; Shimadzu, Kyoto, Japan) equipped with a fused-silica column (SP2330; Supelco, Bellefonte, PA).

*Fast atom bombardment mass spectrometry (MS) analysis of LPA.* The structures of LPA were confirmed by fast atom bombardment MS using a JEOL JMS-SX102A mass spectrometer (Tokyo, Japan). A 2:1 mixture of thioglycerol and dithiothreitol/dithioerythritol (3:1) was used as the matrix, as described previously (30).

*GC/MS analysis of the trimethylsilyl (TMS) derivative of LPA.* LPA were treated with 50 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and 50 µL of pyridine at 60°C for 30 min and converted to the triTMS derivative essentially according to the method of Tokumura *et al.* (49). The electron impact (70 eV) mass spectra of the triTMS derivatives of LPA were obtained using a JEOL JMS-SX102A mass spectrometer (accelerating voltage, 10 kV; ionizing current, 300 µA) coupled with a gas chromatograph equipped with a fused-silica column (DB-1, 30 m × 0.25 mm i.d., 0.25 µm thickness; J&W Scientific, Folsom, CA). The column temperature was increased from 270 to 320°C at the rate of 70°C/min, and the temperature of the injection port was 300°C (30).

*Generation of LPA in ovo during incubation.* Fresh unfertilized eggs were incubated at 37°C for 24 h in an automatic incubator (Showa, Urawa, Japan). LPA were extracted and purified from egg yolk and white. The fatty acyl moiety of acyl LPA was determined by GC.

*Enzymatic formation of acyl LPA from exogenously added LPC.* Radiolabeled LPC (5 nmol, 50,000 dpm), 0.1 mL of egg white, and 0.1 mL of 0.1 M Tris-HCl buffer (pH 7.4) were incubated at 37°C for 0–3 h. The reaction was stopped by the addition of chloroform and methanol (1:2, vol/vol). Lipids were extracted by a modified method of Bligh and Dyer (48) where HCl was added to acidify the mixture. LPC and LPA were purified by two-dimensional TLC developed first with chloroform/methanol/25% ammonia (65:35:5, by vol) and second with chloroform/acetone/methanol/acetic acid/water (4.5:2:1:1.3:0.5, by vol). The radioactivities were determined in a liquid scintillation counter.

*Analysis of fatty acid composition of LPC in egg white.* Total lipids were extracted from egg white by the method of Bligh and Dyer (48). LPC was purified by two-dimensional TLC developed first with chloroform/methanol/25% ammonia (65:35:5, by vol) and second with chloroform/acetone/methanol/acetic acid/water (4.5:2:1:1.3:0.5, by vol). LPC was extracted from the silica gel by the method of Bligh and Dyer (48). The fatty acyl moiety of LPC was analyzed as the fatty acid methyl esters by GC.

## RESULTS

First, we examined whether LPA, growth factor-like lipids, were present in fresh hen eggs. Table 1 summarizes the experimental results. We found that high amounts of acyl LPA were present in both egg yolk (44.23 nmol/g) and egg white (8.81 nmol/g). We also confirmed that both egg yolk and egg white contained small amounts of alkyl LPA in addition to acyl LPA.

**TABLE 1**
**Amounts of Acyl LPA and Alkyl LPA in Hen Egg Yolk and Egg White**

| Subclass | Egg yolk | Egg white |
|---|---|---|
| | nmol/g (%) | |
| Acyl LPA | 44.23 ± 9.48[a] (97.6 ± 0.6)[b] | 8.81 ± 2.24[a] (99.8 ± 0.1)[b] |
| Alkyl LPA | 1.09[c] (2.4 ± 0.6)[b] | 0.02[c] (0.2 ± 0.1)[b] |
| Total | 45.32 (100.0) | 8.83 (100.0) |

[a]The fatty acyl moieties of lysophosphatidic acid (LPA) obtained from egg yolk and egg white were converted to fatty acid methyl esters and analyzed by gas chromatography. Acyl LPA (17:0) was added as an internal standard before the extraction of total lipids as described in the Materials and Methods section. The values are the means ± SD of four (egg yolk) or nine (egg white) determinations.
[b]The ratio of alkyl LPA to acyl LPA was determined by mild alkaline hydrolysis of LPA obtained from egg yolk and white without the addition of acyl LPA (17:)), and is expressed as the mean percentage ± SD of three determinations.
[c]The amount (nmol/g) of alkyl LPA was calculated from the amount of acyl LPA and the ratio of alkyl LPA to acyl LPA.

Figure 1 illustrates the molecular species compositions of acyl LPA obtained from hen egg yolk and white. As shown in Figure 1A, the fatty acyl moiety of acyl LPA present in egg yolk consisted mainly of saturated fatty acids such as 16:0 and 18:0. The levels of polyunsaturated fatty acid-containing species were low. Noticeably, the molecular species composition of acyl LPA obtained from egg white was quite different from that of acyl LPA obtained from egg yolk. As shown in Figure 1B, the major fatty acyl constituents of acyl LPA in egg white were 18:2n-6 and 20:4n-6. The levels of saturated fatty acid-containing species were very low. Hen eggs contain 15.8 ± 1.9 g of yolk/egg (mean ± SD of eight determinations) and 34.5 ± 4.1 g of white/egg (mean ± SD of eight determinations); it was calculated that 90% of the saturated species of egg acyl LPA is localized in the egg yolk and that 93% of the polyenoic species of egg acyl LPA is localized in the egg white.

We then examined the structures of LPA obtained from egg yolk and white by fast atom bombardment MS. Figure 2 shows the negative ion mode mass spectrum of LPA obtained from

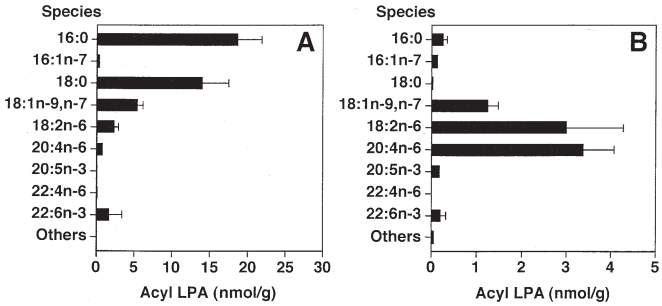


**FIG. 1.** Molecular species compositions of acyl lysophosphatidic acid (LPA) obtained from egg yolk (A) and egg white (B). The fatty acyl moiety of acyl LPA was analyzed as the fatty acid methyl esters by gas chromatography as described in the Materials and Methods section. The values are the means ± SD of four (egg yolk) or nine (egg white) determinations.

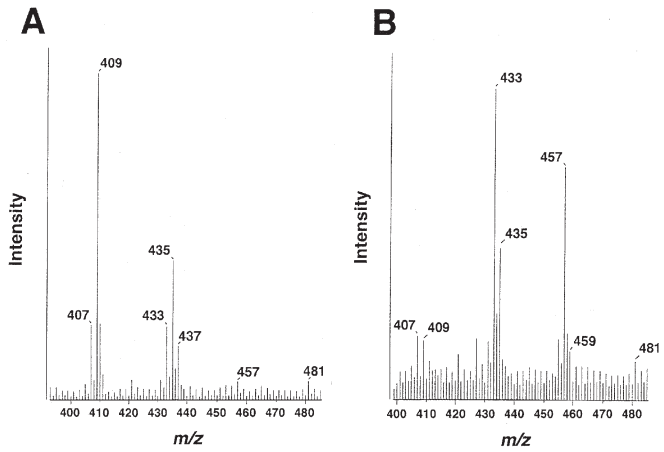


**FIG. 2.** Negative ion mode mass spectrum of LPA obtained from egg yolk (A) and egg white (B). LPA obtained from egg yolk and egg white were analyzed by fast atom bombardment mass spectrometry as described in the Materials and Methods section. For abbreviation see Figure 1.

egg yolk (Fig. 2A) and egg white (Fig. 2B): *m/z* 407 for acyl LPA (16:1n-7) ($[M - H]^-$), *m/z* 409 for acyl LPA (16:0) ($[M - H]^-$), *m/z* 433 for acyl LPA (18:2n-6) ($[M - H]^-$), *m/z* 435 for acyl LPA (18:1n-9,n-7) ($[M - H]^-$), *m/z* 437 for acyl LPA (18:0) ($[M - H]^-$), *m/z* 457 for acyl LPA (20:4n-6) ($[M - H]^-$), *m/z* 481 for acyl LPA (22:6n-3) ($[M - H]^-$).

We further confirmed the structures of LPA by GC/MS as triTMS derivatives without hydrolytic pretreatment. The GC/MS data provide further evidence that various molecular species of acyl LPA are actually present in egg yolk and white (data not shown). We also confirmed that small amounts of alkyl LPA (16:0) are present in egg yolk and white by GC/MS analysis (data not shown).

We next examined whether any changes in the levels of acyl LPA occur during the incubation of the eggs. Figure 3 shows the levels of acyl LPA in egg yolk and white before and after 24-h incubation at 37°C. Changes were not apparent in the levels of acyl LPA in egg yolk before and after incubation. In contrast, the level of acyl LPA in egg white was augmented 1.5-fold after 24-h incubation. We confirmed that the levels of two major species of acyl LPA, i.e., 18:2n-6- and 20:4n-6-containing species, were both markedly elevated after incubation [from 2.66 ± 0.65 to 4.20 ± 1.08 nmol/g and from 3.64 ± 0.80 to 5.00 ± 1.02 nmol/g, respectively (means ± SD of four determinations)], whereas the levels of saturated species such as the 16:0-containing species remained unchanged [from 0.28 ± 0.10 to 0.27 ± 0.06 nmol/g (the mean ± SD of four determinations)].

*In ovo* generation of polyunsaturated fatty acid-containing acyl LPA in egg white during the incubation strongly suggests the occurrence of both enzyme activity and the substrate involved in the synthesis of polyunsaturated fatty acid-containing acyl LPA. To investigate this issue, we examined the enzyme activity. Tokumura *et al.* (32,33) previously demonstrated that acyl LPA was produced from LPC through the action of a lysophospholipase D in rat blood plasma; therefore, we examined whether similar enzyme activity was present in hen egg white. As shown in Figure 4, egg white contained an enzyme activity catalyzing the formation of acyl LPA from corresponding LPC. The amounts of the newly formed acyl LPA increased with time at least up to 3 h. We confirmed that the enzyme activities, estimated using polyenoic LPC (1-20:4n-6 and 2-20:4n-6) as the substrates, were considerably higher than those estimated with saturated LPC (1-16:0).

Finally, we examined whether polyunsaturated fatty acid-containing LPC, a putative substrate for the synthesis of polyunsaturated fatty acid-containing acyl LPA, was present in egg white. As shown in Figure 5, substantial amounts of 20:4n-6-, 18:2n-6-, and 18:1n-9,n-7-containing species, besides a small amount of 16:0-containing species, occurred in egg white: the sum of the 20:4n-6-containing species and the 18:2n-6-containing species accounted for as high as 57.2% of the total. Thus, it is evident that both the enzyme activity and the substrate are present in egg white. Taken together, at least part of the polyunsaturated fatty acid-containing acyl LPA generated in egg white is derived from the corresponding polyunsaturated fatty acid-containing LPC through a phospholipase D-type reaction.

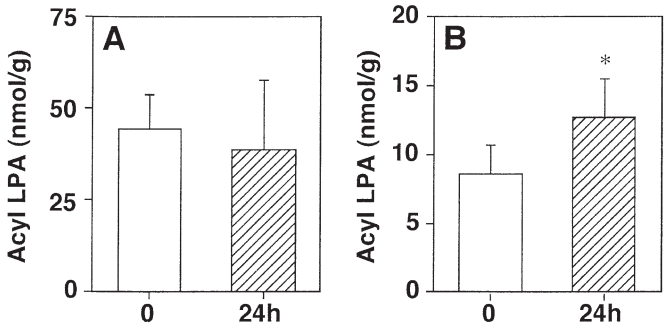


**FIG. 3.** The levels of acyl LPA in egg yolk (A) and egg white (B) before and after incubation at 37°C for 24 h. Fresh eggs were incubated at 37°C for 24 h. LPA were extracted and analyzed as described in the Materials and Methods section. The values are the means ± SD of four determinations. *$P < 0.02$ [compared with control (Student's *t* test)]. For abbreviation see Figure 1.

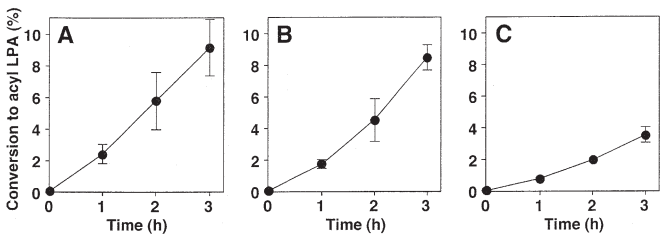


**FIG. 4.** Enzymatic formation of acyl LPA from exogenously added lysophosphatidylcholine (LPC). Radiolabeled LPC (50,000 dpm), 0.1 mL of egg white, and 0.1 mL of 0.1 M Tris-HCl buffer (pH 7.4) were incubated at 37°C for 0–3 h. Lipids were extracted and purified as described in the Materials and Methods section. The enzyme activity was calculated from the radioactivity of LPA and that of LPC. (A) LPC (1-[$^3$H]20:4n-6); (B) LPC (2-[$^3$H]20:4n-6); (C) LPC (1-[$^{14}$C]16:0). The values are the means ± SD of four determinations. For abbreviation see Figure 1.

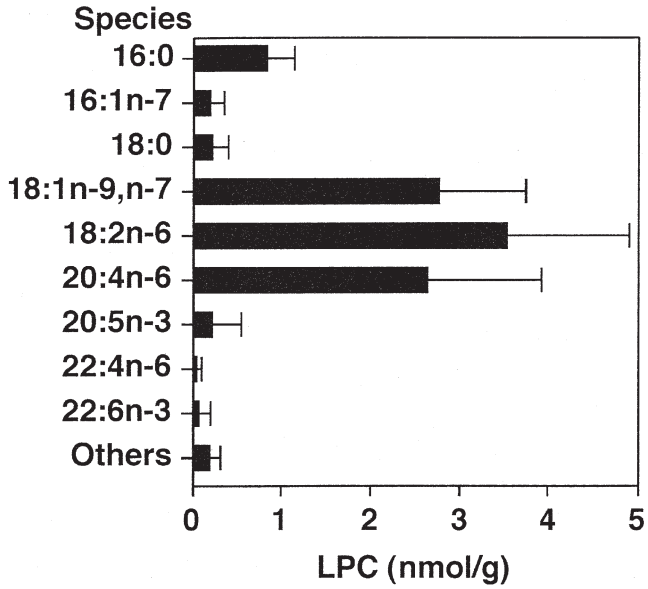


**FIG. 5.** Molecular species composition of LPC obtained from egg white. The fatty acyl moiety of LPC was analyzed as the fatty acid methyl esters by gas chromatography as described in the Materials and Methods section. The values are the means ± SD of six determinations. See Figure 4 for abbreviation.

## DISCUSSION

Naturally occurring LPA include a number of different molecules with different chemical structures in terms of (i) the structure of the aliphatic chain (number of carbon atoms, number and position of double bonds), (ii) the location of the aliphatic chain (*sn*-1, *sn*-2), (iii) the type of bond between glycerol and the aliphatic chain (*O*-acyl, *O*-alkyl, *O*-alkenyl), and (iv) the structure of the backbone (noncyclic, cyclic). Evidence is accumulating that these lipid phosphoric acid molecules compose a novel class of bioactive lipid family. Importantly, the biological activities or potencies of these LPA molecules are considerably different (7,8,11,13,38–45). Several lines of evidence strongly suggest that certain subclasses or analogs of LPA [e.g. lysoplasmenic acid (alkenyl LPA), alkyl LPA, and cyclic phosphatidic acid (cyclic PA)] interact with specific or preferential receptors in addition to other subtypes of LPA receptors, thereby eliciting biological responses (3,44,45). Thus, detailed studies on these subclasses as well as various molecular species of LPA are indispensable for a full understanding of the diverse physiological roles and functions of LPA in animal tissues.

In the present study, we examined in detail the levels as well as the molecular species compositions of LPA in hen egg yolk and white. We found that egg yolk and white contain high amounts of acyl LPA besides small amounts of alkyl LPA (Table 1). Apparently, the levels of acyl LPA found in egg yolk (44.23 nmol/g) and white (8.81 nmol/g) are on the same order as or higher than the levels of acyl LPA required for eliciting biological responses in various animal tissues (1–6). The occurrence of high levels of acyl LPA in hen egg yolk and white is quite noticeable in view of the fact that embryonic cells, such as *Xenopus* oocytes, have been shown to express the LPA receptor (13,22) and that the development of mouse embryos from the four-cell stage to the blastocyst was accelerated markedly in the presence of acyl LPA (19). LPA present in egg yolk may play some essential roles as signaling molecules in addition to the role as metabolic intermediates during the course of differentiation and development of embryos.

A striking observation in the present study was the marked difference between the molecular species composition of egg yolk acyl LPA and that of egg white acyl LPA. Egg yolk acyl LPA consisted mainly of saturated species, whereas egg white acyl LPA consisted mainly of polyenoic species (Fig. 1). Further detailed studies are needed to clarify whether there are specific *in ovo* physiological functions or roles for the saturated species of acyl LPA localized predominantly in the egg yolk and the polyenoic species of acyl LPA localized almost exclusively in the egg white.

The marked difference in the molecular species composition of egg yolk and white acyl LPA may be attributed to the difference in the mechanisms underlying the formation of acyl LPA in egg yolk and white. We found that significant amounts of unsaturated species of acyl LPA were formed in the egg white during the incubation at 37°C (Fig. 3). We obtained evidence that both the enzyme activity (lysophospholipase D) and the substrate (LPC) required for the generation of acyl LPA are present in the egg white (Figs. 4,5). In contrast, acyl LPA was not generated in the egg yolk during the incubation. It is conceivable that acyl LPA was synthesized *de novo* during the process of egg yolk formation in the hen and stored in the egg yolk with other phospholipids such as egg PC.

The lysophospholipase D, which catalyzes the formation of acyl LPA from corresponding LPC, was first identified in rat plasma by Tokumura *et al.* (32,33). This enzyme is specific to lysophospholipids and distinct from other types of phospholipase D, which hydrolyze diacyl phospholipids to generate PA (33). Similar enzyme activity was also detected in human follicular fluids (27). Several lines of evidence suggest that lysophospholipase D plays a crucial role in the generation of acyl LPA, a growth factor-like lipid, in blood plasma and ovarian follicular fluids (27,28,32,33).

Interestingly, this unique lysophospholipase D preferentially utilizes polyunsaturated fatty acid-containing LPC as the substrate (Fig. 4) (27,28,32,33), yet this enzyme is able to metabolize the saturated species of LPC as well at slower rates. In any case, the presence of a lysophospholipase D, which preferentially utilizes polyunsaturated fatty acid-containing LPC in blood plasma, follicular fluids and egg white, strongly suggests that polyunsaturated fatty acid-containing LPA such as acyl LPA (18:2n-6) and acyl LPA (20:4n-6) play some essential role(s) in wound healing, egg maturation, fertilization and development, and differentiation of embryos. Xu *et al.* (37) reported that polyunsaturated fatty acid-containing LPA such as acyl LPA (18:2n-6), acyl LPA (20:4n-6), and acyl LPA (22:6n-3) exhibit much more potent ovarian

cancer-activating activity than saturated or monoenoic fatty acid-containing LPA, although the details still remain to be elucidated. Presumably, the mechanism and/or the site of the action of polyunsaturated fatty acid-containing LPA is considerably different from that of saturated or monoenoic LPA. Bandoh *et al.* (25) recently reported that Edg7 is a specific receptor for unsaturated fatty acid-containing LPA. Further studies are required to clarify the mechanism of the action as well as the exact physiological role(s) of polyunsaturated fatty acid-containing LPA that are abundant in several animal tissues and fluids.

In conclusion, we found that hen egg yolk and white contained high amounts of acyl LPA in addition to small amounts of alkyl LPA. The molecular species composition of egg yolk acyl LPA and of egg white acyl LPA were quite different. Egg yolk acyl LPA, composed mainly of saturated species, and egg white acyl LPA, composed primarily of polyunsaturated species, may possess inherent separate physiological functions *in ovo* during the course of development, differentiation and growth of chick embryos.

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# Fullerene Lipids: Synthesis of Novel Nitrogen-Bridged [60]Fullerene Fatty Ester Derivatives

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**ABSTRACT:** Reactions of methyl 6-azido-hexanoate, 8-azido-octanoate, and 12-azido-dodecanoate with [60]fullerene (**1**) gave the corresponding aza-[60]fullerene ester derivatives (**2a-2c**, 22–35% based on the amount of [60]fullerene reacted). The nitrogen atom is bonded to the [60]fullerene cage to yield a "[5,6]-open" type aza substructure. This was confirmed by the appearance of 30–31 $sp^2$ signals at $\delta_C$ 133–147 in the carbon nuclear magnetic resonance spectra. Reaction of methyl 11-azido-7-undecynoate with [60]fullerene furnished a mixture of aza-[60]fullerene (**2d**, 53%) and aziridine-[60]fullerene (**2e**, 38%) ester derivatives. Compound **2e** was identified as the "[6,6]-closed" type aziridine-[60]fullerene derivative, which displayed 10 $sp^2$ signals in the region $\delta_C$ 140–145 and one signal at $\delta_C$ 85.05 for the $sp^3$ carbons of the cage. Refluxing a solution of compound **2d** in toluene for 50 h gave about 50% yield of compound **2e**, but not vice versa.

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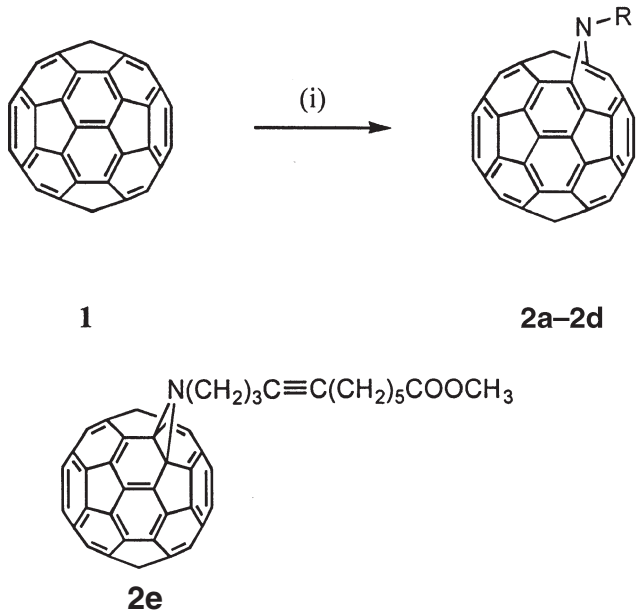


**FIG. 1.** Reagents and conditions for synthesis of nitrogen-bridged[60]-fullerene fatty ester derivatives. (i) $RN_3$, chlorobenzene, reflux for 16 h. 2a, R = $(CH_2)_5COOCH_3$; 2b, R = $(CH_2)_7COOCH_3$; 2c, R = $(CH_2)_{11}$-$COOCH_3$; 2d, R = $(CH_2)_3C{\equiv}C(CH_2)_5COOCH_3$; 2e, R = $(CH_2)_3C{\equiv}C$-$(CH_2)_5COOC_3$.

Studies of the chemical reactivity of [60]fullerene have drawn keen attention since the discovery of the novel $C_{60}$ carbon sphere in 1985 (1). Numerous books and review articles have been published, which describe the syntheses and properties of [60]fullerene derivatives and their potential applications (2–7). Of interest is the inhibition of human immunodeficiency virus-protease activities of water-soluble fullerene derivatives (8–10). We have reported the synthesis of two series of fullerenoid lipid molecules: *viz.*, saturated and unsaturated dialkyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate derivatives (11) and [60]fullerene lipids bearing long-chain saturated or unsaturated esters (12). The incorporation of a [60]fullerene unit into lipid molecules has opened a new frontier to fatty acid chemistry.

Prato *et al.* (13) first reported the reactions of organic azides with [60]fullerene to yield aza-fullerene derivatives, which are potent precursors for the production of nitrogen heterofullerenes. Two comprehensive reviews concerning heterofullerenes have been published recently (14,15). In this paper we describe the reactions of saturated methyl ω-azido fatty esters of various chain lengths and that of a $C_{11}$ acetylenic ω-azido fatty ester with [60]fullerene (Fig. 1). The spectroscopic properties of such novel fatty ester derivatives are reported.

## MATERIALS AND METHODS

Melting points (m.p.) were determined on a heating stage TC92 (Linkam Scientific Instruments Ltd., Waterfield, Surrey, United Kingdom). Infrared (IR) spectra were recorded on a Bio-Rad FTS-165 Fourier transform IR (Bio-Rad Inc., Hercules, CA) spectrometer. Samples were run as neat films on KBr discs. Ultraviolet-visible (UV-vis) spectra of solutions in dichloromethane 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 (TMS) as the internal reference standard. Chemical shifts are given in δ-values in ppm downfield from TMS ($\delta_{TMS}$ = 0 ppm) and *J* constants are given in Hz.

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Abbreviations: APCI, atmospheric pressure chemical ionization; IR, infrared; m.p., melting point; MS, mass spectrometry; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; TLC, thin-layer chromatography; TMS, tetramethylsilane; UV-vis, ultraviolet-visible spectroscopy.

Mass spectral analyses were carried out on a Finnigan MAT-LCQ [atmospheric pressure chemical ionization (APCI)] spectrometer (Finnigan Corp., San Jose, CA). Precoated thin-layer chromatography plates (silica gel 60 $F_{254}$, 20 × 20 cm on glass, E. Merck No. 1,05715) were purchased from Merck (Schuchardt, Germany) and were cut into 1.5 × 6.0 cm strips for general use.

[60]Fullerene was purchased from Materials and Electrochemical Research Corp. (Tucson, AZ). 6-Bromo-hexanoic acid, 8-bromo-octanoic acid, 12-bromo-dodecanoic acid, and 1-pentyn-4-ol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methyl 6-azido-hexanoate, methyl 8-azido-octanoate, and methyl 12-azido-dodecanoate were derived by methylation of the corresponding bromo-acids (with $BF_3$/methanol) followed by chemical substitution of the bromide group by an azide (*via* sodium azide) as described by Alvarez and Alvarez (16).

*Synthesis of methyl 11-hydroxy-7-undecynoate.* 4-Pentyn-1-ol (22 g, 0.26 mol) in tetrahydrofuran (THF: 20 mL) was added to lithium amide [prepared from lithium (5.2 g) in liquid ammonia (500 mL) with a catalytic amount of iron(III) nitrate]. The reaction mixture was stirred for 30 min. 6-Bromohexanoic acid (60 g, 0.30 mol) in anhydrous THF (50 mL) was added over a period of 30 min. The reaction mixture was stirred for a further 16 h, and the liquid ammonia was allowed to evaporate. Dilute HCl (6 M, 400 mL) was added, and the reaction mixture was extracted with diethyl ether (3 × 150 mL). The ethereal layer was washed with brine (100 mL), water (100 mL), and dried over anhydrous $Na_2SO_4$. The filtrate was evaporated, and the residue was refluxed in methanol (200 mL) and $BF_3$/MeOH complex (14% w/w, 15 mL) for 30 min. About 150 mL of the solvent of the reaction mixture was evaporated under reduced pressure distillation. Water (200 mL) was added, and the reaction mixture was extracted with diethyl ether (3 × 200 mL). The ethereal layer was washed with aqueous $NaHCO_3$ (5%, 100 mL), water (2 × 100 mL), dried ($Na_2SO_4$), and filtered. The filtrate was evaporated, and the residue was chromatographed on a silica gel (250 g) column using a mixture of *n*-hexane/diethyl ether, 5:1, vol/vol, to give methyl 11-hydroxy-7-undecynoate (36 g, 46%). TLC ($R_f$): 0.1 ($Et_2O$/*n*-hexane, vol/vol, 1:4); IR (KBr, neat): 3441 (br, O-H str.), 1738 (C=O, str.) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 3.70 (*t*, *J* = 6.6, 2H, 11-*H*), 3.67 (s, 3H, $COOCH_3$), 2.98 (s, 1H, $D_2O$ exchangeable, O-*H*), 2.33 (*t*, *J* = 7.5, 2H, 2-*H*), 2.28–2.22 (*m*, 2H, 9-*H*), 2.18–2.11 (*m*, 2H, 6-*H*), 1.72 (*qn*, *J* = 6.6, 2H, 10-*H*), 1.64 (*qn*, *J* = 7.5, 2H, 3-*H*), 1.22–1.54 (*m*, 4H, 4-*H*, 5-*H*); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 174.25 (C-1), 80.25 (C-7), 79.60 (C-8), 61.36 (C-11), 51.43 ($COOCH_3$), 33.85 (C-2), 31.64 (C-10), 28.54 (C-5), 28.18 (C-4), 24.37 (C-3), 18.45 (C-6), 15.19 (C-9); mass spectrometry (MS) (APCI): 213.2 ($C_{12}H_{20}O_3$, calc. $M^+$ *m/z* = 212.289).

*Methyl 11-bromo-7-undecynoate*. Methyl 11-hydroxy-7-undecynoate (2.7 g, 10.3 mmol) was added to a mixture of triphenylphosphine (3.4 g, 13.0 mmol) and bromine (1.64 g, 10.3 mmol) in $CH_2Cl_2$ (50 mL) at 0–5°C. The mixture was stirred for 20 min, and the solvent was evaporated under reduced pressure. The residue was chromatographed on a silica gel (50 g) column using *n*-hexane/diethyl ether, 95:5, vol/vol (200 mL) as eluant to give methyl 11-bromo-7-undecynoate as a colorless oil (2.39 g, 92%). TLC ($R_f$): 0.5 ($Et_2O$/*n*-hexane, vol/vol, 1:4); IR (KBr, neat): 1738 (C=O, str.) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 3.67 (*s*, 3H, $COOCH_3$), 3.52 (*t*, *J* = 6.6, 2H, 11-*H*), 2.36–2.31 (*m*, 4H, 9-*H*, 2-*H*), 2.15 (*tt*, *J* = 2.4, 6.8, 2H, 6-*H*), 2.00 (*qn*, *J* = 6.6, 2H, 10-*H*), 1.64 (*qn*, *J* = 7.5, 2H, 3-*H*), 1.56–1.36 (*m*, 4H, 4-*H*, 5-*H*); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 174.13 (C-1), 81.14 (C-7), 78.14 (C-8), 51.50 ($COOCH_3$), 33.98 (C-2), 32.61 (C-11), 31.85 (C-10), 28.62, 28.33 (C-5, C-4), 24.48 (C-3), 18.56 (C-6), 17.48 (C-9).

*Methyl 11-azido-7-undecynoate*. A mixture of methyl 11-bromo-7-undecynoate (1.7g, 6.5 mmol) and sodium azide (638 mg, 9.6 mmol) was stirred at 70°C in dimethylsulfoxide (20 mL) for 4 h. Water (60 mL) was added, and the reaction mixture was extracted with diethyl ether (3 × 50 mL). The organic extract was washed with water (20 mL), brine (20 mL), and dried over anhydrous sodium sulfate. The filtrate was evaporated under reduced pressure, and the residue was separated on a silica (30 g) column using a mixture of *n*-hexane/diethyl ether (5:1, vol/vol, 300 mL) to give a colorless oil (1.4 g, 92%). TLC ($R_f$): 0.5 ($Et_2O$/*n*-hexane, vol/vol, 1:4); IR (KBr, neat): 2126 ($N_3$, str.), 1739 (C=O, str.) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 3.67 (*s*, 3H, $COOCH_3$), 3.40 (*t*, *J* = 6.8, 2H, 11-*H*), 2.32 (*t*, *J* = 7.5, 2H, 2-*H*), 2.27 (*tt*, *J* = 2.4, 6.8, 2H, 9-*H*), 2.15 (*tt*, *J* = 2.4, 6.8, 2H, 6-*H*), 1.74 (*qn*, *J* = 6.8, 2H, 10-*H*), 1.64 (*qn*, *J* = 7.5, 2H, 3-*H*), 1.55–1.35 (*m*, 4H, 4-*H*, 5-*H*); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 174.12 (C-1), 81.16 (C-7), 78.42 (C-8), 51.48 ($COOCH_3$), 50.29 (C-11), 33.97 (C-2), 28.65, 28.36, 28.23 ($CH_2$), 24.50 (C-3), 18.57 (C-6), 16.08 (C-9); MS (APCI): 238.2 ($C_{12}H_{19}O_2N_3$, calc. $M^+$ *m/z* = 237.303).

*General procedure for the reaction of methyl ω-azido fatty esters with [60]fullerene as exemplified by the synthesis of 1-(5-carbonylmethoxy-pentyl)-aza-[2′,3′;5,6] [60]fullerene* (**2a**). A solution of methyl 6-azido-hexanoate (308.8 mg, 1.8 mmol) in chlorobenzene (250 mL) was added dropwise through a condenser to a refluxing solution of [60]fullerene (1.0 g, 1.39 mmol) in chlorobenzene (750 mL) under an atmosphere of argon. The reaction mixture was refluxed for a further 16 h. The color of the reaction mixture gradually changed from magenta to dark brown. The solvent was evaporated under reduced pressure to give a residue (1.47 g). A portion of the residue (200 mg) was dissolved in toluene (10 mL) and was loaded onto a silica gel (200 g) column. The column was eluted with a mixture of *n*-hexane/toluene (1:1, vol/vol, 400 mL) to remove unreacted [60]fullerene (66.1 mg). The column was then eluted with toluene (700 mL) to give 1-(5-carbonylmethoxy-pentyl)-aza-[2′,3′;5,6][60]fullerene (**2a**, 14.9 mg). Repeated column chromatographic separation on 200 mg of the residue furnished a total of 504 mg of unreacted [60]fullerene and 109.8 mg of the requisite product (**2a,** 9% yield based on the initial amount of [60]fullerene used or 22% based on the amount of [60]fullerene reacted). Note: 50-mL fractions of eluant were collected during the chromatographic run, and the composi-

tion of each fraction was closely monitored by TLC analysis (using toluene as the developer).

(i) *1-(5-Carbonylmethoxypentyl)-aza-[2',3';5,6][60]fullerene (**2a**).* Dark brown solid: m.p.: >350°C. Yield: 22% based on the amount of [60]fullerene reacted. TLC ($R_f$): 0.6 (toluene); IR (KBr, neat): 2924, 2857, 1736 (C=O, str.) and 526 ($C_{60}$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 3.81 (*t*, *J* = 7.2, 2H, [60]NC$H_2$), 3.71 (*s*, 3H, COOC$H_3$), 2.44 (*t*, *J* = 7.3, 2H, 2-*H*), 2.05 (*qn*, *J* = 7.3, 2H, 5-*H*), 1.87–1.70 (*m*, 4H, C$H_2$). $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 174.11 (C-1), [147.82, 146.84, 145.05, 144.75, 144.56, 144.47, 144.33, 144.30, 144.14 (2C: Intensity of this signal is about twice that of the others), 143.85, 143.66, 143.55, 143.40, 143.22, 143.11, 142.91, 142.81, 142.71, 142.65, 141.45, 140.82, 140.76, 139.23, 138.51, 138.04, 137.86, 137.29, 137.23, 136.22, 135.84, 133.75 ($C_{60}$-$sp^2$)], 51.62 (*C*$H_2$N[60]), 51.42 (COO*C*$H_3$), 34.04 (C-2), 29.26, 29.83, 24.82 (C-3); MS (APCI): 863.5 ($C_{67}H_{13}O_2N$, calc. $M^+$ *m/z* = 863.846).

(ii) *1-(7-Carbonylmethoxyheptyl)-aza-[2′,3′;5,6][60]fullerene (**2b**).* Dark brown solid: m.p.: >350°C. Yield: 27% based on the amount of [60]fullerene reacted. TLC ($R_f$): 0.6 (toluene); IR (KBr, neat): 2920, 2857, 1735 (C=O, str.), 525 ($C_{60}$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 3.80 (*t*, *J* = 7.3, 2H, [60]NC$H_2$), 3.69 (*s*, 3H, COOC$H_3$), 2.36 (*t*, *J* = 7.5, 2H, 2-*H*), 2.02 (*qn*, *J* = 7.7, 2H, 7-*H*), 1.75–1.62 (*m*, 4H, 3-*H*, 6-*H*) 1.53–1.38 (*m*, 4H, C$H_2$); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 174.22 (C-1), [147.88, 147.07, 145.10, 144.79, 144.60, 144.50, 144.37, 144.33, 144.20 (2C: Intensity of this signal is about twice that of the others), 143.89, 143.70, 143.59, 143.44, 143.26, 143.15, 142.95, 142.85, 142.77, 142.68, 141.50, 140.90, 140.79, 139.26, 138.56, 138.09, 137.88, 137.35, 137.31, 136.28, 135.91, 133.81 ($C_{60}$-$sp^2$)], 51.76 (*C*$H_2$N[60]), 51.47 (COO*C*$H_3$), 34.13 (C-2), 29.71, 29.59, 29.15, 27.16, 24.96 (C-3); MS (APCI): 891.5 ($C_{69}H_{17}O_2N$, calc. $M^+$ *m/z* = 891.900).

*(iii) 1-(11-Carbonylmethoxyundecyl)-aza-[2′,3′;5,6][60]-fullerene (**2c**).* Dark brown solid: m.p.: >350°C. Yield: 35% based on the amount of [60]fullerene reacted. TLC ($R_f$): 0.6 (toluene); IR (KBr, neat): 1738 (C=O, str.), 1460, 1428, 1174, 525 ($C_{60}$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 3.80 (*t*, *J* = 7.3, 2H, [60]NC$H_2$), 3.67 (*s*, 3H, COOC$H_3$), 2.31 (*t*, *J* = 7.5, 2H, 2-*H*), 2.02 (*qn*, *J* = 7.7, 2H, 7-*H*), 1.65–1.58 (*m*, 4H, 3-*H*, 10-*H*), 1.46–1.25 (*m*, 12H, C$H_2$); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 174.35 (C-1), [147.85, 147.11, 145.07, 144.75, 144.56, 144.46, 144.38, 144.28, 144.17 (2C: Intensity of this signal is about twice that of the others), 143.84, 143.65, 143.54, 143.40, 143.21, 143.10, 142.90, 142.81, 142.72, 142.62, 141.45, 140.88, 140.73, 139.19, 138.50, 138.00, 137.83, 137.31, 137.22, 136.20, 135.85, 133.74 ($C_{60}$-$sp^2$)], 51.73 (*C*$H_2$N[60]), 51.46 (COO*C*$H_3$), 34.14 (C-2), 29.63, 29.61, 29.59, 29.48, 29.29, 29.18, 27.32, 24.98 (C-3); MS (APCI): 949.1 ($C_{73}H_{25}O_2N$, calc. $M^+$ = 948.008).

*Reaction of methyl 11-azido-7-undecynoate with [60]fullerene.* A mixture of methyl 11-azido-7-undecynoate (100 mg, 0.42 mmol), [60]fullerene (400 mg, 0.56 mmol), and chlorobenzene (350 mL) was refluxed for 16 h under argon. The reaction mixture was evaporated under reduced pressure. The residue was dissolved in toluene (50 mL) and was loaded onto a silica gel (150 g) column. The unreacted [60]fullerene (360 mg) was eluted with a mixture of *n*-hexane/toluene (1:1, vol/vol, 400 mL). A second fraction (brown in color) was eluted with toluene (500 mL) to give compound **2d** (27.8 mg, $R_f$ = 0.5 (toluene), yield of 5.3% based on the amount of [60]fullerene used or 53% based on the amount of [60]fullerene reacted). A third fraction (red in color) was eluted with toluene (500 mL) to give compound **2e** (19.6 mg, $R_f$ = 0.4 (toluene), 3.8% yield based on the amount of [60]fullerene used or 38% based on amount of [60]fullerene reacted).

*(i) 1-(10-Carbonylmethoxy-4-decynyl)-aza-[2′,3′;5,6]-[60]fullerene (**2d**).* Dark brown solid: m.p.: >350°C. Yield: 53% based on the amount of [60]fullerene reacted. TLC ($R_f$): 0.5 (toluene); UV-vis ($CH_2Cl_2$, $\lambda_{max}$, nm): 430, 548; IR (KBr, neat): 1725 (C=O, str.), 1557, 1428, 1077, 525 ($C_{60}$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 3.91 (*t*, *J* = 6.9, 2H, 11-*H*), 3.67 (*s*, 3H, COOC$H_3$), 2.60 (*tt*, *J* = 2.3, 6.9, 2H, 9-*H*), 2.33 (*t*, *J* = 7.5, 2H, 2-*H*), 2.21 (*qn*, *J* = 6.9, 2H, 10-*H*), 2.18 (*tt*, *J* = 2.3, 6.8, 2H, 6-*H*), 1.65 (*qn*, *J* = 7.5, 2H, 3-*H*), 1.55–1.42 (*m*, 4H, C$H_2$); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 174.15 (C-1), [147.79, 146.66, 145.04, 144.72, 144.55, 144.46, 144.32 (2C: Intensity of this signal is about twice that of the others), 144.15, 144.12, 143.83, 143.63 (2C: Intensity of this signal is about twice that of the others), 144.39, 143.20, 143.10, 142.90, 142.79, 142.70, 142.64, 141.40, 140.80, 140.73, 139.20, 138.50, 138.01, 137.84, 137.32, 137.23, 136.19, 135.84, 133.70 ($C_{60}$-$sp^2$)], 81.15 (C-7), 79.30 (C-8), 51.52 (COO*C*$H_3$), 50.49 (C-11), 34.01 (C-2), 28.96, 28.74, 28.44, 24.52 (C-3), 18.72 (C-6), 16.75 (C-9); MS (APCI): 930.2 ($C_{72}H_{19}O_2N$, calc. $M^+$ *m/z* = 929.949).

*1-(10-Carbonylmethoxy-4-decynyl)-aziridino-[2',3';6,6][60]fullerene (**2e**).* Dark brown solid: m.p.: >350°C. Yield: 38% based on the amount of [60]fullerene consumed; TLC ($R_f$): 0.4 (toluene); UV-vis ($CH_2Cl_2$, $\lambda_{max}$ nm): 424, 492; IR (KBr, neat): 1738 (C=O, str.), 1429, 1349, 1173, 1072, 526 ($C_{60}$) $cm^{-1}$; $^1H$ NMR ($CDCl_3$, $\delta_H$): 3.80 (*t*, *J* = 6.9, 2H, 11-*H*), 3.67 (*s*, 3H, COOC$H_3$), 2.67 (*tt*, *J* = 2.3, 6.9, 2H, 9-*H*), 2.34 (*qn*, *J* = 6.9, 2H, 10-*H*), 2.33 (*t*, *J* = 7.5, 2H, 2-*H*), 2.19 (*tt*, *J* = 2.3, 6.9, 2H, 6-*H*), 1.66 (*qn*, *J* = 7.5, 2H, 3-*H*), 1.55–1.32 (*m*, 4H, C$H_2$); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 174.15 (C-1), [145.19, 145.14, 144.65, 144.56, 143.81, 143.10, 142.90, 142.30, 142.15, 140.79 ($C_{60}$-$sp^2$)], 85.05 ($C_{60}$-$sp^3$), 81.35 (C-7), 79.46 (C-8), 51.54 (COO*C*$H_3$), 49.95 (C-11), 34.03 (C-2), 29.05, 28.71, 28.47, 24.56 (C-3), 18.73 (C-6), 16.82 (C-9); MS (APCI): 930.2 ($C_{72}H_{19}O_2N$, calc. *m/z* = 929.949).

## DISCUSSION

Two major difficulties were encountered in the synthesis work. First, the yields of the products from the reactions between ω-azido fatty esters and [60]fullerene were very low (a range from 5–9% based on the amount of [60]fullerene initially used or 22–35% based on the amount of [60]fullerene

reacted). In fullerene chemistry it is the practice to calculate the yields based on the amount of [60]fullerene consumed rather than on the actual amount of [60]fullerene used at the start of the reaction. Second, the separation and purification of the products by silica column chromatography were a very tedious exercise, but unavoidable. The optimal reaction condition was to reflux 1.3 molar equivalent amounts of ω-azido fatty ester with 1 molar equivalent of [60]fullerene for 16 h in chlorobenzene in high dilution. Attempts to increase the relative molar amount of ω-azido fatty ester in the reaction led to the production of [60]fullerene derivatives with multiaddends. This result complicated the separation process even further. All purified products (aza-fullerene derivatives) appeared as dark brown solids with m.p. higher than 350°C and are soluble in diethyl ether, benzene, toluene, chloroform, or dichloromethane. However, these derivatives could not be crystallized from any of these solvents.

Despite the fact that a mixture of [60]fullerene and aza-[60]fullerene fatty ester derivatives appeared to be readily separated on an analytical silica TLC plate, the removal of unreacted [60]fullerene from the isolated crude product could only be accomplished by eluting the silica column with a large quantity of *n*-hexane/toluene (1:1, vol/vol). The requisite product could then be isolated using toluene as the eluant. The column chromatographic separation process was closely monitored by TLC analysis of subfractions (50 mL) of eluant, in order to ensure high purity of the desired aza-[60]fullerene ester derivatives.

Attempts to use preparative high-performance liquid chromatography or preparative TLC to isolate aza-[60]fullerene fatty ester derivatives were unsuccessful. The reactions of saturated ω-azido fatty esters (6:0, 8:0, and 12:0) with [60]fullerene gave aza-[60]fullerene ester derivatives (**2a–2c**) with a "[5,6]-open" type aza substructure in the cage (13,17). The presence of the "[5,6]-open" aza substructure in these products was evident from the $^{13}C$ NMR spectral analysis, which showed the appearance of 30–31 $sp^2$ carbon signals in the region of $\delta_C$ 133–147. All carbon atoms of the [60]fullerene cage were therefore of the olefinic nature ($sp^2$ hybridized).

In the reaction between methyl 6-azido-hexanoate and [60]fullerene, the mass spectral analysis (APCI) of the isolated product (**2a**) showed a weak molecular ion peak at *m/z* = 863.5, which corresponded to the molecular formula of $C_{67}H_{13}O_2N$ (calc. *m/z*: 863.846). This result indicated the presence of a single fatty ester addend attached to the [60]fullerene unit. The IR spectrum confirmed the presence of the ester function and the [60]fullerene cage by the absorption bands at 1734 and 526 $cm^{-1}$, respectively. The proton spectrum of compound **2a** further confirmed the presence of the aza-ester addend from the proton shift of the methylene group adjacent to the nitrogen atom at $\delta_H$ 3.81. The carbon shift of the methylene group adjacent to the nitrogen atom appeared at $\delta_C$ 51.62. These shift values agreed with data reported by Prato *et al.* (13) and those by Hawker *et al.* (18) for aza-fullerene derivatives. The assignment of the shifts of the various carbon atoms of the fatty ester moiety was readily achieved. The reactions of methyl 8-azido-octanoate and methyl 12-azido-dodecanoate with [60]fullerene also gave the corresponding "[5,6]-open" type aza [60]fullerene derivatives (**2b, 2c**).

The reaction of methyl 11-azido-7-undecynoate with [60]fullerene furnished two products (compounds **2d** and **2e**), which were isolated in the pure form by silica column chromatography. The molecular ions of compounds **2d** and **2e** (APCI) were identical ($M^+$, *m/z* = 930.2, calc. 929.949 for $C_{72}H_{19}O_2N$), which indicated that these products were monoadducts and of the same molecular weight. The $^{13}C$ NMR spectrum of compound **2d** showed 30 signals in the region of $\delta_C$ 133–147, which confirmed the "[5,6]-open" type of aza substructure of the [60]fullerene cage. The presence of the acetylenic bond [$\delta_C$ 16.75 (C-9), 18.72 (C-6), 79.30 (C-8), 81.15 (C-7)] and the ester group [$\delta_C$ 51.52, $\delta_H$ 3.67] in the addend (fatty ester moiety) was readily confirmed by NMR spectroscopic analysis.

Compound **2e** showed a very different $^{13}C$ NMR spectrum compared to that obtained for compound **2d**. Instead of displaying a multitude of $sp^2$ signals at $\delta_C$ 133–147, compound **2e** gave only 10 $sp^2$ signals in the region of $\delta_C$ 140–145. This reduction in the number of signals showed that the molecule was more "symmetrical" in its structure than those of compounds **2a–2d**. Moreover, in the carbon spectrum of compound **2e**, a signal at $\delta_C$ 85.05 showed the presence of $sp^3$ carbon atoms in the cage (19). From these shift data, compound **2e** contained an aziridine system where the nitrogen atom straddled the carbon atoms of two six-membered rings (hexagons) of the fullerene cage to yield a "[6,6]-closed" type aziridino adduct.

Many reactions between various azides or diazo compounds with [60]fullerene have been reported (13,17–25). Banks *et al.* (19) and Diederich *et al*. (20) found that the "[6,6]-closed" aziridino adduct and the "[5,6]-open" type aza-fullerene appeared to be more stable than the other two possible types ("[6,6]-open" aza-fullerene and "[5,6]-closed aziridino adduct, respectively).

It is interesting that only [5,6]-open monoadducts were found in the reactions of [60]fullerene with saturated ω-azido fatty esters, but both [5,6]-open and [6,6]-closed adducts were obtained substantially when methyl 11-azido-7-undecynoate reacted with [60]fullerene. In order to find out whether compound **2d** could have been derived from **2e** or vice versa, these derivatives were individually refluxed in toluene. It was found that compound **2d** was partially converted to compound **2e** (about 50% as indicated by TLC and NMR analyses) after 50 h of reflux. However, compound **2e** remained unchanged after refluxing in a toluene solution for 50 h. An attempt to heat compound **2e** to a higher temperature (132°C, refluxing a solution of chlorobenzene) led to decomposition of the compound. These experiments showed that the rearrangement reaction (from a "[5,6]-open" aza-fullerene to a "[6,6]-closed" aziridino adduct) appeared to be unidirectional. To explain this unexpected result, we propose that

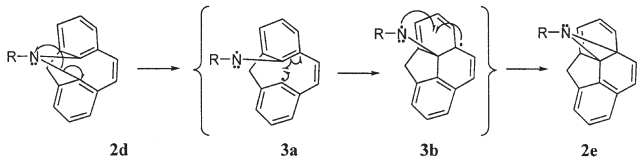


**FIG. 2.** Partial structure of [60]fullerene and the rearrangement of the carbon radical intermediate.

heating of the "[5,6]-open" adduct causes one of the C-N bonds to cleave homolytically to form a very unstable singlet nitrene-carbon free radical intermediate (**3a**). The stability of the intermediate is enhanced by the presence of an acetylenic bond in the alkyl chain. This situation resembles the stabilizing effect of a nitrogen atom on free radical fragments encountered during the electron bombardment of picolinyl esters of unsaturated fatty acids during mass spectrometric analyses (26). To compensate for a more thermodynamically stable intermediate, the nitrene free radical intermediate (**3a**) undergoes ring closure at the 5,6-junction to yield a nitrene with a tertiary carbon free radical (**3b**). Recombination of the singlet nitrene with the tertiary carbon free radical forms the "[6,6]-closed" aziridino adduct (**2e**). The presence of an acetylenic bond in the alkyl chain is therefore vital to stabilize the first step of nitrene free radical formation (Fig. 2). This plausible mechanism appears to be in line with a suggestion made by Banks *et al.* (19) on the mechanistic origin of minor closed "[5,6]-isomers" (aziridino fullerenes).

In conclusion, we found that reactions of saturated ω-azido fatty esters with [60]fullerene furnished mainly [60]fullerene fatty ester derivatives of the "[5,6]-open" type of aza-fullerene, while the acetylenic ω-azido fatty ester gave a mixture of [60]fullenene ester derivatives containing a "[5,6]-open" aza-fullerene and a "[6,6]-closed" aziridino adduct. The presence of the acetylenic group in the fatty ester moiety appeared to affect the isomerization process of a "[5,6]-open" aza substructure to a "[6,6]-closed" aziridino system of the fullerene unit.

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# A New Diacylgalactolipid Containing 4*Z*-16:1 from the Marine Cyanobacterium *Oscillatoria* sp.

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**ABSTRACT:** A new diacylgalactolipid was isolated from the marine cyanobacterium *Oscillatoria* sp., and the structure was elucidated as (2*S*)-3-*O*-β-D-galactopyranosyl-1-*O*-(9*Z*,12*Z*-octadecadienoyl)-2-*O*-(4*Z*-hexadecenoyl)glycerol by enzymatic partial hydrolysis using lipase and physicochemical evidence, which included determining the double bond position in the hexadecenoic acid moiety.

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Cyanobacteria are recognized as a rich source of biologically active natural products, including a variety of glycolipids (1,2). Extracts of several species of *Oscillatoria* have already yielded glycolipids (3) and bioactive peptides (4). As part of an effort to discover biologically active natural products, we report the isolation of a new diacylgalactolipid **1**, along with the known diacylgalactolipids II and IV (3), from the lipophilic extract of the marine cyanobacterium *Oscillatoria* sp.

## EXPERIMENTAL PROCEDURES

*Culture*. The marine cyanobacterium, *Oscillatoria* sp. (strain # KMCC CY-11), was obtained from the Korea Marine Microalgae Culture Center, Pukyong National University. The strain was cultured for 28 d at 23°C in an f/2 medium (5) with aeration (filtered air, 0.3 L/min) under cool-white fluorescent illumination of 5000 lux. After 4 wk, the algal mass was harvested by centrifugation at 10,000 × *g*, filtration on filter paper, and lyophilization. Yields of lyophilized cells were in the range of 100 ± 25 mg/L of culture.

*Isolation of diacylgalactolipid **1**.* The lyophilized algal mass (10.0 g) was extracted with $CH_2Cl_2$/MeOH (1:1, vol/vol) at room temperature and concentrated under reduced pressure to yield an extract (3.0 g). From the extract (3.0 g), seven fractions were obtained by means of silica gel column chromatography (CC) by eluting with *n*-hexane/EtOAc (100% → 0%, vol/vol) and then EtOAc/MeOH (100% → 0%, vol/vol). The eluent with *n*-hexane/EtOAc gave fractions 1-3, and subsequent elution with EtOAc/MeOH yielded fractions 4-7. Fraction 5, containing diacylgalactolipid **1** was eluted with EtOAc/MeOH (5:1, vol/vol). Fraction 5 (140 mg) containing **1** was decolorized by activated-carbon CC using MeOH/$CH_3COCH_3$ (100% → 0%, vol/vol) as the eluent, and purified by high-performance liquid chromatography (YMC, Kyoto, Japan; column 4.6 × 250 mm, refractive index detector, octadecylsilane-A, MeOH) to furnish **1** (20.0 mg). Properties of **1** include the following: colorless viscous solid; $[\alpha]_D$ −6° (*c* 0.2, $CHCl_3$); infrared (IR) (neat): 3420, 1735, 1640, 1245, 1155, 1075 $cm^{-1}$; see Table 1 for nuclear magnetic resonance (NMR) spectral data of **1**.

Other features of the experimental procedures include the following.

*Gas chromatography–mass spectrometry (GC–MS).* Conditions of analysis: Hewlett-Packard (Palo Alto, CA) HP-5 capillary column, 0.32 mm × 50 m; gradient temperature increases (3°C/min) from 80 to 150°C and (5°C/min) from 150 to 290°C.

*Properties of **1a.*** High-resolution fast atom bombardment mass spectrometry (FABMS) *m/z* 513.3039 $[M + Na]^+$ (calculated for $C_{25}H_{46}O_9Na$, 513.3040); low resolution FABMS *m/z* 513 $[M + Na]^+$; see Table 1 for NMR spectral data.

*Methyl 9Z,12Z-octadecadienoate*. $^1H$ NMR (400 MHz, $CDCl_3$) δ5.35 (4H, *m*), 3.67 (3H, *s*), 2.77 (2H, *dd, J* = 6.5, 6.0 Hz); 2.07 (2H, *t, J* = 7.5 Hz), 2.04 (4H, *dt, J* = 7.0, 6.5 Hz), 1.62 (2H, *m*), 1.31 (*m*), 0.89 (3H, *dd, J* = 7.0, 6.5 Hz).

*Methyl hexadecenoate. m/z* 268 $[M]^+$ (1), 236 $[M - CH_3OH]^+$ (16), 194 $[M - CH_3COOCH_3]^+$ (45), 179 (5), 166 (12), 152 (34), 138 (11), 123 (17), 110 (22), 96 (69), 84 (59), 74 $[CH_3COOCH_3]^+$ (100); $^1H$ NMR (400 MHz, $CDCl_3$) δ 5.42 (1H, *ddd, J* = 10.7, 7.5, 7.0 Hz); 5.33 (1H, *m*), 2.36 (2H, *m*), 2.03 (2H, *dt, J* = 7.0, 6.5 Hz), 1.58 (*br. s*), 1.26 (*br. s*), 0.88 (3H, *dd, J* = 6.9, 6.7 Hz).

*Dimethyldisulfide (DMDS) derivative of methyl hexadecenoate. m/z* 362 $[M]^+$ (3), 215 $[CH_3(CH_2)_{10}CH{=}SCH_3]^+$ (64), 147 $[CH_3OOCCH_2CH_2CH{=}SCH_3]^+$ (29), 115 (39), 87 $[147 - (CH{=}SCH_3)]^+$ (100).

## RESULTS AND DISCUSSION

Diacylgalactolipid **1** was obtained as a colorless viscous solid. The IR spectrum of **1** revealed absorptions indicative of hydroxyl (3420 $cm^{-1}$), ester (1735, 1245 $cm^{-1}$), and glycosidic moieties (1075 $cm^{-1}$). The $^1H$ and $^{13}C$ NMR spectra of **1** showed signals assignable to monogalactopyranosyl diacylglycerol (Table 1). Alkaline hydrolysis of **1** with 3.0% (wt/vol) NaOMe/dry MeOH gave a galactopyranosyl glycerol **1b** and a mixture of fatty acid methyl esters.

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Abbreviations: CC, column chromatography; DMDS, dimethyl disulfide; FABMS, fast atom bombardment mass spectrometry; GC–MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance.

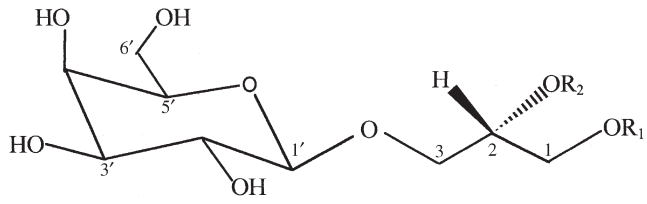
**TABLE 1**
**$^1$H (δ, mult., *J*) and $^{13}$C Nuclear Magnetic Resonance (δ, mult.) Data for Diacyl Galactolipid (1) and 1-*O*-Deacylated Galactolipid (1a)[a]**

| | $^1$H | | $^{13}$C | |
|---|---|---|---|---|
| C# | **1** | **1a** | **1** | **1a** |
| 1 | 4.42 (*dd,* 12.0, 3.0) | 4.21 (*dd,* 7.8, 2.0) | 64.0 (*t*) | 61.7 |
| | 4.22 (*dd,* 12.0, 6.5) | 3.72 (*m*)[d] | | |
| 2 | 5.26 (*m*) | 5.04 (*dddd,* 5.5, 5.2, 4.8, 4.4) | 71.8 (*d*) | 74.8[j] |
| 3 | 3.98 (*dd,* 11.0, 5.5) | 3.96 (*dd,* 10.9, 5.5) | 68.7 (*t*) | 68.8 |
| | 3.73 (*m*)[b] | 3.72 (*m*)[d] | | |
| 1′ | 4.23 (*d,* 7.5) | 4.23 (*d,* 7.3) | 105.4 (*d*) | 105.3 |
| 2′ | 3.50 (*m*) | 3.51 (*m*)[e] | 72.4 (*d*) | 72.4 |
| 3′ | 3.44 (*dd,* 9.5, 3.2) | 3.45 (*dd,* 9.7, 3.2) | 74.9 (*d*) | 74.9[j] |
| 4′ | 3.82 (*d,* 3.2) | 3.81 (*d,* 3.0) | 70.2 (*d*) | 70.3 |
| 5′ | 3.51 (*m*) | 3.51 (*m*)[e] | 76.8 (*d*) | 76.8 |
| 6′ | 3.73 (m)[b] | 3.72 (*m*)[d] | 62.5 (*t*) | 62.5 |
| 1″ | | | 175.0 (*s*)[h] | |
| 9″ | 5.29–5.43 (*m*)[c] | | 131.2 (*d*)[i] | |
| 10″ | 5.29–5.43 (*m*)[c] | | 130.8 (*d*)[i] | |
| 12″ | 5.29–5.43 (*m*)[c] | | 129.0 (*d*)[i] | |
| 13″ | 5.29–5.43 (*m*)[c] | | 128.5 (*d*)[i] | |
| 18″ | 0.90 (*dd,* 7.0, 6.8)[g] | | 14.5 (*q*)[f] | |
| 1‴ | | | 174.2 (*s*)[h] | 174.6 |
| 4‴ | 5.29–5.43 (*m*)[c] | 5.41 (*dt,* 14.0, 7.0) | 132.5 (*d*)[i] | 132.3[i] |
| 5‴ | 5.29–5.43 (*m*)[c] | 5.36 (*ddd,* 12.5, 7.0, 5.5) | 128.9 (*d*)[i] | 128.7[i] |
| 16‴ | 0.91 (*dd,* 7.0, 6.8)[g] | 0.89 (*t,* 7.0) | 14.5 (*q*)[f] | 14.4 |

[a]Recorded in $CD_3OD$ at 300 MHz ($^1$H) and 75 MHz ($^{13}$C).
[b–f]Signals within the same column overlapped.
[g–j]Interchangeable in each column.

The galactopyranosyl glycerol, $[\alpha]_D$ −6° ($H_2O$), was shown to be identical in all respects with (2*R*)-3-*O*-β-D-galactopyranosylglycerol **1b** (3). Therefore, the absolute configuration at C-2 of **1** has been determined to be *S*. The fatty acid composition in diacylgalactolipid **1** was analyzed to be a 1:1 mixture of methyl 9*Z*,12*Z*-octadecadienoate and methyl hexadecenoate by GC–MS, but the latter was not identified in the position of double bond.

To define the locations of these fatty acid residues in diacylgalactolipid **1**, we applied enzymatic hydrolysis (3). The lipase-catalyzed hydrolysis of **1** using Lipase type XIII (Sigma, St. Louis, MO) afforded 1-*O*-deacylated galactolipid **1a** and 9*Z*,12*Z*-octadecadienoic acid, the latter being identified with an authentic sample by GC–MS and $^1$H NMR. Detailed comparisons of the $^1$H and $^{13}$C NMR spectra of **1a** and **1** showed that the signals due to both $H_2$-1 and C-1 were observed at higher fields than those in **1** (Table 1). These data strongly suggested that 9*Z*,12*Z*-octadecadienoic acid residue was on C-1 of the diacylgalactolipid **1**. Alkaline treatment of 1-*O*-deacylated galactolipid **1a** provided (2*R*)-3-*O*-β-D-galactopyranosylglycerol **1b** and methyl hexadecenoate, the latter exhibiting a molecular ion peak at *m/z* 268 corresponding to $C_{17}H_{32}O_2$ and a different spectrum from that of methyl 9*Z*-hexadecenoate in GC–MS. The geometry of the double bond in methyl hexadecenoate was supposed to be *Z* from the coupling constant of olefinic protons [δ 5.42 (1H, *ddd, J* = 10.7, 7.5, 7.0 Hz); 5.33 (1H, *m*)]. Therefore, methyl hexadecenoate was supposed to be an isomer of methyl 9*Z*-hexadecenoate in the double bond position.




**1**: $R_1$ = *9Z,12Z*-octadecadienoyl, $R_2$ = *4Z*-hexadecenoyl
**1a**: $R_1$ = H, $R_2$ = *4Z*-hexadecenoyl
**1b**: $R_1$ = $R_2$ = H

**SCHEME 1**

Treatment of methyl hexadecenoate with DMDS and iodine afforded the disubstituted adduct, resulting from the addition of a molecule of DMDS to a double bond (6,7). The expected adduct showed a molecular ion peak at *m/z* 362 and the fundamental fragments *m/z* 215 and 147, which unequivocally demonstrated the cleavage of the carbon-carbon bond between the two methylthio groups. This characteristic cleavage of the bond between the two carbon atoms linked to sulfur indicates the original position of the double bond and at the same time allows an unequivocal identification of the derivative.

Based on the above evidence, the structure of the diacylgalactolipid **1** was assigned as (2*S*)-3-*O*-β-D-galactopyranosyl-1-*O*-(9*Z*,12*Z*-octadecadienoyl)-2-*O*-(4*Z*-hexadecenoyl)glycerol (Scheme 1).

## ACKNOWLEDGMENTS

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# Triple-Bonded Unsaturated Fatty Acids Are Redox Active Compounds

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**ABSTRACT:** Unsaturated fatty acids with triple bonds are used as inhibitors of unsaturated fatty acid metabolism or cytochrome P450 reactions because they are believed to be chemically inert. In this paper we use *in vitro* cytochrome C reduction to show that two commonly used triple-bonded unsaturated fatty acids are in fact potent electron transfer agents and could affect the multiple cellular systems that are redox-modulated.

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Electron transfer plays an essential role in mitochondia, oxidases, and many other physiological systems. It has been shown that organic acids with double bonds, aside from being metabolized to a series of products, such as prostaglandins and leukotrienes, are also potent electron transfer agents, and indeed this property has been hypothesized as the reason for their being necessary for the function of the electron transport system (1).

Organic acids with triple bonds have been used in biological systems as inhibitors of unsaturated fatty acid metabolism or cytochrome P450 reactions because they are thought to be chemically inert under physiologic conditions (2,3). Because organic acids with triple bonds possess pi electrons, which play a pivotal role in electron transfer, we felt these compounds might also act as electron transfer agents, making them important redox modulators. Pi electrons have been shown to enhance electron transfer in DNA as well as unsaturated fatty acids (1,4). Since they are true catalysts in this reaction they must act by lowering the activation energy for the electron transfer. Two such organic acids with triple bonds, 5,8,11,14-eicosatetraynoic acid (ETYA), a nonmetabolizable arachidonic acid analog, and 17-octadecyonic acid (17-ODYA), a cytochrome P450 inhibitor, were evaluated to examine their potential for redox activity. We assessed the hypothesis that ETYA and 17-ODYA would enhance electron transport as measured by cytochrome C reduction. Organic acids with double bonds, oleic and arachidonic acids, were studied for comparison.

## MATERIALS AND METHODS

ETYA, 17-ODYA, and cytochrome C were obtained from Sigma Chemical Co. (St. Louis, MO). Oleic and arachidonic acids were obtained from Nu-Chek-Prep (Elysian, MN).

A dose-response curve of the reduction of cytochrome C by ferrous iron in the presence of 17-ODYA was obtained (Fig. 1). Each reaction was carried out in 50 mM tris (hydroxy-methyl) aminomethane (TRIS) pH 7.4 and contained 25 μM cytochrome C. A baseline absorbance was taken at 550 nM, ferrous iron (120 μM) was added as an electron donor, and readings were taken at 550 nM at indicated time points. All reactions were carried out at 25°C. Each reaction was carried out in triplicate.

A dose-response curve of the reduction of cytochrome C by ferrous iron in the presence of ETYA was also obtained (Fig. 2). Each reaction was carried out in 50 mM TRIS pH 7.4 and contained 25 μM cytochrome C. A baseline absorbance was taken at 550 nM, ferrous iron (120 μM) was

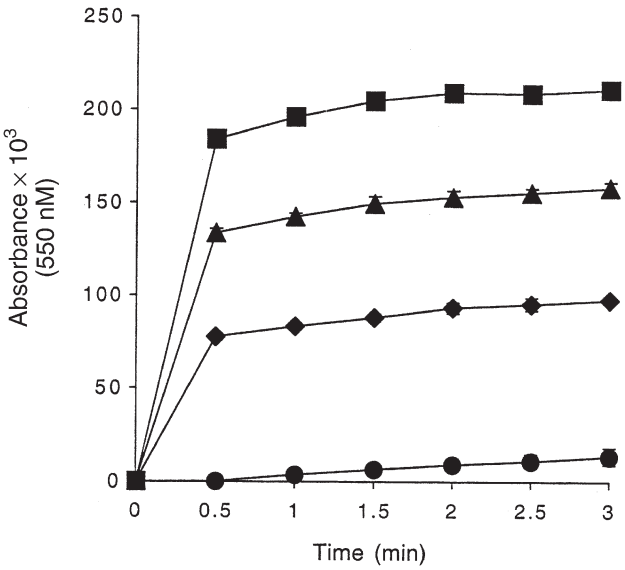


**FIG. 1.** Dose-response curve of acceleration of reduction of cytochrome C by ferrous iron in the presence of 17-octadecyonic acid (17-ODYA). Absorbance was measured at 550 nm. Each data point is mean ± SEM of three separate experiments (some error bars hidden by symbols). ●, Blank; ◆, 20 μM; ▲, 40 μM; ■ 100 μM.

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Abbreviations: ETYA, 5,8,11,14-eicosatetraynoic acid; 17-ODYA, 17-octadecyonic acid; TRIS, tris(hydroxymethyl)aminomethane.

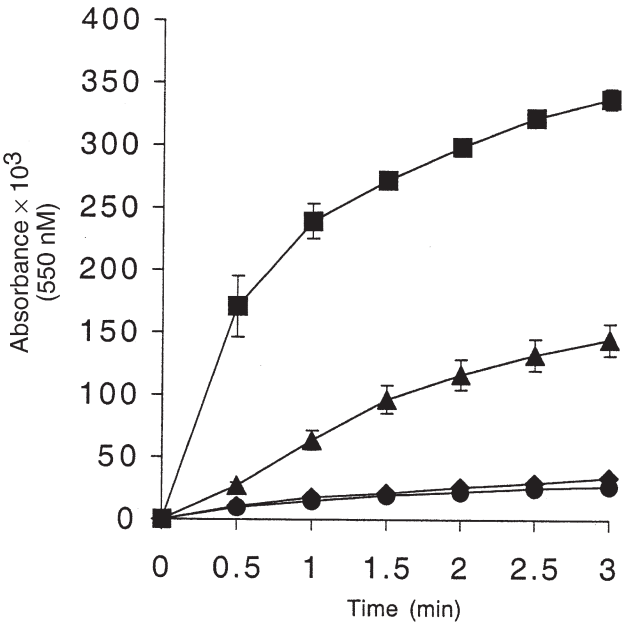


**FIG. 2.** Dose-response curve of acceleration of reduction of cytochrome C by ferrous iron in the presence of 5,8,11,14-eicosatetraynoic acid (ETYA). Absorbance was measured at 550 nm. Each data point is mean ± SEM of three separate experiments (some error bars hidden by symbols). ●, Blank; ◆, 60 μM; ▲, 150 μM; ■ 300 μM.

added, and readings were taken at 550 nM at indicated time points. All reactions were carried out at 25°C. Each reaction was carried out in triplicate.

The effect of oleic acid on cytochrome C reduction was studied in an identical manner (Fig. 3).

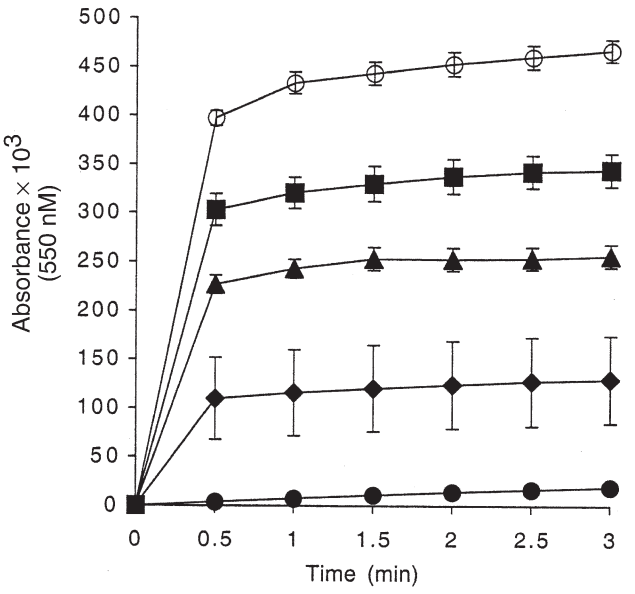


**FIG. 3.** Dose-response curve of acceleration of reduction of cytochrome C by ferrous iron in the presence of oleic acid. Absorbance was measured at 550 nm. Each data point is mean ± SEM of three separate experiments (some error bars hidden by symbols). ●, Blank; ◆, 22.5 μM; ▲, 45 μM; ■ 112 μM; ○, 225 μM.

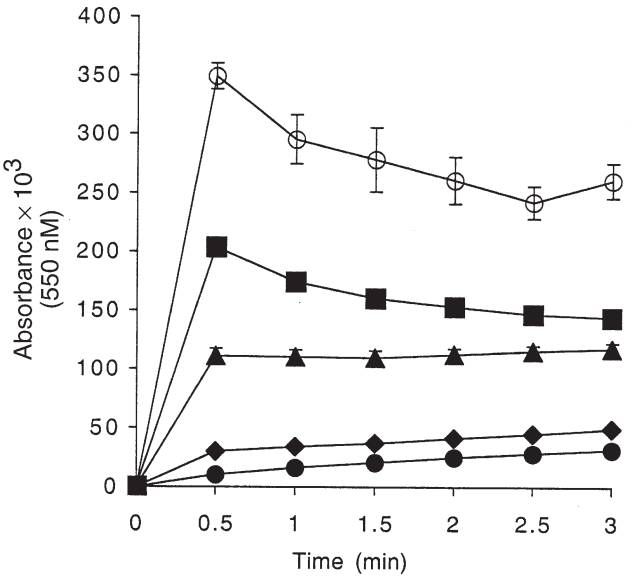


**FIG. 4.** Dose-response curve of acceleration of reduction of cytochrome C by ferrous iron in the presence of arachidonic acid. Absorbance was measured at 550 nm. Each data point is mean ± SEM of three separate experiments (some error bars hidden by symbols). ●, Blank; ◆, 22.5 μM; ▲, 45 μM; ■ 112 μM; ○, 225 μM.

The effect of arachidonic acid on cytochrome C reduction was studied in an identical manner (Fig. 4).

## RESULTS AND DISCUSSION

The dose-response levels indicate that both triple bond organic acids, 17-ODYA and ETYA, are effective electron transfer agents. They are somewhat less potent than double-bonded organic acids with the same degree of unsaturation, i.e., 17-ODYA vs. oleic acid or ETYA vs. arachidonic acid. Because of this property, they could affect any of the multiple cellular systems that have evidence of redox modulation. These include receptor activation (5–10), control of ion channels and pumps (11–17), G protein signaling (18–19), and transcription factor activation (20). Although the concentrations used in this study are slightly higher than the 10 μM used when they are given as inhibitors (2–3), it is known that these fatty acids are lipophilic and concentrate to much higher levels in membranes. Certainly, 20 μM 17-ODYA had a marked effect in terms of promoting the reduction of cytochrome C. It is of interest to note that at higher concentrations (40 μM) ETYA inhibits (as does arachidonic acid) DNA synthesis and in some cell systems produces morphologic changes in the mitochondria similar to those seen with oxidant stress (21,22). If ETYA is acting as an electron transfer agent and molecular oxygen as an electron acceptor, either directly or indirectly *via* a metal, this could produce those morphologic changes. We have previously shown that arachidonic acid can transfer electrons to molecular oxygen (23).

It is important to recognize that the effect of any molecule that is redox active, in addition to having other mechanisms

of action, must be interpreted with caution when evaluating cellular processes.

## ACKNOWLEDGMENTS

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REVIEW

# Fatty Acid Composition of Pinaceae as Taxonomic Markers

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**ABSTRACT:** Following our previous review on *Pinus* spp. seed fatty acid (FA) compositions, we recapitulate here the seed FA compositions of *Larix* (larch), *Picea* (spruce), and *Pseudotsuga* (Douglas fir) spp. Numerous seed FA compositions not described earlier are included. Approximately 40% of all *Picea* taxa and one-third of *Larix* taxa have been analyzed so far for their seed FA compositions. Qualitatively, the seed FA compositions in the three genera studied here are the same as in *Pinus* spp., including in particular the same Δ5-olefinic acids. However, they display a considerably lower variability in *Larix* and *Picea* spp. than in *Pinus* spp. An assessment of geographical variations in the seed FA composition of *P. abies* was made, and intraspecific dissimilarities in this species were found to be of considerably smaller amplitude than interspecific dissimilarities among other *Picea* species. This observation supports the use of seed FA compositions as chemotaxonomic markers, as they practically do not depend on edaphic or climatic conditions. This also shows that *Picea* spp. are coherently united as a group by their seed FA compositions. This also holds for *Larix* spp. Despite a close resemblance between *Picea* and *Larix* spp. seed FA compositions, principal component analysis indicates that the minor differences in seed FA compositions between the two genera are sufficient to allow a clear-cut individualization of the two genera. In both cases, the main FA is linoleic acid (slightly less than one-half of total FA), followed by pinolenic (5,9,12-18:3) and oleic acids. A maximum of 34% of total Δ5-olefinic acids is reached in *L. sibirica* seeds, which appears to be the highest value found in Pinaceae seed FA. This apparent limit is discussed in terms of regio- and stereospecific distribution of Δ5-olefinic acids in seed triacylglycerols. Regarding the single species of *Pseudotsuga* analyzed so far *(P. menziesii),* its seed FA composition is quite distinct from that of the other two genera, and in particular, it contains 1.2% of 14-methylhexadecanoic (anteiso-17:0) acid. In the three genera studied here, as well as in most *Pinus* spp., the $C_{18}$ Δ5-olefinic acids (5,9-18:2 and 5,9,12-18:3 acids) are present in considerably higher amounts than the $C_{20}$ Δ5-olefinic acids (5,11-20:2 and 5,11,14-20:3 acids).

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Abbreviations: Ag-TLC, argentation thin-layer chromatography; anteiso-17:0, 14-methylhexadecanoic; FA, fatty acid; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; TAG, triacylglycerol; UPIFA, unsaturated polymethylene-interrupted fatty acid.

Many attempts have been made to gain some insight into the phylogenetic interrelationships of conifers, because they are the most prominent components of the extant flora, with a very long history and particularly rich fossil record, beginning in pre-Permian time (1). The family Pinaceae already presented a significant history prior to the Late Triassic period (180 million years ago; 2,3). As we were involved in the systematic study of conifer (and more generally gymnosperm) seed fatty acid (FA) compositions, we noted that these data could be of some use as new, original, and supplementary chemometric markers for the taxonomy of this plant group (4–9).

All conifer seeds contain lipids that include part of a series of FA that were considered until recently as "unusual" (10), Δ5-unsaturated polymethylene-interrupted FA (Δ5-UPIFA). These FA have been shown in the meantime not only to be common constituents of seed oils from all Coniferophyte families but also to be characteristic of some Cycadophyte families (10–12). In gymnosperms (Coniferophytes and Cycadophytes), Δ5-UPIFA have the structures 5,9-18:2 (taxoleic); 5,11-18:2 (ephedrenic); 5,9,12-18:3 (pinolenic); 5,9,12,15-18:4 (coniferonic); 5,11-20:1; 5,11,14-20:3 (sciadonic), and 5,11,14,17-20:4 (juniperonic) acids, all ethylenic bonds being in the *cis* configuration. In addition to seeds, these FA also occur in the leaf and wood lipids of Coniferophytes and likely of some Cycadophytes (10,13–16).

The family Pinaceae (Coniferophytinae) contains a total of 11 or 12 genera: *Abies*, *Cathaya*, *Cedrus*, *Keteleeria*, *Larix*, *Nothotsuga*, *Picea*, *Pinus*, *Pseudolarix*, *Pseudotsuga*, *Tsuga*, and *Hesperopeuce*, the latter with an ill-defined taxonomic position (also considered a *Tsuga* species) (17,18). Among these genera, *Pinus* is the largest and most heteromorphic genus. The seed FA compositions available for the most common pine species, totaling approximately one-half of extant species, have been recently reviewed (8). The genera *Larix* and *Picea* are closely related to *Pinus*, although their relationships are still poorly understood. *Pinus*, *Larix*, and *Picea* are sometimes put together along with *Cathaya* and *Pseudotsuga* into a "Pinoid" group, as opposed to an "Abietoid" group that embraces *Abies*, *Cedrus*, *Tsuga*, *Nothotsuga*, *Pseudolarix*, and *Keteleeria* (19). But other subfamily arrangements have been proposed, e.g., Pinoideae (*Pinus*), Laricoideae (*Larix*, *Pseudolarix*, *Cedrus*), and Abietoideae (*Abies*, *Cathaya*, *Keteleeria*, *Picea*, *Pseudo-*

*tsuga*, *Tsuga*); Pinoideae (*Pinus*), Piceoideae (*Picea*), Laricoideae (*Larix*, *Cathaya*, *Pseudotsuga*), Abietoideae (all other genera) (20); or more recently (21), Pinoideae, encompassing three tribes [Pineae (*Pinus*), Abieteae (*Cathaya*, *Picea*, *Tsuga*, *Cedrus*, *Keteleeria*, and *Abies*), and Lariceae (*Larix*, *Pseudotsuga*)], and Pseudolariceae (*Pseudolarix*). Clearly, there is a lack of general agreement as regards to the intergeneric relationships among Pinaceae.

In this study, a compilation of data available on *Picea*, *Larix*, and *Pseudotsuga* seed FA compositions is made, including numerous unpublished seed FA compositions. Species examined here represent approximately 40% of *Picea* and almost one-half of *Larix* species and varieties. Despite the fact that the three genera present rather similar seed FA compositions, principal component analysis and discriminant analysis allow distinction between them, improving our previous analysis of Pinaceae genera (5). This allows preliminary conclusions to be drawn on general features of the quantitative distribution of Δ5-UPIFA and other constituent seed FA in *Picea*, *Larix*, and *Pseudotsuga*.

## EXPERIMENTAL PROCEDURES

*Seeds, oil extraction, and FA methyl ester (FAME) preparation*. Most seeds were purchased from Lawyer Nursery, Inc. (Plains, MT), F.W. Schuhmacher Co., Inc. (Sandwich, MA), and Sandeman Seeds (Pulborough, Great Britain). *Picea abies* seeds from 15 different French orchards and indigenous stands and one location in Poland were kindly donated by Vilmorin S.A. (La Ménitré, France). Seeds were kept at 4°C until use. Lipid extraction, always performed by starting with 10-g samples taken from 15 ± 5 g of powdered seeds, and FAME preparation were performed as described in detail elsewhere for other gymnosperm seeds (4–7). All FAME preparations were made in duplicate and each solution was analyzed once by gas–liquid chromatography (GLC). Generally, FAME were prepared within 24 h after lipid extraction and immediately analyzed.

*Analytical GLC*. All FAME preparations were analyzed by GLC in a Carlo Erba 4130 chromatograph (Carlo Erba, Milano, Italy) equipped with a DB-Wax column (30 m × 0.32 mm i.d., 0.5 μm film; J&W Scientific, Folsom, CA). The oven temperature was 190°C, and the inlet pressure of the carrier gas (helium) was 140 kPa. Occasionally, to confirm some identifications, a CP-Sil 88 column (50 m × 0.25 mm i.d., 0.2 μm film; Chrompack, Middelburg, The Netherlands) was operated with temperature programming in a Carlo Erba HRGC chromatograph from 150 to 185°C at 4°C/min with $H_2$ at 100 kPa. The injector (split mode) and flame-ionization detector were maintained at 250°C for both columns. Quantitative data were calculated by SP 4290 integrators (Spectra Physics, San Jose, CA). In some instances, particularly to detect potential late-eluting components but also to confirm identifications, a Silar 5 CP (50 m × 0.25 mm i.d., 0.2 μm film; Chrompack), fitted in a Hewlett-Packard HP 5890 gas chromatograph (Avondale, PA), was used in the temperature program mode (isothermal for 1 min at 165°C; from 165 to 205°C at a rate of 1°C/min; isothermal at 205°C for 60 min). Nitrogen was the carrier gas, and the injector and detector temperatures were maintained at 230 and 260°C, respectively (22).

*Identification of FAME peaks*. The seed lipids from selected conifer species (23,24) or Ranunculaceae species (25) were used as sources of Δ5-olefinic acid methyl esters with known structures to identify FA from seed lipids by GLC, either by coinjection, comparison of the equivalent chain lengths (DB-Wax column), or retention times (CP-Sil 88 and Silar 5 CP).

*Data analysis*. Principal component analysis was performed with the program STATBOX (Grimmer, Paris, France). The classifications of *Picea* spp. were performed with the program XLSTAT (copyright T. Fahmy, Paris, France). To compare the variability of intra- and interspecific seed FA compositions of *P. abies* and *Picea* spp., respectively, ascending hierarchical classifications were computed using the Ward method. This method consists in minimizing the loss of intraclass inertia at each step. The results are presented as dendrograms (see below) in which the aggregation level can be interpreted as a dissimilarity index between the objects (i.e., *Picea* species, or *P. abies* locations).

## RESULTS AND DISCUSSION

*Comments on Δ5-UPIFA and other FA*. Regarding the genera studied here, most Δ5-UPIFA were initially structurally characterized in *L. kaempferi* [reported as *L. leptolepis* (26)] seed lipids by mass spectrometry coupled with GLC, further in *P. jezoensis* (27), and later supported by GLC in the same two *Larix* species (15). The presence of the 5,9-18:2 and 5,9,12-18:3 acids was recently confirmed by similar techniques in the seeds from *P. glauca engelmannii* ("interior spruce," a hybrid) and *P. glauca* (28). All Δ5-UPIFA reported in the seeds have also been characterized in the leaves of many *Picea* and *Larix* species (14), as well as in the wood of *P. abies* (13,16).

Detailed comments on the resolution of individual FA, including Δ5-UPIFA, have been presented elsewhere (8). All FA reported in the present study are baseline resolved except for *cis*-vaccenic acid, which is not completely resolved from oleic acid. Qualitatively, with respect to both the $C_{18}$ and the $C_{20}$ acid series, the routinely observed FA in the seed lipids from the genera *Picea*, *Larix*, and *Pseudotsuga* are exactly the same as in the genus *Pinus* (8).

The minor FA eluting from 12:0 to 15:0, as well as those eluting after 22:0 acid, are not reported individually in the present study and are included in the category "others" in the tables. On the other hand, the anteiso-17:0 (14-methylhexadecanoic) and 17:1 acids (likely the Δ9 isomer) are included. Anteiso-17:0 acid was unambiguously characterized by mass spectrometry in *Larix leptolepis* (26) as well as in *Pinus* seed lipids (29). In contrast, the 19:0 and "branched" 19:0 acids identified by mass spectrometry in the former species [each accounting for 0.2% of total FA (26)] have not been reported by other authors and are not included in the present study. It is likely that these acids are masked by other more important FA,

owing to similar GLC behavior. No unsaturated $C_{22}$ acids have been reported in *Larix* or *Picea* seed lipids, but a 13-22:1 acid occurs in *Pseudotsuga menziesii* [0.1% (30)].

It is worthwhile to mention the presence of anteiso 19:1, anteiso 5,9-19:2, anteiso 9,12-19:2, and anteiso 5,9,12-19:3 acids, the latter three acids totaling *ca.* 1.8% of total polyunsaturated acids in *P. abies* wood extracts (13). These acids, to our knowledge, have not yet been identified in conifer seeds, although occasionally we noted small unknown peaks in the chromatographic zone where these branched acids are supposed to elute (in the neighborhood of 9,12-18:2 and 9,12,15-18:3 acids). A better insight into these rare minor FA can be obtained by "bidimensional" chromatography that associates GLC and argentation thin-layer chromatography (Ag-TLC) of FAME. The latter analytical procedure, applied to total FAME, allows their fractionation according to the number of ethylenic bonds (provided they all are in the *cis*-configuration) with, however, subtle effects linked to their position along the hydrocarbon chain and to the chain length.

When such isolated fractions are concentrated *ca*. 10 times prior to further GLC analysis, a considerable number of minor components are then observable on chromatograms (results not shown). For *L. decidua* seed lipids (Deluc, L.G., and Wolf, R.L., unpublished results), the anteiso-19:0 acid can be located on chromatograms of the saturated fraction. It accounts for approximately 0.3% of total FA. However, this branched FA, on chromatograms of unfractionated FAME, elutes under the main 9,12-18:2 acid, and cannot be quantitated. In the same way, there are indications of an anteiso-19:1 acid in the 1Δ Ag-TLC fraction (*ca*. 0.3% of total FA) that co-elutes with the 5,9,12-18:3 acid under routine analytical conditions. Such overlaps are the limitations in the accuracy of data presented here, but they should be of very minor importance as regards to our conclusions.

A word should be added concerning juniperonic acid, which apparently does not occur in any significant amounts in *Larix* and *Picea* seed FA when total (4,5, current paper), neutral, or polar (15) lipids are analyzed under routine analytical conditions. Minor amounts (<0.1%) of this FA would, however, occur in one case. On the other hand, applying the bidimensional chromatographic procedure described above to FAME prepared from *L. decidua* seeds allowed unambiguous characterization of juniperonic acid, as well as of its putative metabolic precursor, 11,14,17-20:3 acid, but in trace amounts only, even after concentration of the fraction. Under such conditions, no arachidonic or eicosapentaenoic acids, recently characterized in the seeds and leaves of species from the Araucariaceae family (31,32), could be observed in the species analyzed here. It should, however, be noted that arachidonic acid was reported to be present in the cambium zone of *L. sibirica* (cited in Ref. 33; original article in Russian), though other detailed and thorough studies of *P. abies* wood extracts did not mention such an occurrence (13,16).

*Comments on* Picea*,* Larix*, and* Pseudotsuga *classification and nomenclature.* Several proposals have been made regarding subdivisions of the genus *Picea*, essentially based on morphological characters, e.g., the shape and structure of needles, buds, and cone-scales (34,35). Except for Liu (34), who divided the genus *Picea* into two subgenera (*Omorika* and *Picea*), other systems use "sections," "series," or "phyla." However, according to Wright (36) or Debazac (35), any divisions of the genus *Picea* are unlikely to correspond to well-differentiated evolutive phyla, owing to the large occurrence of natural hybridization. This may be illustrated by the gradual morphological transition from one species to another in the wild, e.g., from *P. abies* to *P. obovata* (*P.* × *fennica*). *Picea abies* can also cross, naturally or experimentally (more or less successfully), with morphologically similar species, separated by wide ranges that, however, are connected by "intermediate" species (Asia: *P. montigena*, *P. likiangensis*, *P. koyamae*), or with morphologically distinct species with neighboring (Europe; *P. orientalis*) or widely separated (North America; *P. mariana*, *P. rubens*, *P. sitchensis*) ranges (36). It was recently (37) inferred from nuclear ribosomal 18S sequence analysis that *P. rubens* and *P. mariana* (North America) are more closely related to the European species *P. omorika* (limited to a small area in Bosnia-Herzegovina) than to other North American *Picea*. Thus, even geographical grouping (35) is questionable.

With respect to *Larix*, tentative divisions of the genus have also been suggested, based primarily on female cone morphology and anatomy (38), but recent chloroplast and nuclear ribosomal DNA fragment analyses were not consistent with such classifications (39,40). Rather, they gave some molecular evidences for clades linked to geographical locations (e.g., *Larix* spp. from Eurasia vs. *Larix* spp. from North America), a situation that would also hold for *Pseudotsuga* (40). However, several *Larix* species can hybridize with one another, e.g., *L. laricina* (North America), *L. decidua* (Europe), and *L. kaempferii* (Japan) (41). The hybrid between the two latter species is known as *L. eurolepis* (35).

Consequently, owing to the lack of agreement, no classifications of the genera *Picea*, *Larix*, and *Pseudotsuga* are adopted here.

Latin as well as trivial names given in Tables 1–3 are from a compilation of descriptions by Wright (36), Debazac (35), and Liu (34), and from tree-seed seller catalogs, corrected wherever possible for synonymy according to Farjon (42). Names reported in original references but not "officially" recognized by Farjon (42) have been modified, e.g., *L. leptolepis* in Takagi and Itabashi (15) and Wolff *et al*. (4) is reported here as *L. kaempferi*. Spelling of names in original references or in tree-seed seller catalogs may also differ from Farjon's recommendations (42), when they are still in usage, e.g., *P. omorika*, instead of *P. omorica*. A few varieties, e.g., *P. pungens* var. *glauca*, or *L. decidua* var. *sudetica*, recognized by foresters or horticulturists but not mentioned by Farjon (42), have also been kept unchanged. For *Pseudotsuga*, some infrageneric taxa are diversely regarded as valid species [e.g., Debazac (35)] or varieties. The nomenclature retained here is mostly that of Farjon (42), but supplementary varieties recognized by tree-seed sellers are also included in Table 3 (e.g., *P. menziesii* var. *caesia*).

**TABLE 1**
**List of *Picea* Species for Which the Seed Fatty Acid Compositions Have (or have not yet) Been Described (including species analyzed in the present study)**

| Species[a] | Trivial name[b] | Reference[c] |
|---|---|---|
| 1. *P. abies* var. *abies* | Norway spruce, common spruce | 4,43, this study |
| 2. *P. abies* var. *acuminata* | — | — |
| 3. *P. alcoquiana* var. *acicularis* | — | — |
| 4. *P. alcoquiana* var. *alcoquiana* | Alcock's spruce | — |
| 5. *P. alcoquiana* var. *reflexa* | — | — |
| 6. *P. asperata* var. *asperata* | Dragon spruce | This study |
| 7. *P. asperata* var. *heterolepis* | — | — |
| 8. *P. asperata* var. *ponderosa* | — | — |
| 9. *P. aurantiaca* | — | — |
| 10. *P. brachytyla* var. *brachytyla* | Sargent spruce | — |
| 11. *P. brachytyla* var. *complanata* | — | — |
| 12. *P. brachytyla* var. *rhombisquamea* | — | — |
| 13. *P. breweriana* | Brewer spruce | This study |
| 14. *P. chihuahuana* | Chihuahua spruce | — |
| 15. *P. crassifolia* | Qinghai spruce | — |
| 16. *P. engelmannii* var. *engelmannii* | Engelmann spruce | 5 |
| 17. *P. engelmannii* var. *mexicana* | — | — |
| 18. *P. farreri* | — | — |
| 19. *P. glauca* var. *albertiana* | Alberta spruce | — |
| 20. *P. glauca* var. *glauca* | White spruce | This study |
| 21. *P. glehnii* | Sakhalin spruce | — |
| 22. *P. jezoensis* spp. *hondoensis* | Hondo spruce | This study |
| 23. *P. jezoensis* spp. *jezoensis* | Yezo spruce | 15 |
| 24. *P. koraiensis* var. *koraiensis* | Korean spruce | This study |
| 25. *P. koraiensis* var. *pungsanensis* | — | — |
| 26. *P. koyamae* | Koyama spruce | This study |
| 27. *P. likiangensis* var. *hirtella* | — | — |
| 28. *P. likiangensis* var. *likiangensis* | Lijiang spruce | This study |
| 29. *P. likiangensis* var. *linzhiensis* | — | — |
| 30. *P. likiangensis* var. *montigena* | — | — |
| 31. *P. likiangensis* var. *rubescens* | — | — |
| 32. *P. mariana* | Black spruce, bog spruce | This study |
| 33. *P. maximowiczii* var. *maximowiczii* | — | — |
| 34. *P. maximowiczii* var. *senanensis* | — | — |
| 35. *P. meyeri* | Meyer spruce | This study |
| 36. *P. morrisonicola* | Taiwan spruce | — |
| 37. *P. neoveitchii* | Veitch spruce | — |
| 38. *P. obovata* | Siberian spruce | This study |
| 39. *P. omorika* | Serbian spruce | 5 |
| 40. *P. orientalis* | Oriental spruce | 5 |
| 41. *P. pungens* | Colorado spruce, blue spruce | 4, this study |
| 42. *P. purpurea* | Purple-coned spruce | — |
| 43. *P. retroflexa* | — | This study |
| 44. *P. rubens* | Red spruce | This study |
| 45. *P. schrenkiana* var. *schrenkiana* | Shrenkiana spruce | — |
| 46. *P. schrenkiana* var. *tianschanica* | Tian-Shan spruce | This study |
| 47. *P. sitchensis* | Sitka spruce | 4 |
| 48. *P. smithiana* | Himalayan spruce | This study |
| 49. *P. spinulosa* | East Himalayan spruce | — |
| 50. *P. torano* | Tigertail spruce | — |
| 51. *P. wilsonii* | Wilson's spruce | This study |

[a]List mostly based on Farjon's *World Checklist and Bibliography of Conifers* (42). Hybrids are not included. See text, however, for synonymy and spelling.
[b]List mostly based on descriptions by Liu (34), Debazac (35), and Wright (36), and on tree seeds sellers' catalogs. The web site http://www.geocities.com/RainForest/Canopy was also consulted.
[c]A dash indicates that the seed fatty acid composition of the species has not yet been established.

*The meaning of seed FA compositions as chemotaxonomic markers*. A study of the variability of the seed FA composition of *P. abies* as a possible function of the geographical origin of the seeds was conducted. For this purpose, seeds from 15 *P. abies* stands located in different regions of France and growing at different altitudes were analyzed. Two lots from each origin were extracted, and each lipid extract was used to prepare FAME for further GLC analysis. The results are

**TABLE 2**
**List of *Larix* Species for Which the Seed Fatty Acid Compositions Have (or have not yet) Been Described (including species analyzed in the present study)**

| Species[a] | Trivial name[b] | Reference[c] |
|---|---|---|
| 1. *L. czekanowskii* | — | — |
| 2. *L. decidua* var. *carpatica* | — | — |
| 3. *L. decidua* var. *decidua* | European larch | 5 |
| 4. *L. decidua* var. *polonica* | — | — |
| 5. *L. gmelinii* var. *japonica* | — | — |
| 6. *L. gmelinii* var. *gmelinii* | Dahurian larch | This study |
| 7. *L. gmelinii* var. *olgensis* | Olga Bay larch | This study |
| 8. *L. gmelinii* var. *principis-rupprechtii* | Prince Rupprecht larch | — |
| 9. *L. griffithii* var. *griffithii* | — | — |
| 10. *L. griffithii* var. *speciosa* | — | — |
| 11. *L. kaempferi* | Japanese larch | 15 |
| 12. *L. laricina* | Tamarack, Eastern larch | This study |
| 13. *L. lyallii* | Subalpine larch | — |
| 14. *L. mastersiana* | — | — |
| 15. *L. occidentalis* | Tamarack, Western larch | This study |
| 16. *L. potaninii* var. *chinensis* | — | — |
| 17. *L. potaninii* var. *himalaica* | — | — |
| 18. *L. potaninii* var. *macrocarpa* | — | — |
| 19. *L. potaninii* var. *potaninii* | — | — |
| 20. *L. sibirica* | Siberian larch | This study |
| 21. *L. sukaczewii* | Siberian larch | This study |

[a]List mostly based on Farjon's *World Checklist and Bibliography of Conifers* (42). Hybrids are not included.
[b]List mostly based on Debazac (35) descriptions, and tree seeds sellers' catalogs. The web site http://www.geocities.com/RainForest/Canopy was also consulted.
[c]A dash indicates that the seed fatty acid composition of the species has not yet been established.

graphically expressed as dendrograms in Figure 1. Intraspecific dissimilarities between *P. abies* from different French stands are visibly of minor importance as compared to interspecific dissimilarities between other *Picea* species. Tillman-Sutela *et al*. (43), who conducted a similar study on *P. abies* from 10 locations in Finland, reached a similar conclusion on the near invariability of *P. abies* seed FA compositions.

It can be inferred from these observations that the seed FA composition of *P. abies* is almost unaffected by edaphic or climatic growing conditions. The very minor differences noted for *P. abies* between France and Finland (Table 4) may as well be linked to differences in analytical procedures and equipment. A similar conclusion regarding the invariability of seed FA was drawn in a study conducted with *Pinus sylvestris* (8). Considering larger distribution areas (Eurasia instead of France), however, showed some small but significant variations for a few FA in *P. sylvestris* seeds from France eastward to Mongolia (8). However, *P. sylvestris* is very heteromorphic [150 "variants" have been described (44)], and at least three varieties are recognized (42). Moreover, the crossability of *P. sylvestris* with Asian pines of the *Sylvestres* subsection is poorly known, and seed FA variations may be linked to introgressions.

**TABLE 3**
**List of *Pseudotsuga* Species for Which the Seed Fatty Acid Compositions Have (or have not yet) Been Described**

| Species[a] | Trivial name[b] | Reference[c] |
|---|---|---|
| 1. *P. japonica* | — | — |
| 2. *P. macrocarpa* | Big cone spruce, big cone Douglas fir | — |
| 3. *P. menziesii* var. *caesia* | Grey Douglas fir | |
| 4. *P. menziesii* var. *glauca* | Blue Douglas fir, Colorado Douglas fir, Rocky Mountain Douglas fir | — |
| 5. *P. menziesii* var. *menziesii* | Douglas fir, Coast Douglas fir | 5,30 |
| 6. *P. sinensis* var. *brevifolia* | — | — |
| 7. *P. sinensis* var. *gaussenii* | — | — |
| 8. *P. sinensis* var. *sinensis* | — | — |

[a]List mostly based on Farjon's *World Checklist and Bibliography of Conifers* (42). See text, however.
[b]List mostly based on Debazac (35) and the web site http://www.geocities.com/RainForest/Canopy.
[c]A dash indicates that the seed fatty acid composition of the species has not yet been established.

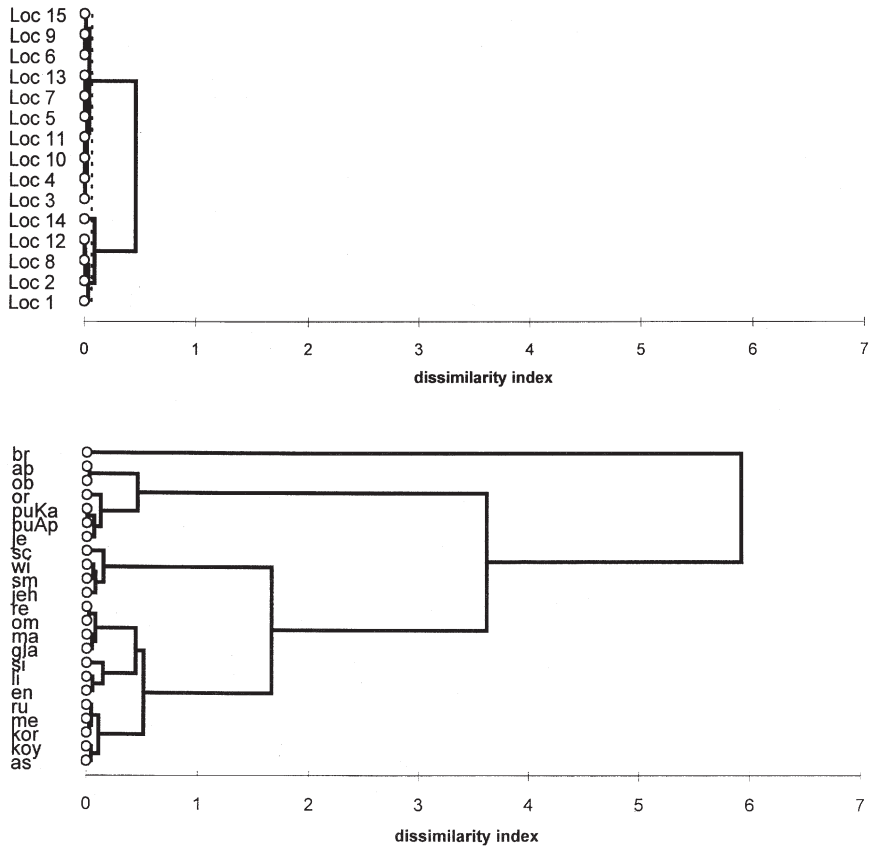


**FIG. 1.** Intraspecific dissimilarity for *Picea abies* from different locations (Loc) in France (upper dendrogram) and interspecific dissimilarity for *Picea* spp. (lower dendrogram). Abbreviations for species correspond to those listed in Table 4.

*Seed FA compositions of* Picea *spp*. Within the Pinaceae family, *Picea* is one of the largest genera (*ca*. 34 species, Table 1) being second to *Abies* and third to *Pinus*. This genus is reported to be rather heteromorphic, but subjected to natural cross-pollination (see above). *Picea* and *Pinus* are most often considered as sister groups, despite a gap of 20 to 70 million years in their fossil history (3,34). *Picea* spp. seeds are rather small, *ca*. 3–6 mm in length [weight of 1000 seeds, 2–9 g (35)]. The oil content is high, in the range 30–40% on a wet weight basis (results not shown). *Picea* spp. seed FA compositions are displayed in Table 4. It is immediately observable that the compositions of species analyzed so far show remarkable similarities. As regards to the species number and the limited variability in seed FA compositions, *Picea* spp. are in sharp contrast with *Pinus* spp. (8).

Total saturated acids in *Picea* spp. are approximately 5% of total FA (data not shown), which is less than in most *Pinus* spp. (*ca.* 10% in most instances). However, in both genera, the prevalence order of individual saturated FA is 16:0 > 18:0 > 20:0 > 22:0, as in *Pinus* spp. As mentioned above, a few branched-chain saturated acids occur. The main visible one is anteiso-17:0 acid, in the narrow range 0.10–0.26%, which is within the range found in most *Pinus* spp. This acid might be an important chemotaxonomic marker, as it does not occur in all conifer families.

The most common unsaturated FA is linoleic acid, with oleic acid being second. Linoleic acid accounts on average for 47.6%, varying in the narrow range 45.3–50.5% of total FA, whereas the corresponding values for oleic acid are 16.5% and 11.4–19.2%, respectively. α-Linolenic acid is a minor component that does not exceed 0.4%. These rankings are similar to those found in most pines (8). The level of *cis*-vaccenic acid in *Picea* seed lipids is relatively high as compared to that in *Pinus* spp. of the *Strobus* subgenus, but not clearly distinct from that occurring in several sections of the *Pinus* subgenus (8).

When considering the distribution profile of Δ5-UPIFA, similarities among the majority of pines are apparent. In particular, the $C_{18}$ Δ5-UPIFA are present in considerably greater proportions than the $C_{20}$ Δ5-UPIFA. Some pine species are not considered here for comparison purposes, e.g., those from the *Parrya* section, or those from the "Mediterranean coast and island" group (45), for reasons discussed elsewhere (mostly because of their exceptionally low total Δ5-UPIFA content) (8). Within the $C_{18}$ Δ5-UPIFA series, the 5,9,12-18:3 acid is always higher than the 5,9-18:2 acid, in the ranges 20.8–26.2%, and 2.1–3.7%, respectively, in *Picea* spp. In *Pinus* spp., the corresponding ranges are somewhat larger (after exclusion of the above-mentioned species, and some others) (8). The minor $C_{20}$ Δ5-UPIFA 5,11-20:2 and 5,11,14-

20:3 acids are always less than 0.09 and 1.24%, respectively, which resemble values found in the haploxyl pine subsection *Strobus*. With regard to sums of Δ5-UPIFA (including the metabolically related 7,11,14-20:3 acid) in *Picea* spp., they are in the range 25.0–30.4% (mean, 28.2%), which are values commonly found in many species of several subsections of the subgenus *Pinus* (e.g., practically all pine species of subsections *Sylvestres*, *Oocarpae*, and *Contortae*), but more seldom in the *Strobus* subgenus (e.g., in *P. strobus*). Bishomopinolenic (7,11,14-20:3) acid, the elongation product of pinolenic acid, is in the range 0.09-0.17%, which would correspond to an elongation rate of the latter acid of 0.6%. This FA is a usual minor component of Pinaceae seed lipids (46).

*Seed FA compositions of* Larix *spp*. This genus apparently has a rather short evolutionary history. No fossil records are known before the Middle or Late Eocene periods (38) or even the Oligocene period (3), and the number of admitted species, not taking into account varieties, is presently limited to 12 (Table 2). As for *Picea* spp., *Larix* spp. seeds are rather small, *ca*. 3–8 mm in length [weight of 1000 seeds, 2–12 g (35)]. Their oil content, relative to the weight of undehulled seeds, varies in the range 9–20% (results not shown).

The seed FA compositions of *Larix* species (Table 5) show remarkable similarities within the genus, and a striking resemblance with *Picea* spp. (*cf.* Table 4). The main FA is 9,12-18:2 acid in both cases, being slightly higher on average in *Larix* spp. than in *Picea* spp., 47.6 vs. 43.3%, respectively. Corresponding values for the second important common FA, 9-18:1 acid, are closer, 16.5 and 17.6%, respectively. The contents of total as well as of individual linear saturated acids in *Larix* spp. are identical to those in *Picea* spp. However, *Larix* and *Picea* spp. fundamentally differ when considering the amount of anteiso-17:0 acid, which in *Larix* is twice that in *Picea* spp. (0.36 vs. 0.18%, respectively).

As for *Picea* spp. and most *Pinus* spp., the $C_{18}$ Δ5-UPIFA are present in *Larix* spp. in higher proportions than the $C_{20}$ Δ5-UPIFA, with 5,9,12-18:3 acid being higher than 5,9-18:2 acid. In *Larix* spp., the range for taxoleic acid is narrower than in *Picea* spp. (2.2–2.6%), and pinolenic acid varies within limits that are definitely higher than in *Picea* spp., 25.8–30.7% vs. 20.8–26.2%. The elongation rate of the latter acid would be higher in *Larix* than in *Picea* spp. (0.9%), leading to a higher percentage of bishomopinolenic acid (mean, 0.24%).

*Seed FA compositions of* Pseudotsuga. Most recent authors (19–21,41,47,48) agree that *Pseudotsuga* is the closest relative to *Larix* within the Pinaceae family. The two genera also share a particular pollination mechanism not observed in other Pinaceae genera (49). This is why this genus is included in the present study. From fossil records, *Pseudotsuga* would have differentiated at the onset of the Cenozoic, slightly earlier than *Larix* (21).

Complete data for *Pseudotsuga* seed FA unfortunately are limited to two analyses of one single species, *P. menziesii*, of unknown variety (possibly the most common one, var. *menziesii*), which are not in full agreement (Table 6). No gross differences with the two preceding genera are noted for linear saturated acids. However, *P. menziesii* appears exceptional as regards to its high anteiso-17:0 acid content, *ca*. 1.2%, which seems unique among Pinaceae genera (29). Older data (50) for Douglas fir seeds indicated the presence of an unknown FA eluting between the 16:1 isomers and 18:0 acid, likely (in retrospect) the anteiso-17:0 acid, in amounts similar to those reported here. Some *Abies* and *Cedrus* species have seed FA containing as high as 0.8–0.9% of anteiso-17:0 acid (29). Whereas the contents of $C_{18}$ Δ5-UPIFA are relatively low, in particular pinolenic acid, the $C_{20}$ Δ5-UPIFA are relatively high, at least when compared to *Picea* and *Larix* species. Based on its seed FA composition, *P. menziesii* thus appears quite distinct from *Larix* spp., but not much more than from *Picea* spp. Obviously, complementary data are needed for *P. menziesii* varieties and for the three other species before a definitive conclusion can be drawn.

*Principal component analysis*. To assess whether the differences noted above between *Picea* and *Larix* spp. seed FA compositions were sufficient to distinguish the two genera on this biochemical basis, data were processed using principal component analysis. For this purpose, data for the species listed in Tables 4 and 5 were used. However, values for the very minor 17:0, 17:1, and 5,11,14,17-20:4 acids were not included as variables in calculations. Figure 2 shows the first two components which explain, respectively, 36.0 and 16.4% of the total inertia. Axis 1 clearly separates the two genera *Picea* and *Larix*. The most explanatory variables correlated with axis 1 can be divided into two groups, of which six are representative of *Larix* spp. These are the 5,9,12-18:3 and its elongation product 7,11,14-20:3; 9,12,15-18:3 and its Δ5-desaturation product 5,9,12,15-18:4; 5,11-20:2; and anteiso-17:0 acids. On the other hand, five FA are representative of *Picea* spp.: 9,12-18:2, its elongation product 11,14-20:2; the Δ5-desaturation product of the latter FA; 5,11,14-20:3; 5,9-18:2; and 20:0 acids.

As part of this study, axis 2 is not relevant to discriminate *Picea* and *Larix* spp. FA correlated with this axis lead to the separation of different species inside both genera. In particular, *P. breweriana* and *L. sukaczewii* are individualized because of a larger proportion of 18:0, 9-18:1 (*P. breweriana*), and 16:0 acids, and a small proportion of 7,11,14-20:3 acid (*L. sukaczewii*). Interestingly, *P. breweriana* is reported to have no close relatives, being locally endemic to southwest Oregon and northwest California in montane to subalpine forests of the Siskiyou Mountains (35). On the other hand, *L. sukaczewii* is reported to be relatively close to *L. sibirica* (35), but their seed FA compositions are rather different (see, e.g., the sums of Δ5-olefinic acids in Table 5). Farjon (42) even considers that *L. sukaczewii* is synonymous with *L. sibirica*, a view that is not supported by our results

*Concluding remarks*. *Picea* species, in contrast to *Pinus* spp., are mostly indistinguishable from one another on the basis of their seed FA compositions. Interspecific variations are even less important than in the *P. sylvestris* complex, which supports the view of Wright (36) that "taxonomically the genus (*Picea*) is more nearly comparable to a single se-

**TABLE 4**
**Fatty Acid Composition (wt% of total fatty acids) of the Seed Lipids from *Picea* spp.**

| Species[a] | 16:0 | 16:1[b] | aiso-17:0[c] | 17:0 | 9-17:1 | 18:0 | 9-18:1 | 11-18:1 | 9,12-18:2 | 9,12,15-18:3 | 20:0 | 11-20:1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. *P. abies* | 2.78 | 0.13 | 0.20 | 0.04 | —[g] | 1.49 | 13.41 | 1.55 | 49.89 | 0.34 | 0.33 | 0.35 |
| 1. *P. abies* (France) | 2.69 | 0.13 | 0.20 | 0.03 | — | 1.50 | 12.98 | 1.41 | 50.15 | 0.25 | 0.28 | 0.32 |
| 1. *P. abies* (Poland) | 3.13 | 0.18 | 0.19 | 0.03 | — | 1.57 | 13.71 | 1.24 | 49.30 | 0.28 | 0.28 | 0.39 |
| 1. *P. abies* (Finland) | 2.18 | 0.09 | — | — | — | 1.52 | 11.37 | 1.76 | 50.52 | 0.31 | 0.27 | 0.32 |
| 6. *P. asperata* | 2.67 | 0.21 | 0.10 | — | — | 1.55 | 17.56 | 1.62 | 46.48 | 0.34 | 0.33 | 0.35 |
| 13. *P. breweriana* | 3.25 | 0.21 | 0.16 | 0.04 | 0.02 | 1.80 | 25.86 | 1.35 | 39.79 | 0.23 | 0.42 | 0.45 |
| 16. *P. engelmanni* | 2.58 | 0.12 | 0.26 | 0.03 | — | 1.28 | 17.28 | 1.51 | 46.39 | 0.41 | 0.34 | 0.42 |
| 20. *P. glauca* var. *glauca* | 2.91 | 0.20 | 0.24 | 0.03 | — | 1.66 | 17.61 | 1.05 | 46.03 | 0.24 | 0.34 | 0.34 |
| 23. *P. jezoensis*[h] | 2.57 | 0.21 | (0.15)[i] | Trace[j] | (Trace)[k] | 1.46 | 15.41 | 0.35 | 49.54 | 0.17 | 0.25 | 0.24 |
| 22. *P. jezoensis* var. *hondoensis* | 3.16 | 0.15 | 0.17 | 0.02 | — | 1.46 | 19.02 | 1.22 | 47.36 | 0.24 | 0.22 | 0.26 |
| 24. *P. koraiensis* | 2.96 | 0.13 | 0.17 | 0.03 | Trace | 1.41 | 16.52 | 1.05 | 47.70 | 0.20 | 0.21 | 0.30 |
| 26. *P. koyamae* | 2.90 | 0.14 | 0.17 | 0.03 | 0.02 | 1.63 | 17.43 | 1.12 | 47.14 | 0.28 | 0.30 | 0.50 |
| 28. *P. likiangensis* | 2.75 | 0.16 | 0.17 | Trace | — | 1.13 | 17.14 | 1.59 | 47.25 | 0.29 | 0.26 | 0.34 |
| 32. *P. mariana* | 2.60 | 0.14 | 0.26 | 0.05 | 0.02 | 1.23 | 18.02 | 0.95 | 45.24 | 0.22 | 0.35 | 0.39 |
| 35. *P. meyeri* | 2.51 | 0.16 | 0.16 | 0.03 | Trace | 1.35 | 16.78 | 1.35 | 47.45 | 0.27 | 0.28 | 0.34 |
| 38. *P. obovata* | 3.08 | 0.14 | 0.18 | 0.03 | Trace | 1.62 | 12.82 | 1.59 | 49.31 | 0.22 | 0.22 | 0.32 |
| 39. *P. omorika* | 2.44 | 0.17 | 0.17 | 0.03 | — | 1.16 | 16.74 | 1.42 | 45.35 | 0.26 | 0.29 | 0.49 |
| 40. *P. orientalis* | 3.09 | 0.09 | 0.22 | 0.03 | — | 1.23 | 16.11 | 0.38 | 49.50 | 0.31 | 0.32 | 0.30 |
| 41. *P. pungens* var. *glauca* (Ap.) | 2.66 | 0.10 | 0.21 | 0.03 | — | 1.22 | 15.12 | 0.95 | 48.47 | 0.37 | 0.27 | 0.36 |
| 41. *P. pungens* var. *glauca* (Ka.) | 2.68 | 0.10 | 0.21 | 0.04 | — | 1.44 | 14.85 | 0.89 | 48.35 | 0.29 | 0.29 | 0.38 |
| 43. *P. retroflexa* | 2.94 | 0.15 | 0.16 | 0.04 | — | 1.31 | 17.33 | 1.58 | 45.28 | 0.28 | 0.21 | 0.28 |
| 44. *P. rubens* | 2.94 | 0.09 | 0.23 | 0.05 | 0.02 | 1.28 | 17.44 | 0.41 | 47.80 | 0.21 | 0.30 | 0.40 |
| 46. *P. schrenkiana* var. *tianshanica* | 3.25 | 0.12 | 0.15 | 0.05 | Trace | 1.77 | 20.45 | 0.96 | 46.10 | 0.33 | 0.33 | 0.36 |
| 47. *P. sitchensis* | 2.94 | 0.13 | 0.17 | 0.04 | — | 1.22 | 16.23 | 0.99 | 48.01 | 0.41 | 0.31 | 0.31 |
| 48. *P. smithiana* | 2.67 | 0.14 | 0.15 | 0.05 | — | 1.23 | 19.05 | 1.88 | 45.97 | 0.23 | 0.23 | 0.42 |
| 51. *P. wilsonii* | 3.08 | 0.14 | 0.17 | 0.07 | — | 1.73 | 19.16 | 1.23 | 46.06 | 0.30 | 0.32 | 0.36 |
| Mean | 2.70 | 0.14 | 0.19 | 0.04 | — | 1.43 | 16.81 | 1.16 | 47.30 | 0.29 | 0.31 | 0.37 |
| SD | 0.53 | 0.04 | 0.04 | 0.01 | — | 0.21 | 3.04 | 0.45 | 2.39 | 0.06 | 0.04 | 0.06 |
| Min. | 2.44 | 0.09 | 0.10 | — | — | 1.13 | 11.37 | 0.35 | 39.79 | 0.17 | 0.21 | 0.24 |
| Max. | 3.25 | 0.21 | 0.26 | 0.07 | 0.02 | 1.80 | 25.86 | 1.88 | 50.52 | 0.41 | 0.42 | 0.50 |

**TABLE 4** (continued)

| Species | 11,14-20:2 | 22:0 | 5,9-18:2 | 5,9,12-18:3 | 5,9,12,15-18:4 | 5,11-20:2 | 5,11,14-20:3 | 7,11,14-20:3 | $\Sigma\Delta5$[d] | Others[e] | Abbreviation[f] | Reference[g] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. *P. abies* | 0.58 | 0.19 | 3.25 | 24.67 | 0.03 | 0.05 | 0.94 | 0.16 | 29.10 | — | — | 4 |
| 1. *P. abies* (France) | 0.71 | 0.10 | 3.23 | 24.99 | 0.03 | 0.03 | 0.96 | 0.16 | 29.40 | — | abFr | This study |
| 1. *P. abies* (Poland) | 0.70 | 0.09 | 3.13 | 24.62 | 0.04 | 0.04 | 0.90 | 0.16 | 28.89 | 0.02 | AbPol | This study |
| 1. *P. abies* (Finland) | 0.69 | 0.11 | 2.98 | 26.21 | — | — | 1.07 | 0.17 | 30.43 | 0.43 | abFi | 43 |
| 6. *P. asperata* | 0.73 | 0.11 | 3.20 | 22.83 | 0.06 | Trace | 1.00 | 0.15 | 27.24 | 0.71 | as | This study |
| 13. *P. breweriana* | 0.54 | 0.18 | 3.39 | 21.55 | 0.04 | Trace | 0.55 | 0.19 | 25.72 | — | br | This study |
| 16. *P. engelmanni* | 0.78 | 0.15 | 2.21 | 24.89 | 0.06 | 0.03 | 0.83 | 0.17 | 28.19 | 0.26 | en | 5 |
| 20. *P. glauca* var. *glauca* | 0.66 | Trace | 3.64 | 24.06 | 0.05 | Trace | 0.68 | 0.13 | 28.56 | 0.13 | gla | This study |
| 23. *P. jezoensis* | 0.44 | Trace | 3.37 | 25.01 | Trace | Trace | 0.71 | — | 29.09 | 0.12 | je | 15 |
| 22. *P. jezoensis* var. *hondoensis* | 0.48 | Trace | 3.50 | 22.02 | Trace | Trace | 0.60 | 0.06 | 26.18 | 0.06 | jeh | This study |
| 24. *P. koyamae* | 0.56 | 0.14 | 4.17 | 22.33 | 0.04 | 0.07 | 0.89 | 0.10 | 27.60 | 0.04 | koy | This study |
| 26. *P. koraiensis* | 0.46 | Trace | 4.57 | 23.40 | Trace | Trace | 0.77 | 0.12 | 28.86 | 0.00 | kor | This study |
| 28. *P. likiangensis* | 0.63 | 0.19 | 2.65 | 23.75 | 0.05 | 0.07 | 1.24 | 0.15 | 27.91 | 0.19 | li | This study |
| 32. *P. mariana* | 0.44 | 0.15 | 4.62 | 24.19 | Trace | 0.09 | 0.97 | 0.08 | 29.95 | — | ma | This study |
| 35. *P. meyeri* | 0.62 | 0.09 | 3.71 | 23.53 | 0.03 | 0.03 | 1.02 | 0.13 | 28.45 | 0.16 | me | This study |
| 38. *P. obovata* | 0.55 | 0.11 | 3.34 | 25.14 | Trace | Trace | 0.70 | 0.07 | 29.25 | 0.56 | ob | This study |
| 39. *P. omorika* | 0.50 | 0.14 | 3.51 | 25.02 | 0.04 | 0.07 | 1.00 | 0.17 | 29.81 | 1.03 | om | 5 |
| 40. *P. orientalis* | 0.41 | 0.18 | 2.71 | 23.54 | 0.06 | 0.08 | 1.09 | 0.16 | 27.64 | 0.19 | or | 5 |
| 41. *P. pungens* var. *glauca* (Ap.)[f] | 0.76 | 0.16 | 3.23 | 24.66 | 0.03 | 0.03 | 1.10 | 0.13 | 29.18 | 0.14 | puAp | 4 |
| 41. *P. pungens* var. *glauca* (Ka.)[f] | 0.75 | 0.14 | 3.50 | 24.59 | 0.06 | 0.06 | 1.10 | 0.10 | 29.41 | 0.18 | puKa | This study |
| 43. *P. retroflexa* | 0.55 | 0.07 | 3.74 | 24.61 | 0.04 | 0.03 | 0.94 | 0.18 | 29.61 | 0.28 | re | This study |
| 44. *P. rubens* | 0.54 | 0.13 | 3.74 | 23.31 | 0.03 | 0.07 | 0.87 | 0.09 | 28.11 | 0.05 | ru | This study |
| 46. *P. schrenkiana* var. *tianshanica* | 0.50 | 0.15 | 4.10 | 20.24 | 0.04 | 0.09 | 0.88 | 0.06 | 25.41 | 0.07 | sc | This study |
| 47. *P. sitchensis* | 0.47 | 0.19 | 2.10 | 25.75 | — | 0.07 | 0.66 | 0.16 | 28.74 | — | si | 4 |
| 48. *P. smithiana* | 0.48 | 0.15 | 3.69 | 21.90 | 0.05 | 0.09 | 1.01 | 0.13 | 26.87 | 0.48 | sm | This study |
| 51. *P. wilsonii* | 0.60 | 0.76 | 3.27 | 20.83 | 0.05 | 0.03 | 0.77 | 0.09 | 25.04 | 0.98 | wi | This study |
| Mean | 0.59 | 0.19 | 3.32 | 23.76 | 0.05 | 0.06 | 0.92 | 0.14 | 28.21 | 0.23 | | |
| SD | 0.12 | 0.19 | 0.60 | 1.61 | 0.01 | 0.02 | 0.17 | 0.04 | 1.49 | 0.30 | | |
| Min. | 0.41 | Trace | 2.10 | 20.83 | Trace | Trace | 0.55 | 0.06 | 25.04 | — | | |
| Max. | 0.78 | 0.76 | 4.62 | 26.21 | 0.06 | 0.09 | 1.24 | 0.19 | 30.43 | 1.03 | | |

[a]Initial nomenclature modified to fit recent recommendations. Abbreviations of origins, Ap. and Ka., Apache and Kaibab National Forests, Arizona. Data for *P. abies* from France and Finland are the means of values obtained from 15 and 10 different locations, respectively. Original data for Finland were given as mol% and have been recalculated as wt%.
[b]Two isomers, 7- and 9-16:1 acids.
[c]14-Methylhexadecanoic, or anteiso-17:0 acid.
[d]Sum of Δ5-olefinic acids, including the 7,11,14-20:3 acid.
[e]Minor and unidentified components.
[f]Abbreviations refer to Figures 1 and 2.
[g]Not detected or not reported.
[h]Reported as *Pinaceae jezoensis*.
[i]Reported as 16:2n-6.
[j]Trace amounts.
[k]Reported as 16:3n-3.

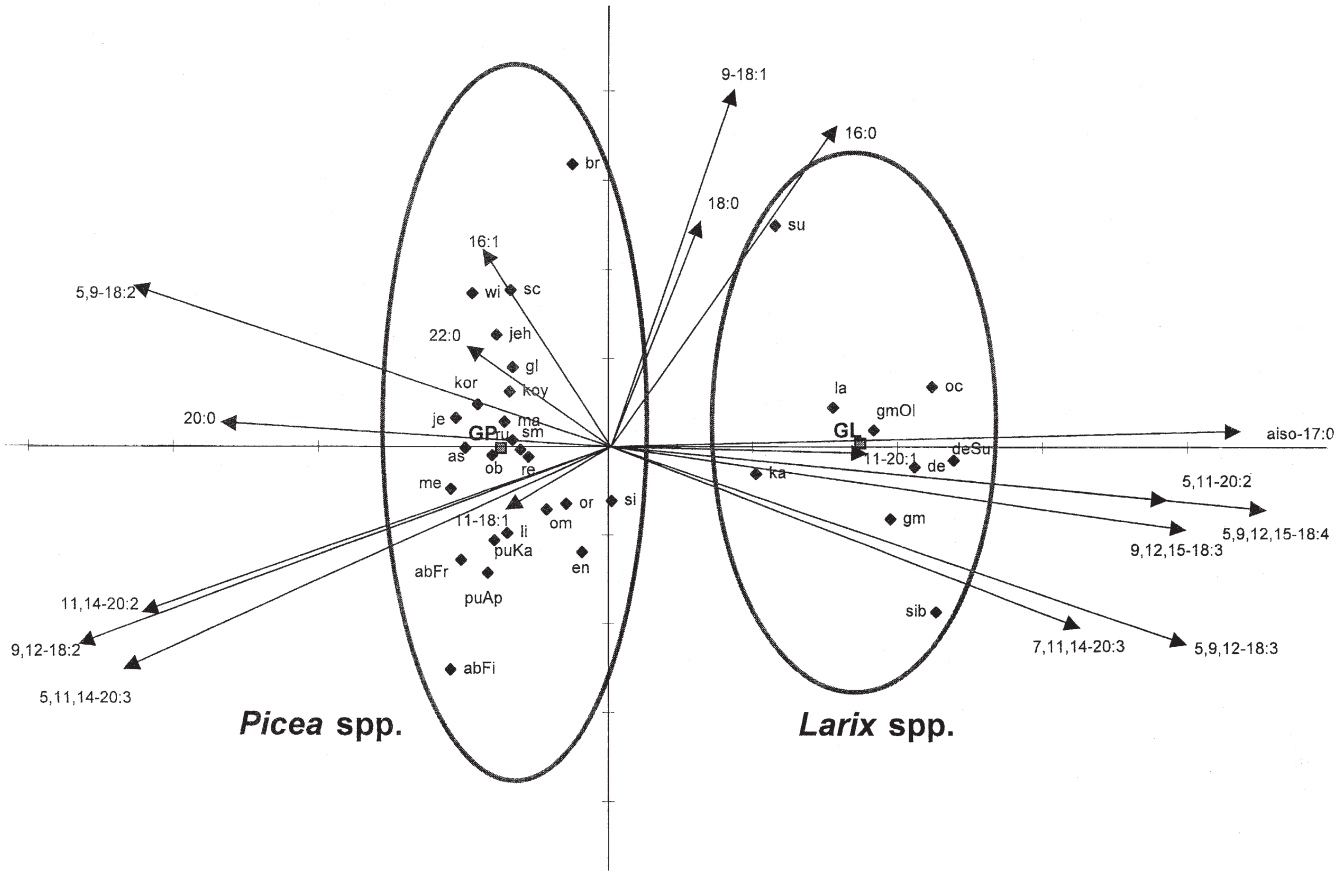


**FIG. 2.** Principal component analysis of *Picea* and *Larix* species and varieties. Abbreviations for species correspond to those listed in Tables 4 and 5. GP and GL, gravity centers.

ries in pine, maple, or ash than to any one of these complete genera." Apparently, the same holds for *Larix* spp. Although *Picea* and *Larix* spp. present on average rather similar seed FA compositions, multicomponent analysis quite clearly distinguishes these two genera. Moreover, our previous statistical analyses of *Picea*, *Larix*, and *Pinus*, though based on a smaller number of species in each genus, showed that *Picea* and *Larix* consistently differed from *Pinus* (5).

This study also confirms our previous observation that total Δ5-UPIFA cannot be higher than one-third of total FA in Pinaceae seeds (51,52). This indeed occurs in the genus *Larix*, for which GLC data were confirmed by $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy (53). A probable explanation for this feature may be the stereospecificity of acylation of these acids to the glycerol backbone of triacylglycerols (TAG). It was shown for a great number of conifers, including some *Larix* and *Picea* species, that Δ5-UPIFA were enriched in the α (external) positions as compared to the β (internal) position of TAG (11). By applying a new regiospecific analysis method (54) that uses GLC of dibutyroyl derivatives of monoacylglycerols generated from TAG by partial deacylation with a Grignard reagent, levels of less than 5% of Δ5-UPIFA (a value close to the limit of detection by $^{13}C$ NMR spectroscopy) were shown to occur in the *sn*-2 position of TAG from *L. gmelinii* var. *olgensis* and *P. shrenkiana* seeds (Destaillats, F., Angers, P., Wolff, R.L., and Arul, J., unpublished data). Somewhat lower values were reported for *P. jezoensis* (15) and *L. decidua* (55). No data, however, are available yet for *P. menziesii*. In a smaller number of cases, including *L. decidua*, stereospecific analysis of conifer seed TAG has shown that Δ5-UPIFA are esterified mostly (*ca.* 90%) to the *sn*-3 position (56), independently of the chain length and the number of ethylenic bonds. Other studies on the seed TAG distribution profile of two *Pinus* species, *P. koraiensis* (57,58) and *P. pinaster* (57), have shown that TAG molecular species containing two or three Δ5-UPIFA are scarce or absent. It is thus probable that Δ5-UPIFA are mostly restricted to the *sn*-3 position of TAG, with the consequence that they cannot be higher than one third or so of total FA.

Incidentally, it should be noted that *Sciadopitys verticillata* (Sciadopityaceae) seed lipids contain in small amounts 2-monoacylglycerols, part of which are esterified with sciadonic acid (59). This would suggest that Δ5-olefinic acids can indeed esterify the *sn*-2 position of TAG, although it cannot be excluded that 2-sciadonoyl-glycerol is an artifact that derives from *sn*-3 monoacylglycerols through isomerization, e.g., during oil extraction.

**TABLE 5**
**Fatty Acid Composition (wt% of total fatty acids) of the Seed Lipids from *Larix* spp.**

| Species[a] | 16:0 | 16:1[b] | aiso-17:0[c] | 17:0 | 9-17:1 | 18:0 | 9-18:1 | 11-18:1 | 9,12-18:2 | 9,12,15-18:3 | 20:0 | 11-20:1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. *L. decidua* | 2.80 | 0.12 | 0.43 | 0.04 | —[h] | 1.46 | 18.76 | 0.97 | 43.10 | 0.56 | 0.23 | 0.40 |
| 1. *L. decidua* var. *sudetica* (Slov.) | 3.21 | 0.15 | 0.41 | 0.05 | — | 1.52 | 17.56 | 0.90 | 42.21 | 0.55 | 0.18 | 0.45 |
| 7. *L. gmelinii* | 2.64 | 0.11 | 0.28 | 0.05 | — | 1.56 | 16.80 | 1.11 | 42.84 | 0.41 | 0.17 | 0.40 |
| 8. *L. gmelinii* var. *olgensis* | 3.13 | 0.12 | 0.38 | 0.04 | 0.03 | 1.67 | 18.18 | 1.07 | 42.70 | 0.40 | 0.18 | 0.43 |
| 12. *L. kaempferi* | 2.62 | 0.34 | (0.30)[i] | 0.02 | Trace[j] | 1.26 | 17.64 | 0.59 | 46.02 | 0.36 | 0.16 | 0.38 |
| 12. *L. kaempferi* | 2.62 | 0.14 | 0.38 | 0.04 | — | 1.36 | 18.38 | 1.11 | 45.53 | 0.35 | 0.31 | 0.50 |
| 13. *L. laricina* | 2.79 | 0.08 | 0.29 | 0.05 | 0.02 | 1.48 | 19.37 | 0.65 | 43.55 | 0.37 | 0.21 | 0.29 |
| 16. *L. occidentalis* | 3.35 | 0.19 | 0.54 | 0.06 | 0.02 | 1.72 | 16.30 | 1.50 | 42.56 | 0.51 | 0.16 | 0.37 |
| 21. *L. sibirica* | 2.80 | 0.10 | 0.36 | 0.05 | Trace | 1.30 | 15.03 | 1.21 | 42.35 | 0.40 | 0.24 | 0.44 |
| 22. *L. sukaczewii* | 3.83 | 0.14 | 0.35 | 0.05 | 0.03 | 1.33 | 20.51 | 1.02 | 41.82 | 0.34 | 0.11 | 0.35 |
| Mean | 2.81 | 0.12 | 0.36 | 0.05 | — | 1.45 | 17.65 | 0.99 | 43.26 | 0.44 | 0.22 | 0.41 |
| SD | 0.21 | 0.03 | 0.06 | 0.01 | — | 0.10 | 1.57 | 0.20 | 1.21 | 0.09 | 0.05 | 0.07 |
| Min. | 2.62 | 0.08 | 0.28 | 0.02 | — | 1.26 | 15.03 | 0.59 | 41.82 | 0.34 | 0.11 | 0.29 |
| Max. | 3.83 | 0.34 | 0.54 | 0.06 | 0.03 | 1.72 | 20.51 | 1.50 | 46.02 | 0.56 | 0.31 | 0.50 |

| Species | 11,14-20:2 | 22:0 | 5,9-18:2 | 5,9,12-18:3 | 5,9,12,15-18:4 | 5,11-20:2 | 5,11,14-20:3 | 7,11,14-20:3 | $\sum\Delta5$[d] | Others[e] | Abbreviation[f] | Reference[g] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. *L. decidua* | 0.35 | 0.10 | 2.20 | 27.39 | 0.12 | 0.14 | 0.51 | 0.20 | 30.56 | 0.12 | de | 4 |
| 1. *L. decidua* var. *sudetica* (Slov.) | 0.41 | 0.13 | 2.50 | 28.21 | 0.15 | 0.13 | 0.67 | 0.29 | 31.95 | 0.32 | deSu | This study |
| 7. *L. gmelinii* | 0.38 | 0.13 | 2.55 | 28.84 | 0.17 | 0.14 | 0.61 | 0.25 | 32.56 | 0.56 | gm | This study |
| 8. *L. gmelinii* var. *olgensis* | 0.42 | 0.10 | 2.24 | 27.72 | 0.10 | 0.12 | 0.56 | 0.23 | 30.97 | 0.18 | gmolg | This study |
| 12. *L. kaempferi* | 0.37 | Trace | 2.25 | 27.00 | 0.08 | 0.08 | 0.30 | — | 29.71 | 0.23 | ka1 | 15 |
| 12. *L. kaempferi* | 0.39 | Trace | 2.24 | 25.81 | 0.08 | 0.08 | 0.52 | 0.22 | 28.95 | — | ka2 | 4 |
| 13. *L. laricina* | 0.23 | 0.09 | 2.41 | 27.38 | 0.11 | 0.14 | 0.27 | 0.10 | 30.41 | 0.12 | la | This study |
| 16. *L. occidentalis* | 0.39 | Trace | 2.11 | 28.90 | 0.14 | 0.06 | 0.33 | 0.18 | 31.72 | 0.61 | oc | This study |
| 21. *L. sibirica* | 0.46 | 0.09 | 2.30 | 30.68 | 0.18 | 0.14 | 0.72 | 0.36 | 34.38 | 0.76 | si | This study |
| 22. *L. sukaczewii* | 0.20 | Trace | 3.75 | 25.53 | 0.06 | 0.06 | 0.25 | 0.06 | 29.71 | 0.03 | su | This study |
| Mean | 0.37 | 0.11 | 2.37 | 28.05 | 0.14 | 0.13 | 0.55 | 0.24 | 31.25 | 0.29 | | |
| SD | 0.08 | 0.02 | 0.14 | 1.64 | 0.04 | 0.02 | 0.16 | 0.09 | 1.63 | 0.26 | | |
| Min. | 0.20 | Trace | 2.11 | 25.53 | 0.06 | 0.06 | 0.25 | 0.06 | 28.95 | — | | |
| Max. | 0.46 | 0.13 | 3.75 | 30.68 | 0.18 | 0.14 | 0.72 | 0.36 | 34.41 | 0.76 | | |

[a]Initial nomenclature modified to fit recent recommendations (e.g., *L. kaempferi* was reported as *L. leptolepis*). Abbreviations of origins: Slov., Slovakia.
[b]Two isomers, 7- and 9-16:1 acids.
[c]14-Methylhexadecanoic, or anteiso-17:0 acid.
[d]Sum of Δ5-olefinic acids, including the 7,11,14-20:3 acid.
[e]Minor and unidentified components.
[f]Abbreviations refer to Figure 2.
[g]Not detected or not reported.
[h]Reported as 16:2n-6.
[i]Trace amounts.

**TABLE 6**
**Fatty Acid Composition (wt% of total fatty acids) of the Seed Lipids from *Pseudotsuga menziesii***

| Species | 16:0 | 16:1[a] | aiso-17:0[b] | 17:0 | 9-17:1 | 18:0 | 9-18:1 | 11-18:1 | 9,12-18:2 | 9,12,15-18:3 | 20:0 | 11-20:1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. menziesii* | 3.53 | 0.11 | 1.15 | 0.04 | —[e] | 1.42 | 17.75 | 0.61 | 49.53 | 0.60 | 0.35 | 0.40 |
| *P. menziesii* | 3.5 | 0.3 | 1.3 | 0.1 | Trace[f] | 1.8 | 18.1 | 0.8 | 44.0 | 0.6 | 0.6 | 0.9 |

| Species | 11,14-20:2 | 22:0 | 5,9-18:2 | 5,9,12-18:3 | 5,9,12,15-18:4 | 5,11-20:2 | 5,11,14-20:3 | 7,11,14-20:3 | 5,11,14,17-20:4 | $\Sigma\Delta5^c$ | Others[d] | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. menziesii* | 0.11 | 0.15 | 2.84 | 18.37 | — | 0.29 | 2.04 | 0.11 | — | 23.65 | 0.60 | 4 |
| *P. menziesii* | 0.5 | 0.5 | 2.8 | 20.1 | 0.1 | 0.4 | 1.7 | — | — | 25.1 | 1.9 | 30 |

[a]Two isomers, 7- and 9-16:1 acids.
[b]14-Methylhexadecanoic, or anteiso-17:0 acid.
[c]Sum of Δ5-olefinic acids, including the 7,11,14-20:3 acid.
[d]Minor and unidentified components.
[e]Not detected or not reported.
[f]Trace amounts.

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ARTICLES

# Characterization of Simple and Reproducible Vascular Stenosis Model in Hypercholesterolemic Hamsters

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**ABSTRACT:** The importance of low-density lipoprotein (LDL) in the etiology of atherosclerosis is well recognized. We have established a reproducible stenosis model in hypercholesterolemic hamsters, and the process of arterial stenosis by thrombus or neointima was studied and compared with that in normal hamsters. The level of plasma LDL was 4.6 times higher in hamsters fed a high-cholesterol diet than in hamsters fed normal food. Endothelial injury in right common carotid arteries was induced using a modified catheter. Arterial blood flow was monitored continuously using a Doppler flow probe. Arterial patency after the initiation of injury in high-cholesterol hamsters was significantly changed as compared with that of normal hamsters. Neointima was observed 2 wk after the vascular injury. The neointimal area of high-cholesterol hamsters was significantly larger than that of normal hamsters. To characterize the stenosis in hypercholesterolemic hamsters, we measured platelet aggregation, thrombin time, activated partial thromboplastin time, and proliferating smooth muscle cells (SMC) *in vitro* and *in vivo*. The half-maximal inhibitory concentration value for platelet aggregation induced by thrombin or collagen, the DNA synthesis stimulated by platelet-derived growth factor (PDGF)-BB, and 5-bromo-2-deoxy-uridine labeling indices (proliferating index of SMC *in vivo*) in high-cholesterol hamsters were each significantly higher than the comparable value from normal hamsters. However, specific binding of PDGF-BB in SMC was not different between the two types of hamsters. Furthermore, we investigated the inhibitory effects of probucol or losartan on neointima formation using this model. Probucol, but not losartan, significantly reduced the neointimal area in hypercholesterolemic hamsters. These findings indicated that high levels of plasma LDL strongly contributed to the development of thrombus and neointima formation *via* both up-regulation of platelet aggregation and the enhancement of SMC proliferation. This stenosis model may be useful for the investigation of hypercholesterolemia-associated cardiovascular diseases.

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Hypercholesterolemia increases the risk of developing cardiovascular diseases, and low density lipoprotein (LDL) plays an important role in atherosclerosis (1). Recent studies have shown that LDL stimulates endothelial cells to produce a variety of growth factors that induce the migration and proliferation of granulocytes, macrophages, and endothelial cells (2,3). These studies raise the possibility that increases in LDL content stimulate the production of factors that play an important role in the proliferation of cells and matrix elements found in both atherosclerosis and vascular neointima formation.

On the other hand, both platelets and LDL are intimately involved in the pathogenesis of atherosclerosis (2). Platelet function is directly influenced by lipoproteins, and platelets from patients with hypercholesterolemia display enhanced platelet reactivity (4). Each platelet processes specific high-affinity binding sites for LDL, ranging from 1000 to 8000 copies per platelet with a dissociation constant ($K_D$) value between 40 and 100 nmol/L (5,6). Moreover, the LDL-induced sensitization is accompanied by decreased angular movement in platelet membranes, possibly caused by increased cholesterol transfer (7), and platelets enriched with cholesterol show increased arachidonic acid release and thromboxane $B_2$ formation (8).

Until now, rabbits fed a high-cholesterol diet or Watanabe rabbits (9) have been the main animals used for the investigation of hypercholesterolemia or atherosclerotic lesions of vascular diseases. Other small animals that can be used for this type of study are exogenously hypercholesterolemic rats and hamsters. In rats, a few months are required to establish hypercholesterolemia after the start of a high-cholesterol diet (10,11). On the contrary, only a few weeks are necessary for hamsters to be hypercholesterolemic (11). Recently, we established a simple and reproducible vascular stenosis model in hamsters (12). We investigated the inhibitory effects of antiplatelet compounds (13–16), angiotensin-converting enzyme inhibitors (17), and an angiotensin II receptor antagonist (18) using this model. Vascular stenosis often accompanies injury to endothelial tissues. Progression of stenosis occurs by thrombus formation during the acute phase or neointima formation during the chronic phase after endothelial injury. Our previous observations clearly indicated that the inhibition of both platelet activation and smooth muscle cell (SMC) proliferation or migration strongly reduced the development of vascular stenosis after endothelial injury (13, 18). On the other hand, the likelihood of vascular occlusion in patients is enhanced by the additive effect of other risk factors, such as diabetes and hypercholesterolemia. Therefore, in the present study, this model was applied to examine the effect of plasma LDL levels on platelets and SMC in order to

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Abbreviations: aPTT, activated partial thromboplastin time; BrdU, 5-bromo-2-deoxy-uridine; DAB, diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HDL, high density lipoprotein; IA, intimal area; $IC_{50}$, half-maximally inhibitory concentration; IELA, internal elastic lamina area; LA, lumen area; LDL, low density lipoprotein; PDGF, platelet-derived growth factor; PRP, platelet rich plasma; SEM, scanning electron microscopy; SMC, smooth muscle cell; TEM, transmission electron microscopy; TT, thrombin time.

define easily determined, clinically significant measures of vascular stenosis progression.

## MATERIALS AND METHODS

*Animals*. Male syrian hamsters (SLC, Sizuoka, Japan) weighing 60–80 g were selected and fed either a standard chow (RC4; Oriental Yeast Co., Ltd., Japan) or chow supplemented with 0.5% (w/w) cholesterol. An operation for vascular injury using a modified catheter was carried out 4 wk after the start of the diet regimen. The body weight of hamsters fed a high-cholesterol diet was not statistically significantly different from that of hamsters fed a normal diet (body weight: 120–130 g). All experiments were performed in accordance with institutional guidelines governing animal experimentation.

*Reagents.* Collagen for platelet aggregation was obtained from Nycomed Arzneimittela GmbH (Munich, Germany). [Methyl-$^{3}$H] thymidine and [$^{125}$I] platelet-derived growth factor (PDGF)-BB were purchased from Amersham Japan (Tokyo, Japan). Probucol and losartan are a kind gift from Otuka Co. Ltd. (Tokushima, Japan) and Banyu Co. Ltd. (Tokyo, Japan), respectively. The other chemicals were obtained from Sigma (St. Louis, MO).

*Induction of vascular stenosis and quantitation of neointima formation.* Four weeks after the start of either the regular or the high-cholesterol dietary regimen, hamsters were anesthetized by intraperitonial injection of 50 mg/kg sodium pentobarbital. In brief, the right common carotid artery and the right femoral artery were exposed, and an arterial injury to the right common carotid artery using a 2FG catheter (Portex Ltd., Kent, United Kingdom) with a roughened tip was performed according to the previously described technique (12). Another catheter (i.d. = 0.5 mm, o.d. = 0.8 mm, polyethylene sp3, Natume Co. Ltd., Tokyo, Japan) was connected to the right femoral artery for monitoring blood pressure and pulse rate using a pressure transducer (AP601G; Nihon Koden, Tokyo, Japan) during experiments on day 0 (initiation of vascular injury). Blood flow in the carotid artery was continuously monitored for 90 min on day 0 using a Doppler flow probe (model PDV-20; Crystal Biotech Co. Ltd., Tokyo, Japan) positioned proximally to the injured area of the carotid artery. Our previous histological observations revealed that a platelet-rich thrombus was obviously established when the blood flow was zero (12). After the recovery from anesthesia, animals were kept in individual cages. Three, 5, 7, and 14 d after vascular injury, hamsters ($n$ = 7 each) in each group were anesthetized and perfused transcardially with saline. The common carotid artery was excised, divided into several sections, and frozen. The tissue samples were cross-sectioned, and stained with hematoxylin (Sigma Chemical Co.) to determine the intimal area. Part of these samples were used for the stain of lipids using oil red. The total areas of the internal elastic lamina (IELA) and lumen (LA) were measured using a computerized image graphic analysis system. The measurement was performed in triplicate for each sample. The average of three intimal area (IA = IELA – LA) determinations was then expressed as a percentage of IELA.

*Measurement of serum cholesterol.* Blood samples (0.3 mL) from hamsters fed either a regular or a high-cholesterol diet were collected in each time period *via* the jugular vein after the animals had been anesthetized with ether. The samples were treated with 3.15% sodium citrate. High density lipoprotein cholesterol (HDL) and LDL cholesterol were determined by enzymatic assays of blood samples taken at 0 (before the start of diet), 2, 4, and 6 wk after the start of feeding regimens These measurement were performed with Nipro cholesterol determination kits (Nipro Co. Ltd., Osaka, Japan).

*Platelet aggregation.* At the end of the experiments, 4.0 mL of blood was collected from every hamster in each group by heart puncture into sodium citrate (3.15%, final concentration) and centrifuged for 10 min at 155 × $g$ to obtain platelet-rich plasma (PRP). Platelets were counted and adjusted to $4 \times 10^8$ cells/mL (final concentration), and then platelet aggregation was induced by collagen (1.0–10.0 μg/mL) or ADP (0.5–5.0 μM) using PRP. Washed hamster platelets were prepared as described previously (19). Washed platelet aggregation was induced by thrombin (0.0001– 0.3 unit/mL). Platelet aggregation was followed using an aggregometer (Aggrecorder II, DA-3220; Kyotodaiichi-Chemical, Kyoto, Japan) at 37°C with a stirring speed of 800 rpm. All measurements were performed in triplicate.

Ex vivo *anticoagulant studies.* After the separation of PRP for platelet aggregation, the remaining blood samples were further centrifuged for 10 min at 1550 × $g$ to obtain platelet-poor plasma (PPP). The activated partial thromboplastin time (aPTT) and thrombin time (TT) were determined using standard clinical laboratory procedures.

*Electron microscopic observation.* In separate experiments, several samples taken from hamsters fed a high-cholesterol or normal diet were used for histological observations by means of scanning or transmission electron microscopy. Hamsters were anesthetized and perfused transcardially with saline 2 wk after the initiation of vascular injury. The common carotid artery was excised and fixed with 2.0% glutaraldehyde in 50 mM sodium phosphate buffer for 30 min. Each segment was cut open longitudinally to allow visual inspection for scanning electron microscopy (SEM) or cross-sectioned for transmission electron microscopy (TEM) as described (12).

*Proliferation index of SMC* in vivo. Proliferating SMC were identified by *in vivo* DNA labeling with the thymidine analog 5-bromo-2-deoxy-uridine (BrdU) (12). BrdU (50 mg/kg) was injected subcutaneously 1, 8, 16, and 24 h prior to removal of the carotid artery. Following carotid artery removal 1, 3, 5, 7, or 14 d after injury from both control and cholesterol-supplemented hamsters ($n$ = 4, each time point), frozen cross sections were prepared from these arteries. BrdU-positive cells were detected with a murine monoclonal antibody (Sigma), followed by treatment with goat antimouse immunoglobulin-antibodies conjugated to peroxidase. The complexes were then stained with diaminobenzidine (DAB). The BrdU labeling index was calculated using the following formula: (nuclei stained positive by DAB)/(total nuclei stained by hematoxylin) × 100. Animals were sacrificed by an overdose of sodium pentobarbital at the end of the experiment.

*Cell culture and measurement of DNA synthesis*. Vascular SMC were isolated from the thoracic aorta of hamsters fed either a normal or supplemented diet. The cells were cultured over several passages using the method of Ross (20). SMC were grown to confluency in 7500-$mm^2$ culture flasks in Dulbecco's modified Eagle's medium [DMEM; Gibco BRL, Grand Island, NY; 5% fetal calf serum (FCS), 100 μg/mL streptomycin, 100 U/mL penicillin, 4 μmol/L glutamine] at 37°C under humidified 5% $CO_2$/95% air. The cells were then treated with 0.25% trypsin in phosphate-buffered saline (pH = 7.4), washed, and counted. A total of $8 \times 10^4$ isolated cells were cultured as above. Cell culture medium was replaced every 2 d. On day 8, cells were detached with trypsin and counted. The cultured cells were stimulated with 5% FCS or various doses of angiotensin II ($10^{-6}$–$10^{-8}$ M), thrombin (0.001–0.03 units/mL), vasopressin ($10^{-6}$–$10^{-8}$ M), endothelin I ($10^{-6}$–$10^{-8}$ M), or PDGF-BB (1.0–100 ng/mL) in 1 mL of DMEM at 37°C for 24 h. Six hours before harvest, the cells were pulse-labeled with [methyl-$^3$H]thymidine (0.5 μCi/dish). Incubation was terminated by adding 1 mL of 10% trichoroacetic acid, and radioactivity in the acid-insoluble materials was determined by using a Beckman LS-6000IC liquid scintillation spectrometer.

*[$^{125}$I]PDGF binding*. Cultured cells were subjected to a binding assay that was essentially as described (21). Briefly, the cells were treated at 37ºC in 10 mM HEPES-buffered Dulbecco's Eagle's medium (1.0 mL, pH = 7.4; Gibco BRL). The cells were incubated with or without a 1,000-fold molar excess of nonradioactive PDGF-BB for 20 min at 37ºC. At the end of the incubation, the cells were thoroughly washed with cold phosphate-buffered saline and solubilized using 0.1% sodium dodecyl sulfate (1.0 mL). The radioactivity of the lysate was then determined using a Wallac 1480 WIZARD 3″ automatic gamma counter (Turku, Finland).

*The effect of probucol or losartan on neointima*. To further define this stenosis model in pharmacological experiments, we investigated the effect of probucol or losartan using this model. Hamsters fed a high-cholesterol diet were divided into seven groups, a control group ($n$ = 10), three groups treated with probucol (twice a day, p.o.) at doses of 30.0, 60.0, or 120.0 mg/kg per day ($n$ = 6 each), and three groups treated with losartan (twice a day, p.o.) at doses of 2.0, 6.0, or 20.0 mg/kg per day ($n$ = 6 each). Oral administration of each compound was started 2 h before the initiation of endothelial injury by a modified catheter and continued for the next 2 wk. Fourteen days after vascular injury, the common carotid artery of hamsters in each group was treated as mentioned in the paragraph on quantitation of neointima formation. At the end of observation period, blood pressure was monitored for 10 min. A catheter (i.d. = 0.5 mm, o.d. = 0.8 mm, polyethylene sp3, Natsume Co. Ltd.) connected to a pressure transducer (AP601G; Nihon Koden) was inserted into the left femoral artery for 10 min. After the measurement of blood pressure, a blood sample was taken for platelet aggregation and measurements of coagulation factors (TT and aPTT).

*Statistics.* All data are presented as the mean ± SEM. The statistical significance of the data was determined by analysis of variance followed by the Student-Newman-Keuls test.

## RESULTS

*Alteration of plasma cholesterol levels.* The mean plasma LDL level in all animals before beginning a specific dietary regimen was <0.5 g/L. After 4 wk of a high-cholesterol diet, the LDL level had increased to ~2 to 2.5 g/L and remained elevated to the end of the observation period. In contrast, there was no significant difference in plasma HDL levels between hamsters fed normal and cholesterol-supplemented diets (Table 1).

*Acute thrombus formation and vascular patency.* Vascular patency is shown in Table 2. Blood flow in the carotid artery in normal hamsters ($n$ = 28) was interrupted in 6.4 ± 0.6 min after the initiation of vascular injury by a catheter. After the blood flow was zero, spontaneous cyclic reflow and reocclusion were observed in 21 hamsters during the observation period of 90 min. The other arteries were not reperfused during the observation period. In the group of high-cholesterol hamsters, the time to occlusion was significantly shortened to 4.8 ± 0.4 min and only four arteries showed spontaneous cyclic reocclusion and reflow during the observation period ($n$ = 28).

*Neointima formation in response to endothelial injury.* All hamsters developed concentric intimal lesions in response to endothelial denudation by a catheter. The ratios of time-dependent vascular stenosis by neointima formation are shown in Figure 1. In hamsters fed a high-cholesterol diet, the extent of vascular stenosis was significantly greater compared with that of hamsters fed a normal diet.

*Platelet aggregation and hemostasis analysis*. The half-maximally inhibitory concentration ($IC_{50}$) values for platelets induced to aggregate by collagen, ADP, and thrombin in ham-

**TABLE 1**
**Plasma Cholesterol Levels (g/L) in Hamsters[a]**

| | Diet group | | Normal group | |
|---|---|---|---|---|
| | LDL | HDL | LDL | HDL |
| Pretreatment | 0.50 ± 0.07 | 0.49 ± 0.08 | 0.47 ± 0.08 | 0.50 ± 0.06 |
| 2 wk | 1.12 ± 0.41 | 0.46 ± 0.09 | 0.49 ± 0.06 | 0.48 ± 0.05 |
| 3 wk | 1.92 ± 0.31* | 0.45 ± 0.06 | 0.49 ± 0.06 | 0.50 ± 0.07 |
| 4 wk | 2.34 ± 0.22* | 0.50 ± 0.07 | 0.49 ± 0.02 | 0.49 ± 0.09 |
| 6 wk | 2.33 ± 0.29* | 0.55 ± 0.07 | 0.52 ± 0.07 | 0.48 ± 0.07 |

[a]Hamsters (4 wk old) were allotted to a high-cholesterol diet group or to the normal diet group. Pretreatment means before beginning a dietary regimen; each week means the time after the start of a particular regimen. Vascular injury in each hamster occurred at 4 wk. *$P < 0.05$ vs. control (normal hamsters in each time course).

**TABLE 2**
**Time to Occlusion and Vascular Patency Status in Hamsters[a]**

| | Normal hamsters | High-cholesterol hamsters[b] |
|---|---|---|
| Time to occlusion (min) | 6.4 ± 0.8 | 4.8 ± 0.4* |
| PP | 0 | 0 |
| CR | 21 | 4** |
| PO | 7 | 24** |

[a]Vascular patency was judged at the end of the observation period. Carotid arterial patency was expressed as persistent occlusion (PO) when no reperfusion was observed at all, as cyclic flow reduction (CR) when the arterial reflow alternately showed stops and flows, and persistent patency (PP) when the arterial flow was maintained until the end of observation period. Data correspond to the number of arteries in each group ($n$ = 28 animals in each group).
[b]*$P < 0.05$; **$P < 0.01$. Time to occlusion data represented mean ± SEM.

**TABLE 3**
**Platelet Aggregation and Hemostatic Analysis of Hamsters[a]**

| | Normal hamster | High-cholesterol hamsters |
|---|---|---|
| ADP | 6.6 ± 0.9 μM | 4.9 ± 1.9 μM |
| Collagen | 7.8 ± 1.1 μg/mL | 3.8 ± 0.2 μg/mL* |
| Thrombin | 0.061 ± 0.1 unit/mL | 0.002 ± 0.0004 unit/mL** |
| TT | 15.4 ± 1.1 s | 13.6 ± 2.3 s |
| aPTT | 58.2 ± 6.8 s | 52.2 ± 4.8 s |

[a]The values of the half-maximally inhibitory concentration for platelet aggregation induced by ADP, collagen (PRP), or thrombin (washed platelets) in hamsters fed a normal diet or a high-cholesterol diet. aPTT, activated partial thromboplastin time; TT, thrombin time. Data represent mean ± SEM. *$P < 0.05$; **$P < 0.01$.

sters fed a normal or a high-cholesterol diet are shown in Table 3. When platelets were induced by thrombin or collagen, the $IC_{50}$ values in high-cholesterol hamsters decreased compared with the values for hamsters fed a normal diet. In terms of hemostatic values, no significant difference was observed between normal and high-cholesterol hamsters. However, these values in high-cholesterol hamsters were slightly shortened as compared with those from normal ones.

*Histological observation.* According to observations obtained through TEM, foam cells and extracellular lipids in neointima were clearly observed in hamsters fed a high-cholesterol diet (Fig. 2). When neointima formation was established 2 wk after vascular injury, the vascular surface in injured area was not smooth; however, repaired endothelial cells were completely covered with neointima. Blood elements were not observed on vascular surfaces in hamsters fed a normal diet (Fig. 3A). On the other hand, platelets were locally adhered on neointimal vascular surfaces in hamsters fed a high-cholesterol diet 2 wk after vascular injury even if endothelial cells were recovered with the vascular surface in the injured area (Figs. 3B,3C).

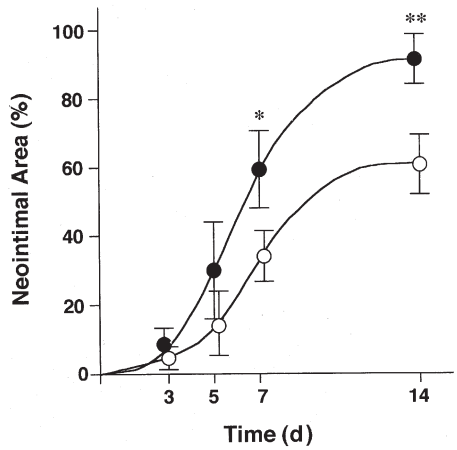



**FIG. 1.** The development of neointima in hamsters fed a normal or a high-cholesterol diet shown as percentage of luminal stenosis. Vascular injury occurred 4 wk after the start of regular (open circles) or high-cholesterol diet (closed circles), and neointima was measured 3, 5, 7, or 14 d after the vascular injury. *$P < 0.05$; **$P < 0.01$. The error bars represent SEM.

*Proliferation of SMC* in vivo. Figure 4 shows the percentage of proliferating SMC on days 1, 3, 5, 7, and 14 after vascular injury. The increased level of plasma cholesterol caused a significant increase in SMC proliferation, measured on days 1, 3, 5, and 7. These differences represented increases of 21.4, 27.3, 11.1, and 14.8% in proliferation index, respectively.

*Proliferation index* in vitro. The amount of DNA synthesis induced by various agonists is shown in Figure 5A. DNA synthesis is markedly increased in high-cholesterol hamsters when SMC are stimulated by FCS or PDGF. Dose-dependent alterations of DNA synthesis induced by PDGF are shown in Figure 5B. DNA synthesis in response to PDGF-BB stimulation of SMC in hamsters fed a high-cholesterol diet is significantly higher than in hamsters fed a normal diet. When SMC were stimulated by the other stimulation factors (thrombin, vasopressin, endothelin I, or angiotensin II), DNA synthesis was not changed in SMC from either group of hamsters.

*[$^{125}$I]PDGF binding in SMC.* The binding affinities in both groups of hamsters were unchanged (Fig. 6).

*Effects of probucol or losartan on neointima.* Figure 7 shows the inhibitory effects of probucol or losartan on neoin-

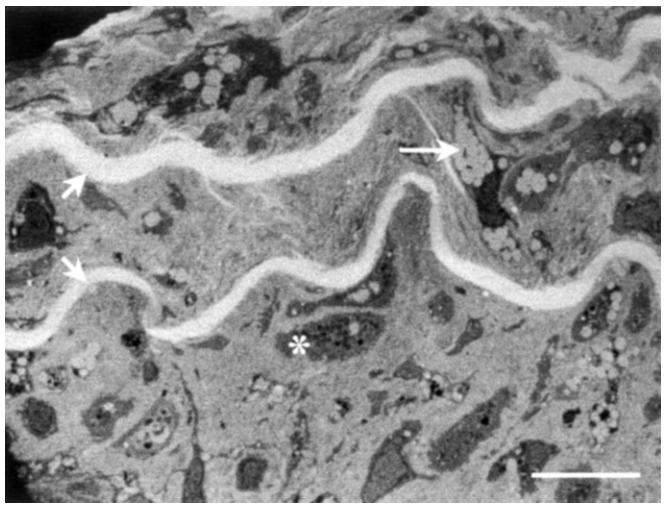
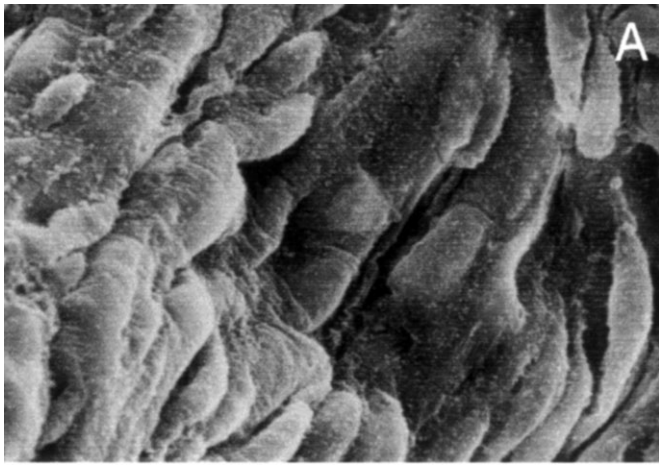
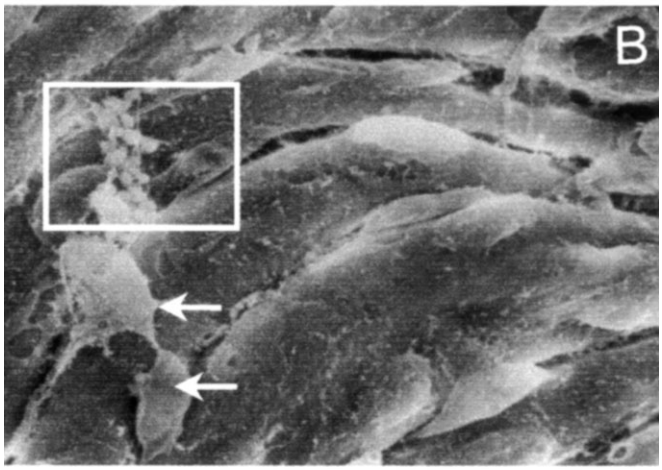


**FIG. 2.** A transmission electron micrograph of neointima formation 14 d after vascular injury in hamsters fed a high-cholesterol diet. Internal elastic lamina (IEL) can be clearly observed (small arrows) and lipid particles (large arrow) and foam cells (asterisk) are observed in media (during IEL) and newly formed intima. The scale bar indicates 200 μm.



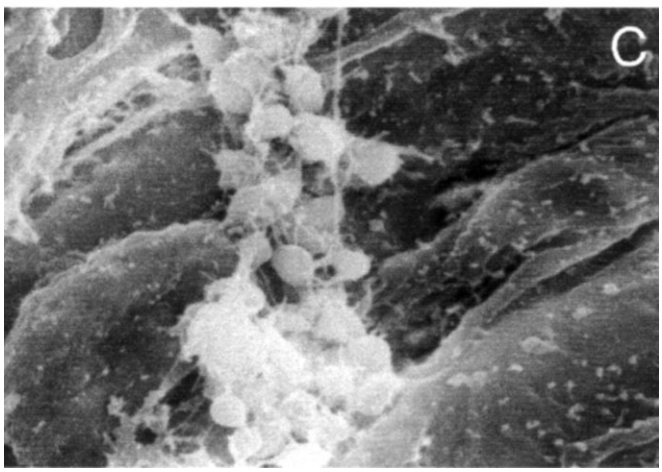




**FIG. 3.** Scanning electron micrographs from hamsters fed a normal or a high-cholesterol diet 14 d after the initiation of vascular injury. (A) Neointima formation on injured area. The luminal surface is entirely covered with newly formed endothelial cells, which are morphologically different from native ones and irregularly oriented. Blood elements are not observed in this area. (B) Locally activated platelets (a window) and monocytes (arrows) are adhered on irregularly oriented vascular surface. (C) A high magnification of a window of panel B. Adherent platelets consist of microthrombus formation.

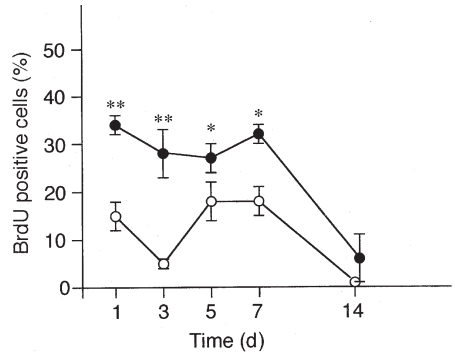
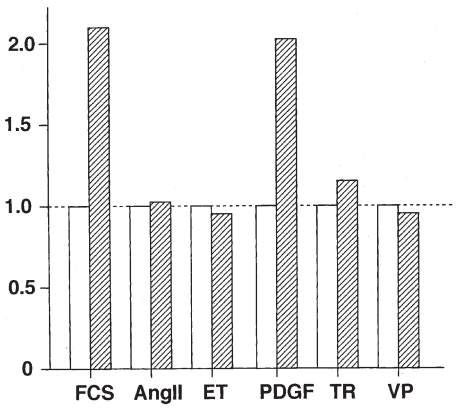



**FIG. 4.** Smooth muscle cell proliferation ($n = 4$, each time point) measured as the 5-bromo-2-deoxy-uridine (BrdU) index (%) following vascular injury in hamsters fed a normal (○) or a high-cholesterol diet (●). $^*P < 0.05$; $^{**}P < 0.01$ vs. control (normal hamsters). The error bars represent SEM.



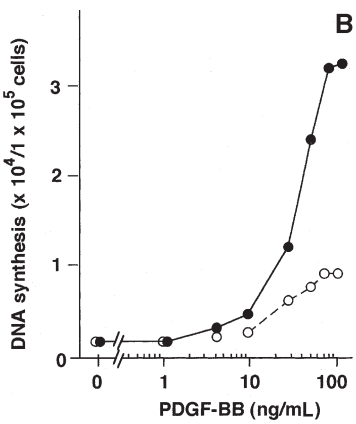




**FIG. 5.** The amount of DNA synthesis induced by various agonists (A) and dose-response line plot of DNA synthesis induced by platelet-derived growth factor (PDGF)-BB (B). (A) The cultured cells from either normally fed hamsters (open bars) or high-cholesterol-fed hamsters (hatched bars) were stimulated with 5% fetal calf serum (FCS), $10^{-6}$ M angiotensin II (AngII), 0.03 unit/mL thrombin (TR), $10^{-6}$ M vasopressin (VP), $10^{-6}$ M endothelin I (ET), or 30 ng/mL PDGF-BB (PDGF) in 1 mL of Dulbecco's modified Eagle's medium. Alteration of DNA synthesis in high-cholesterol hamsters shows a ratio vs. normal hamsters. (B) Dose-response line plot: DNA synthesis induced by PDGF-BB. Each plot presents data from hamsters with normal (open circle) or high-cholesterol diets (closed circles). Each point represents the mean of duplicate cultures.

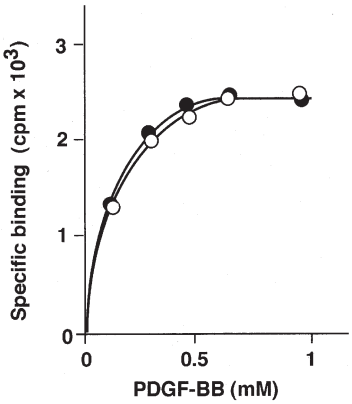



**FIG. 6.** Specific binding of PDGF-BB using explanted vascular smooth muscle cells from hamsters fed a normal or a high-cholesterol diet. Each plot presents data from hamsters with normal (open circle) or high-cholesterol diets (closed circles). Each point represents the mean of duplicate cultures. For abbreviation see Figure 5.

tima formation. The treatment with probucol at a dose of 120 mg/kg/d significantly reduced the neointimal area in hypercholesterolemic hamsters. However, losartan slightly decreased the neointimal area at the highest dose. Photomicrographs of typical neointima formation 2 wk after arterial injury of hamsters either treated with probucol at a dose of 120 mg/kg/d or not, are shown in Figure 8. Formation of neointima including a lot of lipids was observed in hamsters not treated with probucol. On the contrary, neointimal areas and points of lipid accumulation were clearly reduced by treatment with probucol. Platelet aggregation and coagulation factors were not different in hamsters fed a high-cholesterol diet compared with those fed a normal diet. Blood pressure was slightly decreased when the highest dose of losartan was used to treat hamsters.

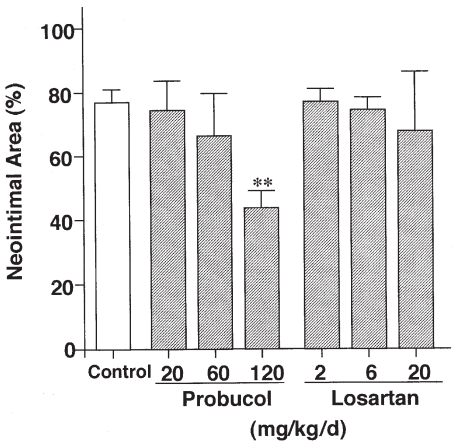



**FIG. 7.** This graph shows dose-dependent inhibition of neointima formation in hamsters fed a high-cholesterol diet by treatment with probucol or losartan. The compound was administered orally twice a day from 2 h before until 14 d after carotid artery injury; control ($n$ = 12) and treated animals ($n$ = 6) were analyzed at day 14. The ratio between neointimal area and the area within the internal elastic lamina was determined on three cross sections of carotid arteries. $^{**}P < 0.01$ vs. control. The error bars represent SEM.

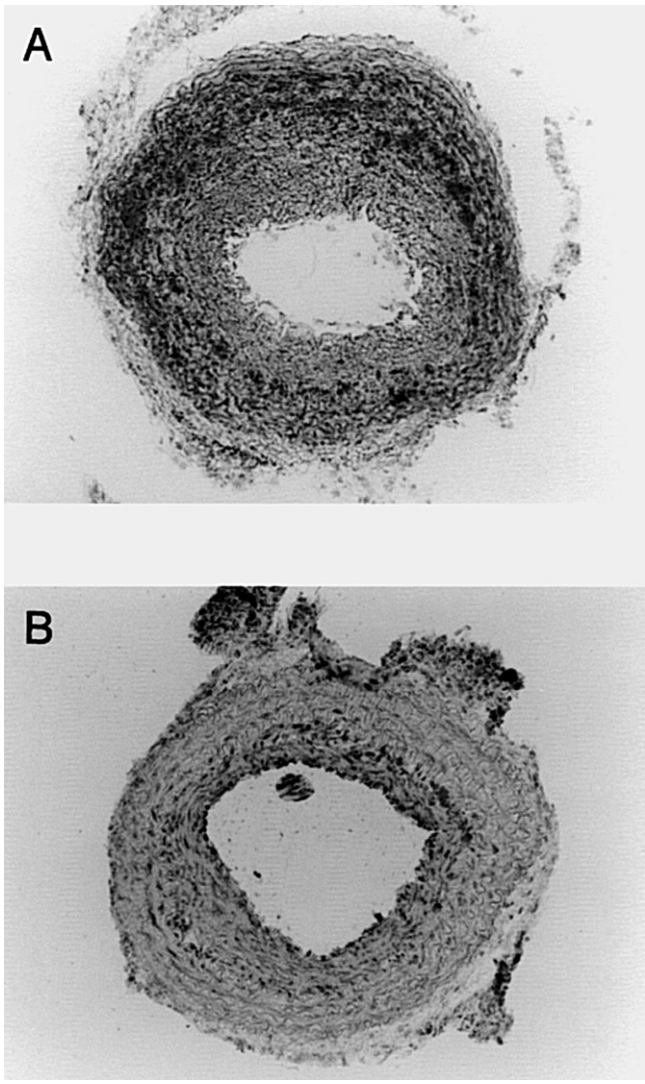



**FIG. 8.** Photomicrographs show neointima formation in damaged carotid arteries of hypercholesterolemic hamsters either treated with 120.0 mg/kg/d probucol (B) or not treated (A). Lipids were stained bright red. The tissue was counterstained with hematoxylin. A packed neointima was observed in both samples, however, the stenosis area in tissue from animals treated with probucol was diminished and lipids in stenosis clearly decreased.

## DISCUSSION

This study demonstrates that high plasma LDL levels in hamsters fed a high-cholesterol diet are associated with marked increases in the development of thrombus and neointima formation, as compared to normally fed hamsters, in injured carotid arteries, which we have established and used to demonstrate the inhibitory effects of several kinds of compounds on vascular stenosis (12–18). Furthermore, these phenomena are mainly characterized by the enhancement of platelet activation induced by thrombin and of SMC proliferation induced by PDGF-BB.

A high arterial LDL level is an important factor in the development of vascular stenosis. Several molecular mechanisms have been suggested to account for the correlation of elevated LDL serum levels with the development of chronic vascular

diseases (22,23). In our experiments, the plasma LDL level of hamsters fed a high cholesterol diet (2.24 ± 0.32 g/L) 2 wk after the start of the diet regime was 4.6 times higher than that of hamsters fed a normal diet (0.49 ± 0.02 g/L), but the HDL plasma level was not significantly different. These levels are similar to those found in pathologic conditions, such as familial combined hyperlipidemia (24), nephrotic syndrome (25) (LDL = 1.6 to 2.0 g/L), and especially homozygous familial hypercholesterolemia (LDL = 3.0 to 5.5 g/L) (26,27).

Vascular stenosis was related mainly to thrombus development in the acute phase or to neointima formation in the chronic phase after endothelial injury. In the present experiments, the development of thrombus formation of high-cholesterol hamsters *in vivo* was clearly accelerated, because the time to occlusion and vascular patency in high-cholesterol hamsters were significantly changed as compared with those of normal hamsters. This phenomenon was mainly attributable to up-regulation of platelet activation since $IC_{50}$ platelet aggregation values significantly decreased in high-cholesterol hamsters, but coagulation factors were not different in both hamsters. Our previous data indicated that platelets play a major role in the development of thombus formation since activated platelets adhere to injured vascular surfaces in the days immediately after injury (13,14). Moreover, LDL may induce a state of hypersensitivity in the platelets that contributes to the high rate of thrombosis formation observed in patients (7,28).

Our findings also showed that neointima formation in injured carotid arteries of hamsters fed a high cholesterol diet was significantly greater than in hamsters fed a normal diet. The number of proliferating SMC *in vivo* measured at each time point after vascular injury in hamsters fed a high-cholesterol diet was also significantly higher compared with hamsters fed a normal diet. LDL particles, trapped in the extracellular matrix of the vessel wall, are well known to undergo substantial structural and chemical modification in response to many kinds of stimuli (29). In our histological observations using TEM, lipid particles can be found in vascular neointima formation. Furthermore, to define the findings of increased neointima lesion in high-cholesterol hamsters, we studied the DNA synthesis using explanted SMC induced by various agonists. These results indicated that PDGF plays a key role in arterial stenosis by neointima after endothelial injury. When PDGF-BB were applied to SMC from hamsters fed a high-cholesterol diet, DNA synthesis increased markedly compared with synthesis in hamsters fed a normal diet. LDL is capable of increasing PDGF production and expression of PDGF receptors in human vascular SMC (30). Therefore, we performed binding experiments using $^{135}$I-labeled PDGF-BB on SMC from hamsters fed a high-cholesterol diet or a normal diet. The results indicated there were no differences in binding assay in SMC between hamster populations. According to these findings, we speculated that the responsibility of post-PDGF receptors, such as intracellular signaling, could play an important role in the enhancement of neointima formation in hypercholesterolemia. Moreover, PDGF is an α granule component (31) that is released during the platelet reaction induced by collagen or ADP (32), or when platelets adhere to sites of injured blood vessels, as seen in our findings using SEM. Therefore, up-regulation of sensitivity in platelets in high-cholesterol hamsters also plays a role in the enhancement of neointima formation. Indeed, our previous findings indicated that platelets play a significant role in the development of neointima formation since antiplatelet compounds improved SMC proliferation during the acute phase of vascular injury (13,14).

To define the utility of this model in pharmacological experiments, we used losartan and probucol to treat hamsters fed a high-cholesterol diet, because probucol inhibited aortic cholesterol accumulation (33) and reduced neointima formation (34) and losartan also reduced neointima formation (35). In these experiments, probucol significantly reduced the neointimal area and attenuated lipid formation in vascular SMC. Indeed, foam cell lesions in hypercholesterolemic hamsters were decreased by treatment with probucol (33). On the contrary, losartan did not show significant effects on neointima formation even though our previous study showed that losartan significantly reduced neointima formation in normal hamsters (18). These findings indicated that probucol greatly affects the development of neointima formation promoted by hypercholesterolemia, but not the inhibition of angiotensin II by losartan.

Finally, we speculate that the physiological importance of such responses to the development of neointima formation in hypercholesterolemia may be modeled as follows: First, after endothelial injury, activated platelets adhere to and aggregate at damaged endothelial surfaces. Next, these platelets are sensitized by high levels of plasma LDL. The production of locally secreted PDGF by activated platelets increases proliferation of SMC in the region of the injury. The continuous production of PDGF may play a very significant role in the amplified development of neointima in hypercholesterolemia. This is especially relevant since the other known stimulants (angiotensin II, vasopressin, or endothelin) did not increase DNA synthesis in SMC from high-cholesterol hamsters. This model is a sensitive one for pharmacological experiments.

In conclusion, high levels of plasma LDL lead to intimal hyperplasia *via* up-regulation of platelet aggregation and enhancement of proliferating SMC. Further research is necessary to determine whether this LDL-induced hyperplasia occurs in humans with elevated LDL levels. If so, antiplatelet and antithrombin drugs could be part of a new supportive therapeutic concept in the treatment of hypercholesterolemia-associated cardiovascular diseases. This model could be a useful tool for investigation of this field since hamsters are smaller and permit a reduction in the amount of drug required.

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# Effects of Dietary Defatted Squid on Cholesterol Metabolism and Hepatic Lipogenesis in Rats

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**ABSTRACT:** Male Sprague-Dawley rats were fed a cholesterol-free (Exp. 1) or cholesterol-supplemented (Exp. 2) diet containing 20% casein (control group) or 15% defatted squid and 5% casein (defatted squid group), as protein, for 14 d. Serum and hepatic cholesterol concentrations were lower in rats fed defatted squid than in those fed casein in both cholesterol-free (–20%, $P < 0.05$ and –15%, $P < 0.05$, respectively) and cholesterol-supplemented (–25%, $P < 0.05$ and –15%, $P < 0.05$, respectively) diets. Hepatic triglyceride concentration was lower in the defatted squid than in the control groups in both cholesterol-free (–51%, $P < 0.05$) and cholesterol-supplemented diets (–38%, $P < 0.01$). The activities of cytosolic fatty acid synthase and the NADPH-generating enzymes, malic enzyme and glucose-6-phosphate dehydrogenase, in the liver were lower in the defatted squid than in the control groups in both cholesterol-free (–21%, $P < 0.01$, –33%, $P < 0.05$, and –33%, $P < 0.01$, respectively) and cholesterol-supplemented diets (–34%, $P < 0.05$, –57%, $P < 0.05$, and –67%, $P < 0.05$, respectively). The activity of mitochondrial carnitine palmitoyltransferase in the liver was comparable between the control and defatted squid groups. The activity of $Mg^{2+}$-dependent phosphatidate phosphohydrolase in the liver cytosol was lower in the defatted squid (–9%, $P < 0.05$) than in the control groups only in the cholesterol-free diet. Fecal excretion of total steroids was stimulated by the feeding of defatted squid in both cholesterol-free (+77%, $P < 0.005$) and cholesterol-supplemented diets (+29%, $P < 0.01$). These results suggest that the nonlipid fraction of squid exerts a hypocholesterolemic effect by increasing the excretion of total steroids in feces. The fraction also induces a triglyceride-lowering activity in the liver by decreasing hepatic lipogenesis.

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An inverse association between seafood consumption and death from coronary heart disease has been reported (1,2). This effect is ascribed primarily to reductions of serum triglyceride and cholesterol and to suppression of platelet aggregation by two n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, occurring in seafoods (3–5). The Japanese frequently eat seafoods other than fishes, for example, shellfish, crustaceans, mollusks. Because these seafoods contain both n-3 PUFA and cholesterol, the effect of dietary seafoods on cholesterol metabolism is complicated. Childs *et al.* (6) reported that six seafoods—clam, oyster, scallop, crab, shrimp, and lobster—do not have a profound hypercholesterolemic effect in normal subjects. Recently, we observed (7,8) that dietary short-necked clam, shrimp, squid, and octopus reduced the concentrations of serum and liver cholesterol in mice. Squid and octopus are consumed mainly in Japan and are also eaten in the Far East, Southeast Asia, and Mediterranean area. The hypocholesterolemic effect of these seafoods was ascribed mainly to the increased output of fecal steroids. It was also observed that dietary seafoods, in particular squid, effectively reduced liver triglyceride concentrations in mice (8). However, mechanisms of the triglyceride-lowering action were not investigated in the study. The hypolipidemic effect of the seafoods are not necessarily attributable to the constituent n-3 PUFA, because the contents of n-3 PUFA in the seafoods apparently are too low to exert the cholesterol- and triglyceride-lowering action. However, information on the effect of the nonlipid fraction of seafoods other than fish on lipid metabolism is seldom obtained.

In the present study, the effect of delipidated squid on cholesterol and triglyceride metabolism was elucidated in more detail in Sprague-Dawley rats, in which knowledge of lipid metabolism is well established (9–13). In one study, cholesterol and sodium cholate were supplemented in the diets in order to investigate the effect of squid on hypercholesterolemia.

## MATERIALS AND METHODS

*Materials.* Fresh squid (*Tadarodes pacificus*) were obtained from a fish market. The tentacle of squid was removed. An edible fraction of squid was minced and lyophilized. Freeze-dried squid was defatted by a mixture of hexane/ethanol (4:1, vol/vol). Crude protein and fat contents in defatted squid, which were assayed by the Kjeldahl method and the Soxhlet method with diethyl ether, were 80.0 and 0.03%, respectively. Defatted squid contained 5.91% ash.

*Animals and diets.* Male Sprague-Dawley rats, 4 wk old and weighing about 110 g (Seac Yoshitomi, Co., Fukuoka, Japan),

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Abbreviations: CPT, carnitine palmitoyltransferase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; HDL, high density lipoprotein; PAP, phosphatidate phosphohydrolase; PUFA, polyunsaturated fatty acids; VLDL, very low density lipoprotein.

**TABLE 1**
**Dietary Regimens (%)**

| Ingredient | Cholesterol-free diets Experiment 1 | | Cholesterol-supplemented diets Experiment 2 | |
|---|---|---|---|---|
| | Control | Defatted squid | Control | Defatted squid |
| Casein | 22.2[a] | 5.5[a] | 22.2[a] | 5.5[a] |
| Defatted squid | — | 18.8[a] | — | 18.8[a] |
| Fat (corn oil) | 5.0 | 5.0 | 5.0 | 5.0 |
| Mineral mixture (Harper mixture)[b] | 4.0 | 4.0 | 4.0 | 4.0 |
| Vitamin mixture (Harper mixture)[b] | 1.0 | 1.0 | 1.0 | 1.0 |
| Cellulose powder | 4.0 | 4.0 | 4.0 | 4.0 |
| Choline chloride | 0.2 | 0.2 | 0.2 | 0.2 |
| Cholesterol | — | — | 0.5 | 0.5 |
| Sodium cholate | — | — | 0.125 | 0.125 |
| Sucrose | to 100 | to 100 | to 100 | to 100 |

[a]The protein content was adjusted to 20% on an isonitrogenous basis.
[b]Harper mixture (14).

were housed individually and kept in an air-conditioned room at 22–25°C in a 12 h light/12 h dark cycle (0800 to 2000). They were given one of the four experimental diets shown in Table 1 and water *ad libitum* for 14 d. The level of dietary protein in the experimental diets was adjusted to 20% on an isonitrogenous base. Since the nutritional value of squid is somewhat low, 15% defatted squid protein and 5% casein were served as the protein source in the defatted squid diet. Casein was the sole protein source in the control diet. Corn oil was added at the 5% level. In Experiment 1, the diets did not contain cholesterol, but in Experiment 2, 0.5% cholesterol and 0.125% sodium cholate were supplemented in the diets. The other experimental conditions were the same between the experiments. Feces were collected for 2 d at the end of experiments. After 7 h of fasting, rats were killed by withdrawing blood from the abdominal aorta, and livers were immediately excised.

All animal studies were carried out under the guidelines for animal experiments in Faculty of Agriculture, Graduate School of Kyushu University (Fukuoka, Japan) and Law No. 105 and Notification No. 6 of the government of Japan.

*Lipid analyses.* Serum lipids were assayed enzymatically using commercial kits (Cholesterol C-II Test, Triglyceride G-Test, Phospholipid B-Test; Wako Pure Chemicals, Osaka, Japan; and HDL-C · 2-Daiichi; Daiichi Chemicals, Tokyo, Japan). Liver lipids were extracted by the method of Folch *et al.* (15), and concentrations of cholesterol, triglyceride, and phospholipid were measured as described elsewhere (16). Fecal neutral and acidic steroids were measured by gas–liquid chromatography using an OV-17 (Chromatotec, Tokyo, Japan) column (17) and an AN-600 (Chromatotec) column (18), respectively.

*Measurements of lipogenic enzyme activity.* The enzyme activities of fatty acid synthase (FAS) (19), glucose-6-phosphate dehydrogenase (G6PDH) (20), malic enzyme (21), phosphatidate phosphohydrolase (PAP) (22), and carnitine palmitoyltransferase (CPT) (23) were determined as described.

*Statistical analysis.* Data were inspected by Student's *t*-test.

## RESULTS

There were no differences in body weight gain, food intake, and relative liver weight between the control and the defatted squid groups in both cholesterol-free and cholesterol-supplemented diets (Table 2).

The concentration of serum cholesterol was significantly lower in rats fed defatted squid diet than in rats fed control diet in both the cholesterol-free and cholesterol-supplemented groups (–20 and –25%, respectively, $P < 0.05$) (Table 3). The concentration of high density lipoprotein (HDL)-cholesterol was higher in the control group than in the defatted squid group when cholesterol was added to the diets (+34%, $P < 0.05$). However, there was no difference in the very low density lipoprotein (VLDL)- and low density lipoprotein-cholesterol level between the control and defatted groups in both cholesterol-free and cholesterol-supplemented diets. The ratio of total cholesterol to HDL-cholesterol was comparable between the groups irrespective of the addition of cholesterol to the diets. The concentration of serum triglyceride was comparable between the control and defatted squid groups in both cholesterol-free and cholesterol-supplemented diets. There was no significant difference in the concentration of serum phospholipid between the corresponding groups.

The concentration of hepatic cholesterol was significantly lower in the defatted squid group than in the control group of cholesterol-free and cholesterol-supplemented diets (–15%, $P < 0.05$) (Table 4). Liver triglycerides were reduced in the defatted squid groups in cholesterol-free (–51%, $P < 0.05$)

**TABLE 2**
**Effects of Dietary Defatted Squid on Body Weight Gain, Food Intake, and Liver Weight in Rats[a]**

| | Cholesterol-free diets Experiment 1 | | Cholesterol-supplemented diets Experiment 2 | |
|---|---|---|---|---|
| | Control | Defatted squid | Control | Defatted squid |
| Initial body weight (g) | 109 ± 4 | 109 ± 3 | 110 ± 3 | 110 ± 3 |
| Body weight gain (g) | 108 ± 5 | 118 ± 9 | 99 ± 4 | 101 ± 2 |
| Food intake (g/d) | 19.3 ± 0.5 | 20.0 ± 0.8 | 18.7 ± 0.5 | 18.8 ± 0.4 |
| Liver weight (g/100 g body weight) | 5.46 ± 0.17 | 5.41 ± 0.18 | 6.15 ± 0.21 | 6.13 ± 0.23 |

[a]Mean ± SE of six rats.

**TABLE 3**
**Effects of Dietary Defatted Squid on the Serum Lipid Levels in Rats[a]**

| | Cholesterol-free diets Experiment 1 | | Cholesterol-supplemented diets Experiment 2 | |
|---|---|---|---|---|
| | Control | Defatted squid | Control | Defatted squid |
| Cholesterol (mg/dL) | 122 ± 7 | 98.1 ± 7[b] | 213 ± 16 | 159 ± 15[b] |
| HDL-cholesterol (mg/dL) | 59.5 ± 1.1 | 52.5 ± 3.4 | 35.8 ± 3.1 | 26.8 ± 2.7[b] |
| VLDL- + LDL-cholesterol (mg/dL) | 62.3 ± 6.5 | 45.7 ± 4.1 | 177 ± 18 | 131 ± 17 |
| Total cholesterol/HDL-cholesterol (%) | 2.05 ± 0.10 | 1.87 ± 0.04 | 5.95 ± 0.87 | 5.93 ± 1.17 |
| Triglyceride (mg/dL) | 204 ± 16 | 183 ± 22 | 120 ± 6 | 99.8 ± 12 |
| Phospholipid (mg/dL) | 238 ± 6 | 231 ± 13 | 235 ± 12 | 206 ± 19 |

[a]Mean ± SE of six rats.
[b]Significantly different from the control group at $P < 0.05$. Abbreviations: HDL, high density lipoprotein; VLDC, very low density lipoprotein; LDL, low density lipoprotein.

and in cholesterol-supplemented diets (–38%, $P < 0.01$). The concentration of liver phospholipid was the same between the groups in both experiments.

The activities of enzymes related to fatty acid synthesis—FAS, malic enzyme, and G6PDH—in liver cytosol were significantly lower in the defatted group than in the control group in both cholesterol-free (–21%, $P < 0.01$, –33%, $P < 0.05$, and –33%, $P < 0.01$, respectively) and cholesterol-supplemented diets (–34%, $P < 0.05$, –57%, $P < 0.05$, and –67%, $P < 0.01$, respectively) (Table 5). There was no difference in the activity of hepatic mitochondrial CPT, the rate-limiting enzyme of mitochondrial β-oxidation, between the control and defatted squid groups. The activity of $Mg^{2+}$-dependent PAP in liver microsomes, the rate-limiting enzyme of triglyceride synthesis, was comparable in cholesterol-free and cholesterol-supplemented diets, but the activity in cytosol was lower in the defatted group than in the control group in cholesterol-free diets (–9%, $P < 0.05$).

As shown in Table 6, fecal weight was higher in the groups fed defatted squid than in the control groups, and the difference was significant in the cholesterol-free diet (+29%, $P < 0.05$). Feeding of defatted squid significantly increased the excretion of neutral steroids in cholesterol-free diet (+86%, $P < 0.005$). In cholesterol-supplemented diet, the excretion of acidic steroids was significantly increased (+85%, $P < 0.0005$). Consequently, total steroid excretion was higher in the defatted squid groups than in the control groups in both cholesterol-free (+77%, $P < 0.005$) and cholesterol-supplemented diets (+29%, $P < 0.01$).

## DISCUSSION

The present study revealed that feeding the nonlipid fraction of squid affects both cholesterol and triglyceride metabolism differently compared with that of casein. Because a large portion (approximately 80%) of the nonlipid fraction is protein, the effect is considered to be mainly exerted by protein.

Dietary defatted squid lowered serum and liver cholesterol concentrations in both cholesterol-free (Exp. 1) and cholesterol-supplemented (Exp. 2) diets compared with the control diets. Because the excretion of neutral steroids into feces was higher in the defatted squid group of rats fed a cholesterol-free diet (Exp. 1), it is conceivable that intestinal absorption of cholesterol was inhibited in the defatted squid group. Therefore, the reduction of hepatic cholesterol in this group is ascribed to the reduced cholesterol absorption. In contrast, dietary defatted squid did not increase fecal excretion of neutral steroids, but it enhanced the excretion of acidic steroids in Experiment 2. Dietary cholesterol is known to stimulate bile acid synthesis in the liver (24). Madani *et al.* (25) reported that the activity of hepatic cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis, was lower in rats fed soybean protein than in rats fed casein in a cholesterol-free diet, but the activity was higher in rats fed soybean protein than casein in a cholesterol-supplemented diet. Therefore, we think that defatted squid stimulates bile acid synthesis more than casein does in a cholesterol-supplemented diet, and hence more bile acids were secreted into the intestinal lumen and fecal output was enhanced. Because the increased

**TABLE 4**
**Effects of Dietary Defatted Squid on the Concentration of Liver Lipids in Rats[a]**

| | Cholesterol-free diets Experiment 1 | | Cholesterol-supplemented diets Experiment 2 | |
|---|---|---|---|---|
| | Control | Defatted squid | Control | Defatted squid |
| Cholesterol (mg/g) | 3.91 ± 0.15 | 3.32 ± 0.15[b] | 60.7 ± 2.3 | 51.4 ± 3.7[b] |
| Triglyceride (mg/g) | 11.4 ± 1.2 | 5.64 ± 0.38[b] | 28.9 ± 4.3 | 17.9 ± 2.1[b] |
| Phospholipid (mg/g) | 12.6 ± 1.7 | 9.11 ± 0.99 | 17.0 ± 1.5 | 16.3 ± 2.1 |

[a]Mean ± SE of six rats.
[b]Significantly different from the control group at $P < 0.05$.

**TABLE 5**
**Effects of Dietary Defatted Squid on the Activities of Fatty Acid Synthase, Malic Enzyme, Glucose-6-Phosphate Dehydrogenase, Carnitine Palmitoyltransferase, and Phosphatidate Phosphohydrolase in Rat Liver[a]**

| | Cholesterol-free diets Experiment 1 | | Cholesterol-supplemented diets Experiment 2 | |
|---|---|---|---|---|
| | Control | Defatted squid | Control | Defatted squid |
| | (nmol/min/mg protein) | | | |
| Liver cytosol | | | | |
| Fatty acid synthase | 42.8 ± 2.2 | 33.7 ± 0.7[b] | 41.4 ± 5.7 | 27.2 ± 2.0[b] |
| Malic enzyme | 127 ± 9 | 84.5 ± 12.7[b] | 60.4 ± 14.2 | 26.2 ± 3.2[b] |
| Glucose-6-phosphate dehydrogenase | 122 ± 7 | 81.4± 10.2[b] | 40.5 ± 9.1 | 13.4 ± 0.8[b] |
| Mitochondria | | | | |
| Carnitine palmitoyltransferase | 1.25 ± 0.3 | 1.19 ± 0.5 | 1.02 ± 0.21 | 0.93 ± 0.28 |
| $Mg^{2+}$-dependent phosphatidate phosphohydrolase[c] | | | | |
| Microsomes | 18.6 ± 1.1 | 18.3 ± 1.0 | 15.5 ± 0.7 | 13.8 ± 0.7 |
| Cytosol | 13.6 ± 0.4 | 12.4 ± 0.4[b] | 10.6 ± 0.4 | 11.1 ± 0.4 |

[a]Mean ± SE of six rats.
[b]Significantly different from the control group at $P < 0.05$. The activities of the fatty acid synthase, malic enzyme, and glucose-6-phosphate dehyrogenase were measured in the liver cytosol fraction. Carnitine palmitoyltransferase activity was measured in liver mitochondria.
[c]The assay was performed in the presence of 3.45 mM $MgCl_2$.

bile acid concentration in the lumen can accelerate micellar solubility of cholesterol, there is a possibility that it weakens the inhibitory effect of cholesterol absorption by defatted squid. This may be why the feeding of defatted squid did not accelerate fecal excretion of neutral steroids in Experiment 2. Although more studies will be necessary on this point, a similar inconsistency has been reported on the effect of soybean protein on fecal excretion of neutral and acidic steroids. That is, dietary soybean protein increased fecal excretion of neutral (26,27) or acidic (28,29) steroids or both (30,31).

Defatted squid lowered serum cholesterol concentration, without modifying the ratio of total cholesterol to HDL-cholesterol, compared with the control group in both cholesterol-free and cholesterol-supplemented diets. In our previous experiment in mice (8), defatted squid did not reduce the serum cholesterol concentration. Causes of the differences in the effect of defatted squid on serum cholesterol level between our previous and present experiments are obscure. However, since the decrease in liver cholesterol level followed the feeding of defatted squid in our previous study (8), serum cholesterol level may be reduced by the prolongation of the feeding period. Also, there is a possibility that the difference in species between mice and rats is involved.

Feeding of defatted squid reduced hepatic triglyceride concentration in the present and previous studies (8). There was no precise information on the effects of squid on hepatic lipogenesis. In the present results, since defatted squid suppressed the activities of FAS, malic enzyme, and G6PDH, it is considered that defatted squid reduced the rate of fatty acid synthesis more effectively than casein. Iritani *et al.* (32) reported that the activities of G6PDH, malic enzyme, and FAS in rat liver were lower in the feeding of plant proteins, soybean protein, and gluten than in the feeding of animal protein, casein, and fish protein. The present study has shown for the first time that squid protein suppresses hepatic fatty acid synthesis compared with casein. The activities of CPT, an indicator of mitochondrial β-oxidation, and $Mg^{2+}$-dependent PAP, the key enzyme in the synthesis of triglyceride (33), were not appreciably modified in rats fed defatted squid. Therefore, the reduction of hepatic triglyceride level in the feeding of defatted squid is thought to be mainly induced by the reduction of the activities of FAS and NADPH-generating enzymes.

**TABLE 6**
**Effects of Dietary Defatted Squid on Fecal Steroid Excretion in Rats[a]**

| | Cholesterol-free diets Experiment 1 | | Cholesterol-supplemented diets Experiment 2 | |
|---|---|---|---|---|
| | Control | Defatted squid | Control | Defatted squid |
| Feces (g/d)[c] | 1.12 ± 0.07 | 1.44 ± 0.11[b] | 1.24 ± 0.07 | 1.42 ± 0.10 |
| Neutral steroids[d] (mg/d) | 12.4 ± 0.7 | 23.1 ± 2.9[b] | 47.2 ± 3.9 | 50.4 ± 1.2 |
| Acidic steroids (mg/d) | 2.41 ± 0.40 | 3.18 ± 0.52 | 18.6 ± 2.1 | 34.5 ± 1.3[b] |
| Total steroids (mg/d) | 14.9 ± 1.0 | 26.3 ± 2.8[b] | 65.7 ± 5.4 | 84.9 ± 1.4[b] |

[a]Mean ± SE of six rats.
[b]Significantly different from the control group at $P < 0.05$.
[c]Dry weight.
[d]Neutral steroids = cholesterol + coprostanol.

Serum triglyceride was not influenced by the feeding of defatted squid in the present study. We previously observed that dietary EPA and DHA reduced hepatic triglyceride concentration by suppressing the activities of enzymes involving fatty acid synthesis in rat liver (12). In contrast to the present study, these n-3 PUFA also decreased serum triglyceride concentration. However, serum triglyceride concentration was not always determined only by fatty acid synthesis in the liver. The activity of lipoprotein lipase is also a major determinant of serum triglyceride concentration. We observed that dietary fish oil increased lipoprotein lipase activity in adipose tissue, suggesting that chylomicron- and VLDL-triglyceride clearance from serum is accelerated in fish oil feeding (13). Therefore, there is a possibility that the effect of defatted squid on lipoprotein lipase may be different from that of fish oil. Further investigation is necessary in this point.

In summary, the present studies suggest the possibility that nonlipid components of squid decrease serum and liver cholesterol and liver triglyceride levels through the stimulation of fecal steroid excretion and the suppression of hepatic lipogenesis in rats.

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# α-Tocopherol Affects the Urinary and Biliary Excretion of 2,7,8-Trimethyl-2(2′-carboxyethyl)-6-hydroxychroman, γ-Tocopherol Metabolite, in Rats

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**ABSTRACT:** In this study, we investigated a change in the excretory content of 2,7,8-trimethyl-2(2′-carboxyethyl)-6-hydroxychroman (γ-CEHC), a γ-tocopherol (γ-Toc) metabolite, in rat urine and bile by using a new high-performance liquid chromatography–electrochemical detection (HPLC–ECD) method. In this determination, CEHC [α- and γ-CEHC, where α-CEHC = 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman] in the biological specimens were treated with 3 N methanolic HCl to hydrolyze conjugates and to promote esterification. The methylated samples were extracted by *n*-hexane/water (1:2). The analyses of the methyl esters of α-CEHC and γ-CEHC were performed by an HPLC–ECD using an ODS-3 column at 35°C. The mobile phase was acetonitrile/water (45:55, vol/vol) containing 50 mM sodium perchlorate. After rat urine and bile samples, respectively, were methylated as described above, methylated biliary metabolites were identified by liquid chromatography–mass spectrometry as methyl esters of γ-CEHC. Furthermore, we examined the differences in the excretion of γ-CEHC between rat urine and bile after an oral administration of γ-Toc or α- + γ-Toc by the above HPLC method. In the γ-Toc group, each vitamin E-deficient rat was given 0.5 mL of a stripped corn oil preparation containing 10 mg of γ-Toc. In the α- + γ-Toc group, the rat was given 10 mg of α-Toc and 10 mg of γ-Toc. The content of γ-CEHC in rat urine from the α- + γ-Toc group was increased more in comparison to the γ-Toc group at 18–36 h after oral administration. Moreover, the content of γ-CEHC in rat bile in the α- + γ-Toc group was increased more in comparison to the γ-Toc group at 6–18 h after oral administration. Therefore, we have suggested that γ-CEHC was shifted mainly to urinary excretion after γ-CEHC had been excreted into the bile. Furthermore, we assume that α-Toc may affect the metabolism of γ-Toc to γ-CEHC in the body.

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Abbreviations: AsA, ascorbic acid; α-CEHC, 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman; γ-CEHC, 2,7,8-trimethyl-2(2′-carboxyethyl)-6-hydroxychroman; δ-CEHC, 2,8-dimethyl-2(2′-carboxyethyl)-6-chromanol; CEHC-Me, CEHC-methyl ester; ECD, electrochemical detection; HPLC, high-performance liquid chromatography; α-, γ-, or δ-Toc, α-, γ-, or δ-tocopherol; TOC-3, tocotrienol; TQ, tocopherylquinone; α-TTP, α-tocopherol transfer protein.

There are eight different naturally occurring forms of vitamin E: four tocopherols (Toc) (α-, β-, γ-, δ-) and four tocotrienols (Toc-3) (α-, β-, γ-, δ-). α-Toc is known to be a major antioxidant in protecting cellular membranes (1).

Orally administered Toc analogs (especially α- and γ-Toc) are absorbed equally well from the small intestine without discrimination. After uptake into the intestinal cells, Toc analogs are secreted into chylomicrons. Chylomicron remnants are subsequently catabolized during circulation by lipoprotein lipase. After uptake of chylomicron remnants by the liver, Toc analogs are discriminated by α-Toc transfer protein (α-TTP) in liver, which transports α-Toc, circulated in plasma, and then transported to each tissue. On the other hand, γ-Toc remains in liver. Hosomi *et al*. (2) examined the structural characteristics of vitamin E analogs required for recognition by α-TTP. Relative affinities (*RRR*-α-Toc = 100%) calculated from the degree of competition were as follows: β-Toc, 38%; γ-Toc, 9%; δ-Toc, 2%; α-Toc acetate, 2%, α-Toc quinone, 2%; *SRR*-α-Toc, 11%; α-tocotrienal, 12%; trolox, 9%. From these results, they proposed that the affinity of vitamin E analogs for α-TTP, which may determine their plasma levels, is a major determinant of their biological activity. The question has been raised regarding what happens to Toc remaining in liver. Kayden and Traber (3) pointed out that the different *RRR*-α-Toc remaining in liver (i.e., γ-Toc or *SRR*-α-Toc) will probably be excreted in the bile.

The following mechanism for α-Toc metabolism has been suggested: α-Toc is first oxidized to α-tocopherylquinone (-TQ); it is then reduced to α-tocopherylhydroquinone, then the side chain is degraded. Finally, these are found in urine as Simon metabolites, which were first found in rabbit urine (4), in which they are present mainly as glucuronide conjugates. However, in 1995, Schultz *et al*. (5) reported that 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman (α-CEHC) was the major urinary metabolite of α-Toc. They suggested that α-Toc can undergo ω-oxidation without cleavage of the chroman ring and that this pathway will be responsible for generation of the major urinary α-Toc metabolite in healthy humans. On the other hand, we also studied the metabolism of α-Toc stereoisomers in rats by using [5-methyl-

$^{14}$C] *SRR*- or *RRR*-α-Toc. According to these results, the *ca.* 73% of the total radioactivity of both groups was found to be present in α-CEHC when urine samples from rats receiving *SRR*-α-Toc were hydrolyzed with 3 N methanolic HCl and analyzed by HPLC (6). Furthermore, Wechter *et al.* (7) isolated a new endogenous natriuretic factor, LLU-α, from human urine, which was presumably a metabolite of γ-Toc. With respect to δ-Toc, Chiku *et al*. (8) reported that the major metabolite of δ-Toc treated with sulfatase was 2,8-dimethyl-2(2′-carboxyethyl)-6-hydroxychroman. Clearly, urinary metabolites of vitamin E analogs are mainly CEHC forms, but how CEHC forms *in vivo* is not clear. Determination of free-CEHC by using HPLC–electrochemical detection (ECD) after enzyme treatment (9,10) or of CEHC by using a coupled-column HPLC with a fluorometric derivatization (11) have been reported. We tried to establish a new way to convert CEHC into CEHC-methyl esters (CEHC-Me) in biological specimens by HPLC-ECD and to estimate the amounts of CEHC present in rat urine and bile. Furthermore, we examined the effect of α-Toc on γ-Toc metabolism *in vivo*.

## MATERIALS AND METHODS

*Materials. RRR*-α-Toc (α-Toc), *RRR*-γ-Toc (γ-Toc), α-CEHC, and 2,7,8-trimethyl-2(2′-carboxyethyl)-6-hydroxychroman (γ-CEHC) were donated by Eisai Co. (Tokyo, Japan).

*Animals.* Male Sprague-Dawley strain rats (3 wk of age) were purchased from Nippon Clea Co. (Tokyo, Japan). They were initially fed a commercial diet (CE-2; Nippon Clea Co.) for a week to allow them to adapt to the new environment. The rats were then fed a diet deficient in vitamin E (AIN-76; Eisai Co.) for 4 or 8 wk. This diet was composed of 23.68% sucrose, 23.68% glucose, 18.95% vitamin-free casein, 14.21% cornstarch, 4.74% filter paper, 3.32% mineral mixture, 0.95% vitamin mixture except vitamin E, 0.28% DL-methionine, 0.19% choline bitartrate, and 10% stripped corn oil. Animals were housed individually in cages at 22°C and 55% humidity with a 12-h light/dark cycle. The feed and water were supplied *ad libitum*.

*Experimental protocol using vitamin E-deficient rats (Experiments 1 and 2).* In Experiment 1, the vitamin E-deficient rats (fed the diet for 8 wk) were divided into two groups after 17 h of fasting: one was the γ-Toc group and the other the α- + γ-Toc group. Rats of the γ-Toc group ($n$ = 4) were each given orally 0.5 mL stripped corn oil containing 10 mg of γ-Toc, and rats of α- + γ-Toc group ($n$ = 4) were each given orally 0.5 mL stripped corn oil containing 10 mg each of α-Toc and γ-Toc. Rats were then housed individually in metabolic cages; urine was collected into flasks kept cool with dry ice. Urine was collected over 6-h intervals after administration for 48 h.

In Experiment 2, the vitamin E-deficient rats (fed the diet for 4 wk) were divided into two groups and then prepared by a method similar to the one described above. Under nembutal anesthesia, rats administered Toc were subjected to cannulation of bile duct; then the bile was collected into tubes cooled by dry ice. After surgery, bile collection was started at 3 h after oral administration and performed at 3-h intervals for 24 h. Samples of urine or bile were immediately lyophilized and stored at −20°C under nitrogen until CEHC determination. The present study was approved by the animal committee of Ochanomizu University.

*Hydrolysis of conjugated CEHC during acid methylation.* Urine was collected from a human volunteer after supplementation with all-*rac*-α-tocopherol (400 mg for 5 d). Because free-CEHC was completely converted to CEHC-Me by this method, the sample was divided: one was methylated by using this method after the conjugated CEHC had been converted to free-CEHC by treatment with β-glucuronidase; the other was methylated directly with this method. For enzymatic hydrolysis, we used a method of Lodge *et al*. (9). Human urine (2 mL) was hydrolyzed by the addition of 200 μL enzyme solution (4 mg β-glucuronidase in 450 μL of 0.1 M sodium acetate buffer, pH 4.5) and incubation for 4 h at 37°C. After enzyme treatment, samples were lyophilized immediately. This portion of the study was carried out under the guidelines established by the Center for Human Care of Ochanomizu University, Tokyo, Japan.

*Extraction of CEHC from the rat urine and bile.* We tried to establish a simultaneous determination of α- and γ-CEHC. We speculate CEHC are present as unconjugated and conjugated forms *in vivo* (5,8–10). Our methodology determined both unconjugated and conjugated CEHC in samples as CEHC-Me that replaced a conjugated substance or a part of carboxy group with methyl group by methylation. Replacing CEHC-Me can measure stability. Furthermore, the extraction and determination of this method are relatively simple and performed at low cost.

Purified water (10 mL) was added to the tube containing the lypophilized sample. From this was pippetted 0.5 mL into a centrifuge tube containing 0.1 mL of ascorbic acid (AsA) solution (0.5 g/mL) and 1 mL of EDTA solution (0.54 mM). This sample was immediately lyophilized again. After 2 mL of 3 N methanolic HCl had been added to each tube, the contents were methylated with shaking at 60°C for 1 h under $N_2$. After methylation, sample tubes were cooled in ice water, 6 mL of water was then added to each tube, and the medium was shaken vigorously with 3 mL of *n*-hexane for 1 min. This mixture was centrifuged at 3000 rpm for 5 min, and the upper layer was collected and evaporated. The residue was dissolved in 100 μL of 45% acetonitrile/water containing 50 mM sodium perchlorate for the determination by HPLC. Methylated CEHC in biological specimens was also identified by liquid chromatography–mass spectrometry (LC–MS).

*Chromatographic apparatus and conditions.* The HPLC system consisted of JASCO Intelligent HPLC Pump (PU-980), Intelligent Sampler (AS-950-10), Column Oven (860-CO), and integrator (870-IT) (JASCO Co., Tokyo, Japan). The electrochemical detector used was an LC-4C Amperometric Detector (BAS Co., Tokyo, Japan) applying a potential of +0.6 V vs. Ag/AgCl. The analysis of α- and γ-CEHC was performed at 35°C by using an ODS-3 column (5

μm, 250 × 2.1 mm i.d.; GL Science Inc., Tokyo, Japan). The mobile phase was acetonitrile/water (45:55, vol/vol) containing 50 mM sodium perchlorate (pH was adjusted to 3.6 with acetic acid) at a flow rate of 0.2 mL/min.

*Conditions of LC–MS.* LC–MS analysis was performed on a TSQ 7000 LC/APCI MS system (Thermo Quest K.K., Tokyo, Japan). Conditions were as follows: auxiliary gas flow, 10 units; sheath gas pressure, 70 psi; capillary temperature, 150°C; vaporizer temperature, 400°C; corona current, 5 μamps; scan time, 1 s. For LC, Irica PR-18T C18 column (5 μm, 250 × 2.0 mm; Irica Instruments Inc., Kyoto, Japan) was used. The mobile phase was 50% (vol/vol) acetonitrile/water at a flow rate of 0.2 mL/min.

*Statistical analysis.* All results were expressed as mean ± SD. Student's *t*-test was used for a comparison of γ-Toc group and α- + γ-Toc group. Differences were considered significant at $P < 0.05$.

## RESULTS

*Simultaneous determination of α- and γ-CEHC in biological specimens.* Figure 1 shows HPLC chromatograms of methylated α- and γ-CEHC standard. α- and γ-CEHC-Me were completely separated into two peaks by this method. The first peak was γ-CEHC-Me, and the second was α-CEHC-Me.

Calibration curves were prepared with solutions containing authentic samples of both α-and γ-CEHC. One-milliliter aliquots of 0.01, 0.02, and 0.05 μg methanolic α-CEHC (or γ-CEHC)/mL were methylated with methanolic HCl in the presence or absence of 10% methanolic AsA solution under $N_2$. The resultant products were analyzed by HPLC-ECD. For the calibration curves of α- and γ-CEHC-Me, the linear regression equation of α-CEHC-Me without AsA was $y = 1.73 \times 10^7 x - 8.49 \times 10^4$ (correlation coefficient: $r = 0.965$); that of γ-CEHC-Me without AsA was $y = 1.88 \times 10^7 x + 1.22 \times 10^4$ (correlation coefficient: $r = 0.991$). In contrast, the linear regression equation of α-CEHC-Me with AsA was $y = 2.06 \times 10^7 x - 2.40 \times 10^4$ (correlation coefficient: $r = 0.983$), that of γ-CEHC-Me with AsA was $y = 2.02 \times 10^7 x + 1.50 \times 10^4$ (correlation coefficient: $r = 0.992$) (Fig. 2). Accordingly, the standard solutions were methylated in the presence of 10% methanolic AsA solution under $N_2$. The resultant calibration curves were found to be optimal for analysis of the vitamin E metabolites. Also, samples were treated similarly to the procedure described above after lyophilization.

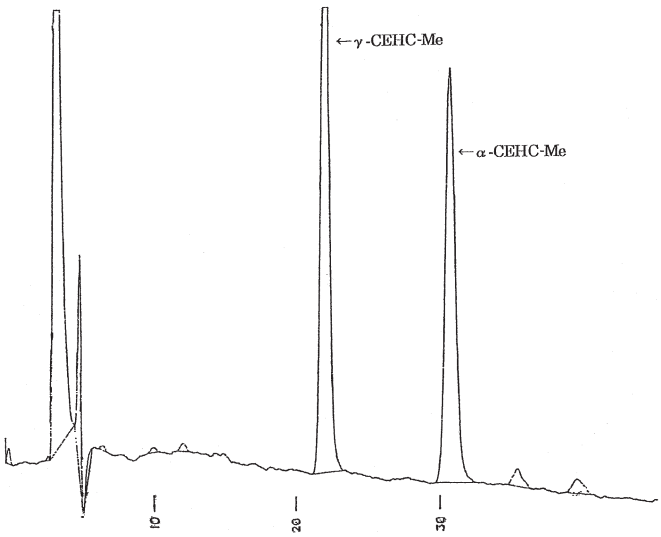


**FIG. 1.** High-performance liquid chromatogram of methylated α- and γ-CEHC standards. α- and γ-CEHC standards were treated with 3 N methanolic HCl, then extracted by *n*-hexane. α-CEHC, 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman; γ-CEHC, 2,7,8-trimethyl-2(2′-carboxyethyl)-6-hydroxychroman.

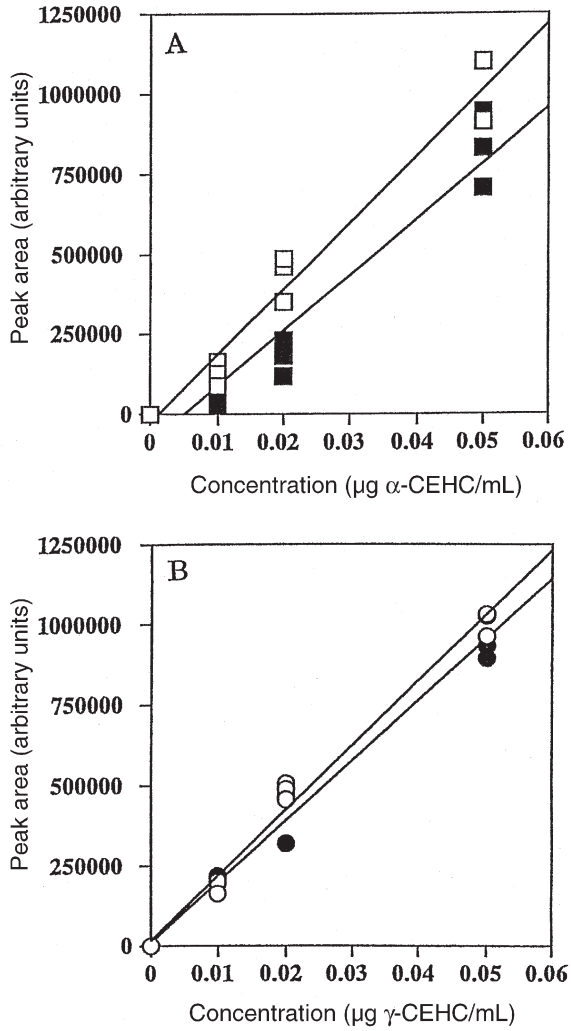


**FIG. 2.** Calibration curves of methylated α- and γ-CEHC. (A) Calibration curves of α-CEHC with AsA (□) and α-CEHC without AsA (■). (B) Calibration curves of γ-CEHC with AsA (○) and γ-CEHC without AsA (●). One milliliter of 0.01, 0.02, or 0.05 μg/mL of α- or γ-CEHC methanolic solution was treated with 3 N methanolic HCl. The extracted solutions were analyzed by high-performance liquid chromatography with electrochemical detection. AsA, ascorbic acid. See Figure 1 for other abbreviations.

Standard samples of α- and γ-CEHC were added to rat urine, and the analytical recoveries of α-and γ-CEHC-Me were measured by this method. A urine sample (0.5 mL) was

**TABLE 1**
**Comparison Between CEHC Methylated After Being Treated with Enzyme and CEHC Methylated Directly[a]**

| μg/mL urine | Methylation with enzyme[b] | Methylation without enzyme[c] | Without/with |
|---|---|---|---|
| α-CEHC-Me | 22.04 ± 1.09 | 22.56 ± 0.68 | 1.023 |
| (CV%) | (4.95) | (3.01) | |
| γ-CEHC-Me | 2.00 ± 0.11 | 2.03 ± 0.09 | 1.020 |
| (CV%) | (5.50) | (4.43) | |

[a]Values are means ± SD of five times. α-CEHC, 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman; γ-CEHC, 2,7,8-trimethyl-2(2′-carboxyethyl)-6-hydroxychroman; CEHC-Me, CEHC-methyl ester.
[b]Urine sample was methylated by using this method after conjugated CEHC was converted to free-CEHC by treatment with β-glucuronidase.
[c]Urine sample was methylated directly by using this method. For experimental details, see the Materials and Methods section.

pipetted into a centrifuge tube containing 1 mL of EDTA solution (0.54 mM). Then α-and γ-CEHC solutions (final concentration, 0.05 μg/mL) were added to each sample followed by 0.1 mL of AsA solution. These were lyophilized and methylated according to the method described above. The analytical recoveries of α- and γ-CEHC were then determined. Endogenous CEHC in rat urine without added standard were not detected by this method. According to this method of standard addition, the analytical recoveries of both α- and γ-CEHC were above 97%. Therefore it was found that free-CEHC was completely converted to CEHC-Me by this method.

*Hydrolysis of conjugated CEHC during acid methylation.* We next examined whether the conjugate is hydrolyzed completely during the acid methylation reaction. Table 1 shows the comparison between CEHC methylated after treatment with enzyme and CEHC methylated directly without treatment. The concentration of CEHC methylated directly in human urine equaled that of CEHC methylated after treatment by enzyme with respect to both α- and γ-CEHC. Therefore, we concluded that the conjugate is hydrolyzed and methylated completely by this method.

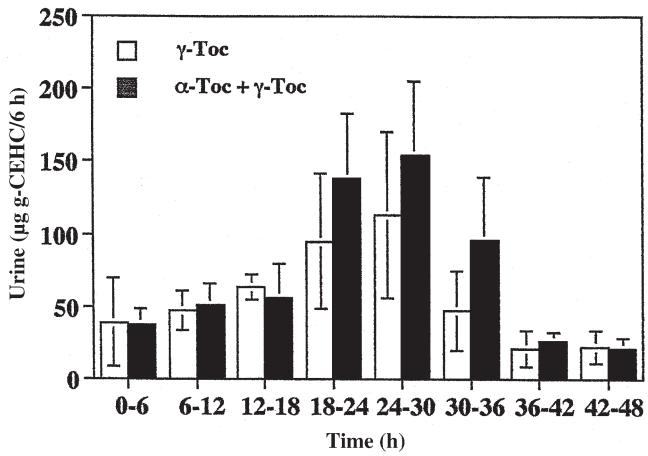


**FIG. 3.** Change in content of γ-CEHC in rat urine with time. Vitamin E-deficient rats were orally administered a single dose of 10 mg γ-Toc (□) or 10 mg α-Toc + 10 mg γ-Toc (■). Values are means ± SD of four rats. α- and γ-Toc, α- and γ-tocopherol. See Figure 1 for other abbreviation.

*γ-CEHC excretion in rat urine (Experiment 1).* We investigated changes of γ-CEHC concentration in rat urine (Fig. 3). It is highest at 24 to 30 h after administration in both the γ-Toc and the α- + γ-Toc groups. γ-CEHC content of rat urine in the α- + γ-Toc group was higher than that of the γ-Toc group from 18 to 36 h after administration. Therefore, we suggest that α-Toc may affect γ-Toc metabolism as evidenced in urine excretion.

*γ-CEHC excretion in rat bile (Experiment 2).* There is no report on the form of CEHC in bile. Therefore, the biliary metabolite of γ-Toc was identified by LC–MS. In Figure 4 are shown the mass spectra of the methylated biliary metabolite and methyl esters of the γ-CEHC standard. The molecular weight of the methylated biliary metabolite was 279, as was the methylated γ-CEHC standard. The methylated biliary metabolite was thus identified as γ-CEHC -Me, and it was clear that the metabolite excreted in rat bile has the basic structure of γ-CEHC. The urinary metabolite was also identified as γ-CEHC-Me by the same method (data not shown).

We next investigated changes in the γ-CEHC content in rat bile (Fig. 5). γ-CEHC content in rat bile was the highest from 6 to 12 h after administration of Toc for both the γ-Toc and the α- + γ-Toc groups. γ-CEHC content of rat bile from the α- + γ-Toc group was higher than that in the γ-Toc group from 6 to 18 h after administration. Therefore we suggest that γ-CEHC excretion is gradually shifted from bile to urine.

## DISCUSSION

In this study, we investigated the difference between γ-CEHC excretion in rat urine and in rat bile by using a new HPLC-ECD method. Stahl *et al.* (10) and Lodge *et al.* (9) reported the determination of α- and γ-CEHC in human serum and urine, respectively, by means of HPLC-ECD. These investigators measured the free-CEHC after its conjugated forms were hydrolyzed by enzymes. On the other hand, we measured the CEHC-Me after both the conjugated and unconjugated forms were methylated by 3 N methanolic HCl. By the methylation of CEHC, it is possible to stabilize them. In regard to the unconjugated form, the analytical recoveries of α- and γ-CEHC-Me were above 97% when the standard samples of α- and γ-CEHC were added to rat urine and then methylated. Therefore, we found that free-CEHC were converted to CEHC-Me quantitatively by using this method. We also found that conjugated CEHC were converted to CEHC-Me completely by using this method (Table 1). Furthermore, we had previously methylated an unlabeled α-CEHC standard with 3 N methanolic HCl, then injected the product into a high-performance liquid chromatograph (6). The α-CEHC standard had a retention time of 6.8 min. After methylation, the retention time was 16.7 min. On the other hand, the retention time of radioactive substances in urine after oral administration of *SRR*-α-[$^{14}$C]Toc was shifted completely to 16.7 min after methylation of these substances; moreover, this radioactive substance was identified as α-CEHC-Me by LC–MS (6). Therefore methylation with 3 N methanolic HCl hydrolyzes conjugates and promotes esterification.

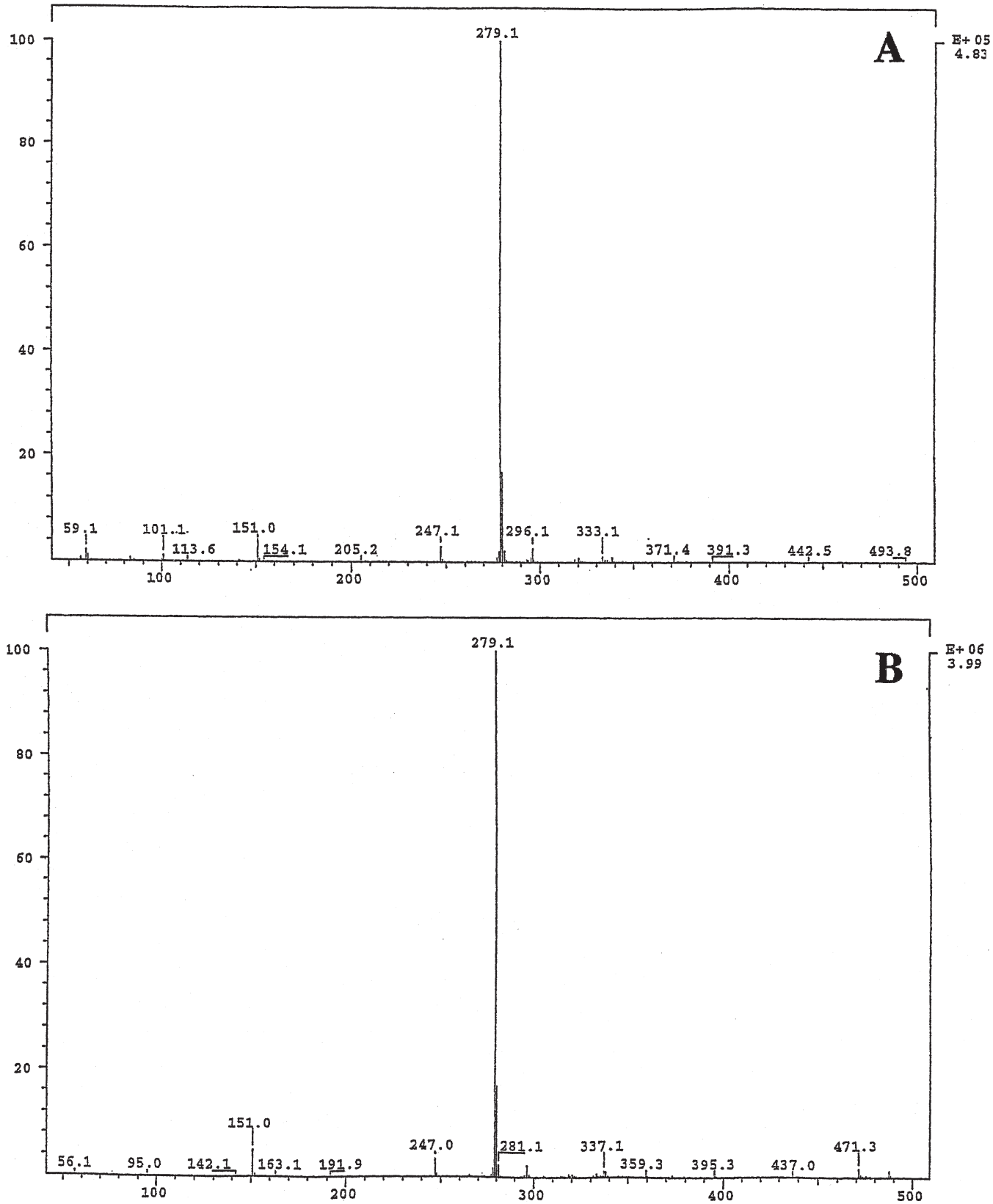


**FIG. 4.** Mass spectra of methylated biliary metabolite of tocopherol and γ-CEHC methyl ester. Bile samples were collected from 12 to 24 h after oral administration of γ-Toc (A) and γ-CEHC standard (B). These samples were treated with 3 N methanolic HCl (see the Materials and Methods section). For abbreviations see Figures 1 and 3.

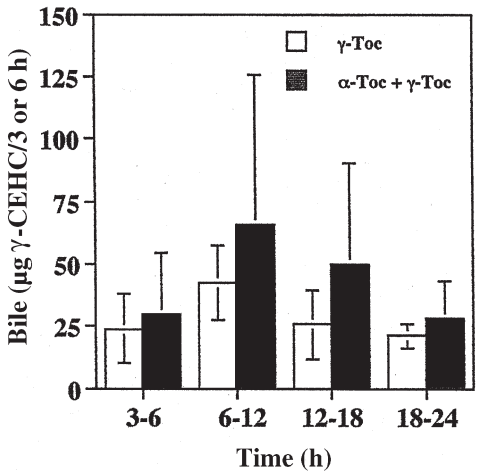


**FIG. 5.** Change in the content of γ–CEHC in bile with time. Vitamin E-deficient rats were orally administered a single dose of 10 mg γ-Toc (□) or 10 mg α-Toc + 10 mg γ-Toc (■). Values are means ± SD of four rats. For abbreviations see Figures 1 and 3.

Each urine and bile sample was collected into a flask being cooled with dry ice, followed immediately by lyophilization. This procedure is carried out to protect against the breakdown of the conjugated CEHC form. However, in the present study, all CEHC (conjugated and unconjugated forms) were converted into methyl ester form. Hereafter, unconjugated forms of CEHC will be measured by this method after methanolic extraction.

The γ-CEHC content in rat urine of both groups was at the highest level from 24 to 36 h after single-dose administration; then it declined to 48 h, and the metabolite may be excreted. The total amount of γ-CEHC excreted in rat urine until 48 h after oral administration was *ca.* 450 μg in the γ-Toc group and *ca.* 580 μg in the α– + γ-Toc group. Therefore we suggest that α-Toc may affect γ-Toc metabolism.

The γ-CEHC content in the bile of both groups was at the highest level from 6 to 12 h after administration; after that it decreased until 24 h after administration. According to these results, we assume that γ-CEHC was shifted to the urinary excretion after part of the γ-CEHC had been excreted into the bile. The total CEHC content of the urine and bile for 24 h was *ca.* 250 μg in γ-Toc group, and *ca.* 280 μg in the α- + γ-Toc group. The content of γ-CEHC in rat bile for 24 h after oral administration was *ca.* 130 μg in the γ-Toc group and *ca.* 190 μg in the α– + γ-Toc group. These results indicate that the content of γ-CEHC in urine was significantly higher ($P \leq 0.05$, *t*-test) than that for bile 24 h after oral administration. Therefore, γ-CEHC clearly was mainly excreted into the urine rather than into the bile.

Swanson *et al.* (12) presumed that γ-CEHC is present in conjugated form in human urine, mainly as glucuronide. On the other hand, Chiku *et al.* (8) presumed that δ-CEHC in a conjugated form in rat urine is found mainly as a sulfate. We assume that the CEHC conjugated form in bile is different from that in urine.

In summary, we focused on the effects of α-Toc on γ-Toc metabolism in the rat. We found that γ-Toc metabolism is affected by α-Toc *in vivo* and demonstrated that the presence of α-Toc accelerates the metabolism of γ-Toc to γ-CEHC.

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# Absorption of Canthaxanthin by the Rat Is Influenced by Total Lipid in the Intestinal Lumen

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**ABSTRACT:** In this study the effect of luminal lipid on the absorption of canthaxanthin (CTX) was investigated using the lymph duct cannulated rat. Treatments were emulsions designed to deliver increasing amounts of olive oil (10, 30, 50, 70, or 90 mg/h) and CTX (12.5 nmol/h). Emulsions were continuously infused into the duodenum for 12 h, and lymph was collected during the final 6 h of infusion for analysis. As the amount of lipid in the emulsion increased, a linear increase in the absorption of CTX was observed. The recovery of CTX in the lymph when infused with 10 mg/h olive oil was 14.2 ± 1.2% and with 90 mg/h was 26.9 ± 5.7%. The efficiency of CTX absorption nearly doubled by increasing the amount of lipid infused with CTX. The correlation between lipid load and CTX absorbed was $r = 0.85$. We conclude that luminal lipid load affects CTX absorption.

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Because of the health benefits associated with the consumption of carotenoid-rich foods, factors influencing carotenoid absorption are of interest. The carotenoids are a class of lipophilic dietary components absorbed from the digestive tract with other dietary lipids. Numerous studies have shown that low-fat diets or the absence of dietary fat results in greatly reduced carotenoid absorption as measured by serum carotenoid and retinol levels (1–6). Therefore, dietary lipid appears to be a key factor influencing carotenoid absorption.

The presence of dietary lipid in the digestive tract promotes the release of bile salts into the intestinal lumen. This is crucial as carotenoids freed from the food matrix gain access to the intestinal brush border membrane by solubilization into bile salt micelles. In the absence of bile salt micelles, there is essentially no carotenoid uptake by enterocytes (7). Once bile salts are present at a concentration above the critical micelle concentration, it is not clear if additional lipid influences carotenoid absorption. A recent review suggests that the amount of dietary fat required to ensure carotenoid absorption is low and may only be 3 to 5 g per meal (8). This low level of dietary lipid appears to be enough to stimulate the release of bile salts into the intestinal lumen. It was further noted that dietary fat above the 3- to 5-g level did not enhance plasma concentrations of carotenoids (8). This would suggest that additional lipid has no effect on carotenoid absorption.

The objective of this study was to determine if increasing the lipid load in the intestine affects carotenoid absorption. The lymph duct cannulated rat, an animal model previously used in our laboratory to study the absorption of non-provitamin A carotenoids, was used (9–11). The carotenoid selected for study was canthaxanthin (CTX) because it is absorbed intact by the rat and has physical properties similar to other xanthophyll-type carotenoids.

## MATERIALS AND METHODS

*Animals and surgical procedure*. The animal protocol used in this study was approved by The University of Connecticut Institutional Animal Care and Use Committee. Male Holtzman albino rats obtained from Holtzman Laboratory Animals (Madison, WI) and weighing 250–300 g at the time of surgery were used. Prior to surgery the animals were housed individually in stainless-steel cages and fed a standard laboratory diet (Purina, Rodent Chow 5001; Purina Mills, St. Louis, MO) *ad libitum*.

Surgery included cannulation of the major mesenteric lymph duct and placement of a feeding tube into the duodenum as previously described (9). Immediately following surgery, the rats were placed in a warm dark environment and allowed to recover for approximately 36 h. During the recovery period the rats had access to water, and a glucose/electrolyte solution (Pedialyte; Ross Laboratories, Columbus, OH) was infused into the duodenum at 2.0 mL/h.

*Treatment emulsions.* On the day of the study a stock solution of CTX (Sigma Chemical, St. Louis, MO) was prepared in dichloromethane. The concentration of CTX was estimated using the extinction coefficient $E$ at 466 nm ($E^{1\%}_{1cm} = 2200$), and purity was verified by spectral characteristics and high-pressure liquid chromatography (HPLC).

Five treatment emulsions designed to deliver increasing amounts of olive oil (10, 30, 50, 70, or 90 mg/h) and 12.5 CTX nmol/h (Sigma Chemical) were used. The emulsions contained olive oil, CTX, and a buffer solution (115 mmol/L NaCl, 5.0 mmol/L KCl, 6.8 mmol/L $Na_2HPO_4$, and 16.5 mmol/L $NaH_2PO_4$), and 10 mmoL/L sodium taurocholate (Sigma Chemical). The emulsions were prepared by placing the olive oil in a round-bottomed flask with an appropriate amount of CTX stock solution. Sodium taurocholate and buffer were added to the olive oil/CTX mix and emulsified by

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Abbreviations: CTX, canthaxanthin; HPLC, high-pressure liquid chromatography.

using a probe sonicator (Branson Sonifier, Model 185; Branson Sonic Power, Danbury, CT).

*Administration of treatment emulsions and collection of lymph.* Three rats per treatment were used. Treatment emulsions were intraduodenally infused at a rate of 2.0 mL/h for 12 h. During this time animals had free access to water and maintained a constant hydration state. After infusing the emulsion for 6 h, lymph was collected for analysis during the final 6 h of infusion. In an earlier study, we had observed that CTX in the lymph reached a plateau or steady-state transport into the lymph by 6 h of continuous intraduodenal infusion (9).

*Carotenoid analysis of lymph.* Prior to HPLC analysis, CTX was extracted from lymph. An aliquot of lymph and dichloromethane/methanol (2:1, vol/vol) containing an internal standard, ethyl-β-apo-8′-carotenoate (Fluka, Ronkonkoma, NY) were placed in a separatory funnel at a solvent-to-lymph ratio of 9:1 (vol/vol) and stored in the dark at 4°C for 4–6 h. The bottom phase was removed and saved. Methanol at 1.5 times and dichloromethane at 6 times the original lymph volume were added to the upper phase. The separatory funnel was again stored for several hours in the dark at 4°C. After phase separation the bottom phase was removed and combined with the original bottom phase. Solvent was removed from the extracted lipids in the combined bottom phase under reduced pressure and redissolved in 500 µL 2-propanol/dichloroethane (1:1 vol/vol). For analysis, aliquots of 20–40 µL were injected into the HPLC in triplicate.

Carotenoid separation by HPLC was with a Waters C18 Resolve column (15 cm × 3.9 mm; Millipore, Milford MA) with an Upchurch C18 guard column (Upchurch Scientific, Oak Harbor, WA) by the method of Barua *et al*. (12). An isocratic mobile phase consisting of acetonitrile/dichloromethane/methanol/*n*-butanol/ammonium acetate (90:15:10:0.1:0.1, by vol) was used at a flow of 1.0 mL/min. The CTX and ethyl-β-8′-carotenoate were identified based on a comparison of retention times with standards and quantified by peak areas at a wavelength of 450 nm. Standard curves for CTX and ethyl-β-8′-carotenoate were developed and used to calculate the concentrations of carotenoids. The recovery of ethyl-β-8′-carotenoate in the lymph averaged 90% and was used to correct for losses of CTX during analysis. Extractions with less than 80% recovery of the internal standard were rejected.

*Fatty acid analysis of treatment emulsions and lymph.* A 200-µL aliquot of the treatment emulsion or lymph was used for fatty acid analysis. Fatty acids in the aliquot were prepared for analysis by direct methylation in methanol/hexane (4:1, vol/vol) in the presence of acetyl chloride (13). A known amount of heptadecanoic acid was added as an internal standard. The fatty acid methyl esters were separated by gas–liquid chromatography on a Supelcowax 10 fused-silica capillary column (30 m, 0.53 mm i.d., Supelco, Bellefonte, PA), and identification of individual fatty acids was based on comparison of retention times with known standards. The total lipid concentration in the treatment emulsions and lymph was calculated based on the peak areas of the fatty acid methyl esters relative to the internal standard.

## RESULTS AND DISCUSSION

The results of the study are presented in Table 1. Lipid in the lymph, calculated from total fatty acid analysis, increased with the amount of lipid infused into the duodenum. Interestingly, when the lowest lipid load (10 mg/h) was infused, the total fatty acid recovered in the lymph averaged 14.5 with a range of 11.6 to 18.3 mg/h. The greater amount of fatty acids in the lymph was due to the absorption of endogenous lipid. Lymph contained myristic acid and arachidonic acid, two fatty acids not present in the treatment emulsion, and a greater proportion of stearic acid in lymph than in the emulsion. When the 90 mg/h lipid load was infused, there was a 93 ± 10.3% recovery of total fatty acids in the lymph. We did not observe any signs of malabsorption.

The absorption of CTX infused at a constant rate of 12.5 nmol/h significantly ($P < 0.05$) increased with the amount of lipid infused into the duodenum. At the lowest lipid load (10 mg/h), the efficiency of CTX absorption was 14.2 ± 1.2% and increased to 26.9 ± 5.7% when 90 mg/h lipid was infused. As seen in Figure 1, the relationship between the amount of lipid infused and the amount of CTX absorbed was linear. For each 10 mg/h increase in lipid intraduodenally infused, there was an additional 0.2 nmol/h CTX recovered in the lymph.

Few studies have directly measured carotenoid absorption. Nevertheless, reports measuring plasma and tissue concentrations have identified factors affecting carotenoid bioavailabil-

**TABLE 1**
**Recovery of Canthaxanthin and Total Fatty Acids in the Mesenteric Lymph of Rats[a]**

| | Lymph measurements | | | |
|---|---|---|---|---|
| Infused lipid (mg/h) | Fatty acids (mg/h) | Percent recovered | Canthaxanthin (nmol/h) | Percent recovered |
| 10 | 14.5 ± 1.2 | 145 ± 19.9 | 1.8 ± 0.2 | 14.2 ± 1.2 |
| 30 | 33.1 ± 2.2 | 110 ± 7.5 | 2.1 ± 0.2 | 16.7 ± 1.1 |
| 50 | 53.9 ± 8.0 | 108 ± 16.2 | 2.6 ± 0.3 | 20.9 ± 2.9 |
| 70 | 63.1 ± 5.7 | 90 ± 6.3 | 2.9 ± 0.5 | 22.8 ± 4.0 |
| 90 | 83.9 ± 7.2 | 93 ± 10.3 | 3.4 ± 0.7 | 26.9 ± 5.7 |

[a]Each value is the mean ± standard deviation from three individual rats. Canthaxanthin was infused into the duodenum at the rate of 12.5 nmol/h. "Infused lipid" is the sum of all fatty acids in the treatment emulsions as measured by gas chromatography.

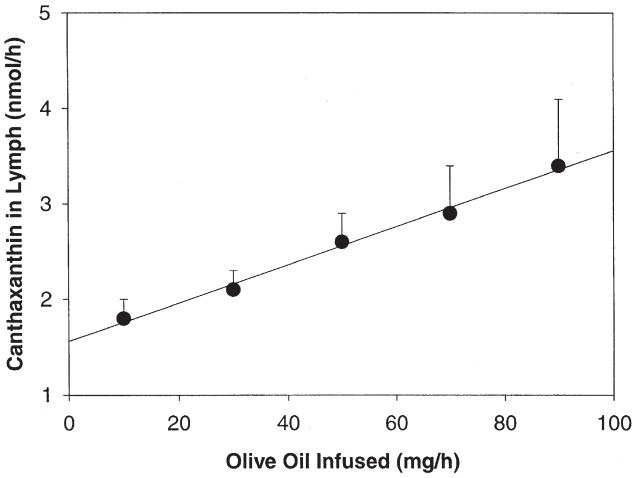


**FIG. 1.** Relationship between lipid load in the duodenum and canthaxanthin recovered in the mesenteric lymph. Canthaxanthin was continuously infused into the duodenum at a level of 12.5 nmol/h for 12 h. Lymph was collected from 6 to 12 h after the initiation of infusion and represents absorption under steady-state conditions. Each point is the mean ± SD from three individual rats. There was a significant ($P < 0.05$) linear relationship between the amount of lipid infused and the recovery of canthaxanthin in the lymph.

ity. A list of these factors have been used to form the mnemonic ***SLAMENGHI*** representing: ***S***pecies of carotenoids, ***L***inkages at the molecular level, ***A***mount of carotenoid, ***M***atrix, ***E***ffectors, ***N***utrient status, ***G***enetics, ***H***ost-related factors, and ***I***nteractions among these variables (14). One ***E***ffector of carotenoid absorption identified in several reports is the amount of dietary fat present (1–6). The precise manner by which dietary fat affects absorption is poorly understood. A key step in carotenoid absorption affected by dietary fat would be the transfer of carotenoid from the food matrix to the bile salt micelle for transport to the intestinal absorptive membrane.

In this study, bile salts and lipid were present in adequate amounts to ensure carotenoid absorption even at our lowest level of lipid infusion. As the lipid load in the duodenum increased there was a significant positive correlation with absorption of CTX into the lymph ($r = 0.85$, $P < 0.05$). This positive relationship may be explained by the increase in lipid digestion products being transferred from emulsion particles to bile salt micelles. We speculate that the greater amount of digested lipid in the bile salt micelle would result in an increased capacity to solubilize carotenoids in the micelle for transport to the brush border membrane and subsequent absorption.

We conclude that in the presence of adequate amounts of bile salts, absorption of carotenoid may further be improved by increasing the amount of lipid consumed. This is not consistent with the observation that increasing dietary fat above 3 to 5 g per meal did not enhance plasma concentrations of carotenoids (8). Additional work is needed to understand the luminal phase of carotenoid absorption.

## ACKNOWLEDGEMENT

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# Cytotoxic Effect of Conjugated Trienoic Fatty Acids on Mouse Tumor and Human Monocytic Leukemia Cells

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**ABSTRACT:** The cytotoxicity of fatty acids from seed oils containing conjugated linolenic acids (CLN) was studied. Fatty acids from pomegranate, tung, and catalpa were cytotoxic to human monocytic leukemia cells at concentrations exceeding 5 μM for pomegranate and tung and 10 μM for catalpa, but fatty acids from pot marigold oil had no effect at concentrations ranging up to 163 μM. The main conjugated fatty acids of pomegranate, tung, catalpa, and pot marigold were *cis*(*c*)9,*trans*(*t*)11,*c*13-CLN (71.7%), *c*9,*t*11,*t*13-CLN (70.1%), *t*9,*t*11,*c*13-CLN (31.3%), and *t*8,*t*10,*c*12-CLN (33.4%), respectively. Therefore, the cytotoxicities of fatty acids from pomegranate, tung, and catalpa were supposed to be due to 9,11,13-CLN isomers. To elucidate the cytotoxicity of these CLN, we separated each CLN isomer from the fatty acid mixtures by high-performance liquid chromatography and analyzed its cytotoxicity. The cytotoxicities of *c*9,*t*11,*c*13-CLN, *c*9,*t*11,*t*13-CLN, and *t*9,*t*11,*c*13-CLN were much stronger than that of *t*8,*t*10,*c*12-CLN. Therefore, the higher cytotoxicity of fatty acids from pomegranate, tung, and catalpa than those from pot marigold would be derived from the different activities of 9,11,13-CLN and 8,10,12-CLN. Since there was little difference in the cytotoxicities of *c*9,*t*11,*c*13-CLN, *c*9,*t*11,*t*13-CLN, and *t*9,*t*11,*c*13-CLN, it is suggested that the *cis/trans* configuration of 9,11,13-CLN isomers had little effect on their cytotoxic effects. The mechanism of the cytotoxicity of the four fatty acids above may involve lipid peroxidation, because the order of toxicity of the fatty acids was consistent with their susceptibility to peroxidation in aqueous phase. This was supported by the decrease in the cytotoxicity of the fatty acids by addition of butylated hydroxytoluene.

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Many papers have been published on the potent beneficial health and biological effects of conjugated linoleic acids (CLA) (1–7). Preliminary studies indicate that CLA is a powerful anticancer agent in the rat mammary tumor model with an effective range of 0.1–1% in the diet (8). The effect of CLA on various human cancer cell cultures has also been examined. CLA inhibited the growth of lung, breast, and colorectal cancer but had no effect on the glioblastoma cell line (9,10). Although the mechanisms for the anticancer effect of CLA have not been fully elucidated, these results suggested that the conjugated diene system may be responsible for this cytotoxic effect.

On the other hand, previous studies showed the occurrence of other types of conjugated polyunsaturated fatty acids (PUFA) in natural products (11–14), and some reports have appeared on the biological properties of these PUFA (15–18). Cornelius *et al.* (15) reported the strong cytotoxic effect of conjugated octadecatetraenoic acid [*cis*(*c*)9,*trans*(*t*)11,*t*13,*c*15-18:4; α-parinaric acid] from garden balsam seed oil on human leukemia cells. They also found that lipid peroxidation was involved in this effect. The cytotoxic effect of conjugated PUFA on human tumor cells also has been found in α-linolenic acid (LN), eicosapentaenoic acid, and docosahexaenoic acid after base treatment (17,18). Furthermore, Dhar *et al.* (16) reported that *c*9,*t*11,*t*13-18:3 from karela seed oil acted as antioxidant in rat tissues.

Conjugated PUFA including CLA usually constitute less than 1% in natural products, however, some kinds of seed oils have much higher contents (40–80%) of conjugated linolenic acids (CLN) (14). α-Eleostearic (*c*9,*t*11,*t*13-CLN), punicic (*c*9,*t*11,*c*13-CLN), catalpic (*t*9,*t*11,*c*13-CLN), and calendic (*t*8,*t*10,*c*12-CLN) acids are reportly found in tung seed oil (68%), pomegranate seed oil (83%), catalpa seed oil (42%), and pot marigold seed oil (62%), respectively.

This paper reports a study of the cytotoxic effect of CLN found in these seed oils. Igarashi and Miyazawa (17) reported the cytotoxicity of alkaline isomerized α-linolenic acid (LN) and tung oil fatty acids on human tumor cells. Isomerized linolenic acid (IsoLN) was a mixture of unreacted linolenic acid and CLN having conjugated dienoic and trienoic double bonds. Tung oil fatty acids were also a mixture of α-eleostearic acid and nonconjugated fatty acids. To better understand the cytotoxicity of CLN, it is important to elucidate the cytotoxic effect of each positional and geometrical CLN isomer. Thus, we separated each CLN isomer from fatty acid mixtures of these seed oils by high-performance liquid chro-

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; BHT, butylated hydroxytoluene; CLA, conjugated linoleic acid; CLN, conjugated linolenic acid; DMEM, Dulbecco's modified Eagle's medium; GC, gas chromatography; HPLC, high-performance liquid chromatography; IsoLN, isomerized linolenic acid; LA, linoleic acid; LN, α-linolenic acid; PUFA, polyunsaturated fatty acid; WST, water-soluble tetrazolium.

matography (HPLC) and analyzed its cytotoxic effect on tumor cells.

## EXPERIMENTAL PROCEDURES

*Oil sources for CLN with conjugated trienes*. Raw materials for preparation of fatty acids containing a particular CLN isomer were tung (*Aleutrites fordii*) seed oil for *c*9,*t*11,*t*13-CLN, pomegranate (*Punica granatum*) seed oil for *c*9,*t*11,*c*13-CLN, catalpa (*Catalpa ovata*) seed oil for *t*9,*t*11,*c*13-CLN, and pot marigold (*Calendula officinalis*) seed oil for *t*8,*t*10,*c*12-CLN (14). Each seed oil, except for tung, was obtained by extraction of ground seeds with *n*-hexane at room temperature. Tung oil was a bottled commercial product.

*HPLC separation of CLN isomer from seed oil methyl esters.* Each seed oil containing CLN was transesterified to its methyl esters with 0.5 M sodium methoxide in methanol. Each CLN isomer was separated from the mixed methyl esters by preparative reversed-phase HPLC (Shimadzu LC 10A system; Shimadzu Seisakusho, Kyoto, Japan) using a mixture of acetonitrile/water (50:10, vol/vol), a flow rate of 8.0 mL/min, a column of 5 μm C-18 (25.0 × 2.0 cm, YMC-pack R&D ODS; YMC, Kyoto, Japan), sample loads of 90–100 mg, and an ultraviolet detector set at 235 nm. Each isomer isolated by HPLC was characterized by capillary gas chromatography (GC) and compared with the reference fatty acid methyl esters from the original seed oil containing the particular CLN isomer (14). The isolated CLN methyl ester showed a purity greater than 99%. The GC analysis was performed on a Shimadzu GC-14B (Shimadzu Seisakusho) equipped with a flame-ionization detector and a capillary column [Omegawax 320 (30 m × 0.32 mm i.d.) or SP-2380 (100 m × 0.25 mm i.d.); Supelco, Bellefonte, PA].

*Isomerization of ethyl α-linolenate*. Ethyl α-linolenate (99+% purity) was purchased from Sigma (St. Louis, MO). The ethyl ester was catalytically isomerized. Potassium *t*-butoxide (Nacalai Tesque, Kyoto, Japan) was used as a catalyst for the isomerization of double bonds in linolenate ester so as to produce conjugation. Reaction was done by stirring linolenate (0.5–1.5 g) with catalyst in dimethylformamide (50 mL) at 30°C for 1 h. After incubation, the reaction mixture was neutralized with 2 N HCl. The reaction products were extracted with *n*-hexane and fractionated by silicic acid column chromatography, eluting with *n*-hexane, diethyl ether/*n*-hexane (5:95, vol/vol), and diethyl ether/*n*-hexane (20:80, vol/vol), respectively. Isomerized ethyl ester was eluted with diethyl ether/*n*-hexane (5:95, vol/vol).

*Fatty acid preparation*. Seed oils, methyl esters of CLN isomer isolated by HPLC, and isomerized α-linolenate were saponified with 0.2 N KOH at room temperature under nitrogen overnight, and unsaponifiable matter, such as tocopherols, was removed by diethyl ether extraction. After acidifying the aqueous solution with 1 N HCl, free fatty acid was extracted with diethyl ether and purified on a silicic acid column (Silicagel 60; Merck, Darmstadt, Germany), eluting with *n*-hexane and a mixture of diethyl ether/*n*-hexane solution (5:95, 10:90, 20:80, 30:70, and 40:60, vol/vol). Free fatty acid was eluted with diethyl ether/*n*-hexane (20:80 and 30:70, vol/vol). This chromatographic purification was done just before use of each fatty acid. The refined free fatty acid sample gave only a single spot on the thin-layer chromatogram with normal-phase silica plates (Merck) developed with diethyl ether/*n*-hexane/acetic acid (40:60:1, by vol). The peroxide value of the free fatty acid was less than 1.0 as determined by the colorimetric iodine method (19). The composition of conjugated dienes and trienes in IsoLN was evaluated by the AOCS Official Method Cd 7-58 (20). CLA (99+% purity; mixture of 9,11- and 10,12-18:2) was obtained from Nu-Chek-Prep (Elysian, MN). Nonconjugated fatty acids, linoleic acid (LA; 99+% purity) and LN (99+% purity), were obtained from Sigma (St. Louis, MO).

The fatty acid composition of seed oils was analyzed by capillary GC after conversion to methyl esters by heating in a sealed tube at 50°C for 1 h with 0.5 M sodium methoxide in methanol. The capillary GC analysis was performed as described above.

*Cell culture conditions*. Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum was obtained from Sanko Chemical (Tokyo, Japan). Penicillin-streptomycin solution was obtained from Sigma. Water-soluble tetrazolium solution (WST-1) was obtained from Dojindo (Kumamoto, Japan).

Mouse fibroblast cell (A31) and its transformed cell (SV-T2) were obtained from Japan Health Sciences Foundation (Tokyo, Japan). The cells ($3 \times 10^4$/mL) were grown in plastic tissue culture dishes in DMEM (10 mL) containing 10% fetal bovine serum, 0.12% $NaHCO_3$, 100 units/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in a humidified $CO_2$ (5%) incubator.

Human monocytic leukemia cells (U-937) were also obtained from the Japan Health Sciences Foundation (Tokyo, Japan). U-937 cells ($1 \times 10^5$/mL) were grown in plastic flasks in RPMI 1640 medium (10 mL) with 10% fetal bovine serum, 0.12% $NaHCO_3$, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. Cells were maintained at 37°C in a humidified $CO_2$ (5%) atmosphere.

The percentage of viability of these cells after incubation was determined by WST-1 assay (21), which is a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction (MTT) assay based on the tetrazolium salt/formazan system. Cells preincubated as described above were seeded at a density of $2 \times 10^3$ cells/well in 96-well microplates and cultured in 100 μL medium/well for 24 h. Each fatty acid was dissolved in 10 μL of 1% dimethyl sulfoxide solution and then added to the culture. Butylated hydroxytoluene (BHT), as an antioxidant, was added to the fatty acid with mixing. After 21 h of incubation, 10 μL of WST-1 solution was added to each well, and the plate was incubated for a further 3 h. Cell viability was then measured spectrophotometrically (Microplate reader, Emax; Molecular Devices, Sunnyvale, CA) and was expressed as a

percentage of the viability obtained in control cultures, which were incubated without the addition of free fatty acids.

*Oxidative stability of free fatty acids in emulsion*. Each fatty acid was mixed homogeneously with Triton X-100 (Nacalai Tesque) in chloroform. After removing the chloroform by gently sweeping with nitrogen, a 0.05 M phosphate buffer (pH 7.4 at 37°C) was added to the mixture. Emulsification was carried out in an ice bath for 3 min with a sonicator (5202 PZT; Ohtake Works, Tokyo, Japan). Oxidative stability was evaluated by analyzing oxygen consumption. For continuously monitoring oxygen uptake by the oxidation of fatty acids in the solution, a model 5300 biological oxygen monitor (YSI, Yellow Springs, OH) was used. As soon as the 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) solution had been added to the substrate solution, the reaction vessel was charged with 3 mL of the reaction solution, and the concentration of dissolved oxygen in the solution was measured. AAPH was obtained from Wako Pure Chemical Ind. (Osaka, Japan). The final concentrations of the substrate fatty acids, Triton X-100, and AAPH were 1.08 mg/3 mL, 0.1 wt%, and 1.0 mM, respectively.

## RESULTS AND DISCUSSION

Igarashi and Miyazawa (17) examined the cytotoxic effect of two kinds of alkaline IsoLN on human tumor cells and showed that the IsoLN consisted of conjugated triene (22.5%) and conjugated diene (47.5%), which was as cytotoxic as tung oil fatty acid, whereas IsoLN consisting of conjugated triene (17.0%) and conjugated diene (66.7%) had little effect. From these results, they demonstrated that the cytotoxicity of IsoLN was attributable to its conjugated triene structure. However, there was little difference in the content of conjugated triene in the two kinds of IsoLN, and more data are required to conclude that conjugated trienoic acid is more cytotoxic than conjugated dienoic acid.

Thus, in this experiment, we prepared two kinds of alkaline IsoLN (IsoLN-1 and -2) with significant differences in the ratio of conjugated diene to conjugated triene and compared the cytotoxic effects of these CLN with other 18-carbon PUFA, namely LA, CLA, and LN. IsoLN-1 and IsoLN-2 were prepared by the alkaline isomerization of ethyl α-linolenate in dimethylformamide followed by the saponification of the isomerized linolenate, but the concentrations of catalyst (potassium *t*-butoxide) and substrate used for IsoLN-1 preparation (17.4 and 34.7 mM, respectively) were different from the case of IsoLN-2 (104.4 and 104.4 mM, respectively). Table 1 shows the compostion of conjugated dienes and trienes in each IsoLN. The change in isomerization conditions produced two kinds of IsoLN (IsoLN-1 and -2) containing different ratios of conjugated trienes to conjugated dienes. For cell viability assay, IsoLN-1 was used in conjunction with a mixture of IsoLN-2 + LN prepared such that IsoLN-1 and IsoLN-2 + LN contained the same overall concentration of conjugated fatty acids (Table 1).

Although LA and LN had no cytotoxic effect on U-937 cells, conjugated fatty acids (CLA, IsoLN-1, IsoLN-2 + LN) reduced cell viability at concentrations up to 324 μM for CLA, 327 μM for IsoLN-1, and 41 μM for IsoLN-2 + LN (Fig. 1). This result indicates the higher cytotoxic effect of conjugated trienes than conjugated dienes and confirms the earlier report (17).

**TABLE 1**
**Composition of Conjugated Dienes and Conjugated Trienes in IsoLN-1, IsoLN-2, and IsoLN-2 + LN[a]**

| Fatty acids | Conjugated dienes (%) | Conjugated trienes (%) | Total conjugated fatty acids (%) |
|---|---|---|---|
| IsoLN-1 | 45.3 | 3.7 | 49.0 |
| IsoLN-2 | 51.6 | 35.6 | 87.2 |
| IsoLn-2 + LN | 28.6 | 19.7 | 48.3 |

[a]IsoLN, isomerized linolenic acid; LN, α-linolenic acid.

As described above, the importance of the conjugated triene structure in the cytotoxicity to tumor cells is confirmed; however, there are many kinds of positional and geometrical isomers of CLN in alkaline IsoLN, and the difference in the cytotoxicity of each CLN isomer remains to be elucidated. Some seed oils contain high contents of different types of CLN isomer (14), and it is interesting to compare the cytotoxic effects of these seed oil fatty acids. The fatty acid profiles of seed oils containing CLN are shown in Table 2. The percentages of the main components are generally in harmony with those described previously (14).

Figure 2 shows the inhibitory effect of these fatty acid mixtures containing CLN on mouse normal (A31) and tumor (SV-T2) cells. Fatty acids from pot marigold had no effect on either cell line, but other kinds of fatty acids from seed oils were cytotoxic to SV-T2 cells at concentrations exceeding 41 μM for pomegranate and tung, and 81 μM for catalpa, respectively. Fatty acids from pomegranate and tung were also cytotoxic to A31 cells at concentrations exceeding 81 and 163 μM, respectively. As shown in Table 2, free fatty acids from

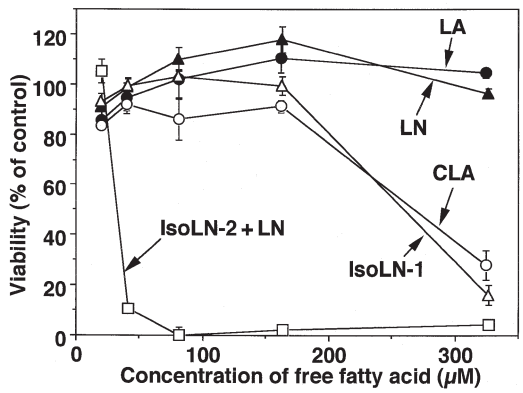


**FIG. 1.** Cytotoxic effect of linoleic acid (LA), conjugated linoleic acid (CLA), α-linolenic acid (LN), isomerized linolenic acid (IsoLN)-1 and IsoLN-2 + LN on U-937 cells. Cells were incubated in RPMI 1640 medium at 37°C in a humidified $CO_2$ (5%) atmosphere. Viability was assessed spectrophotometrically by using water-soluble tetrazolium (WST-1) reagent. Each point is the mean ± SD of three values obtained from separate cultures.

**TABLE 2**
**Fatty Acid Composition of Seed Oils**

| Fatty acid (wt%) | Pot marigold | Pomegranate | Tung | Catalpa |
|---|---|---|---|---|
| 16:0 | 4.1 | 3.1 | 2.6 | 2.8 |
| 18:0 | 2.1 | 2.0 | 2.7 | 1.9 |
| 18:1n-9 | 7.4 | 4.5 | 6.0 | 7.8 |
| 18:2(*c*9,*c*12) | 42.5 | 5.1 | 7.4 | 32.3 |
| 18:2(*t*9,*t*12) | — | — | — | 13.0 |
| 18:3n-3 | 1.0 | — | — | 1.3 |
| 18:3(*t*8,*t*10,*c*12) | 33.4 | — | — | — |
| 18:3(*t*8,*t*10,*t*12) | 4.7 | — | — | — |
| 18:3(*c*9,*t*11,*c*13) | — | 71.7 | — | — |
| 18:3(*c*9,*t*11,*t*13) | — | 2.8 | 70.1 | — |
| 18:3(*t*9,*t*11,*c*13) | — | 5.1 | 1.8 | 31.3 |
| 18:3(*t*9,*t*11,*t*13) | — | 1.6 | 6.2 | 5.7 |
| Mean molecular weight of fatty acid | 278.8 | 278.1 | 278.4 | 279.2 |

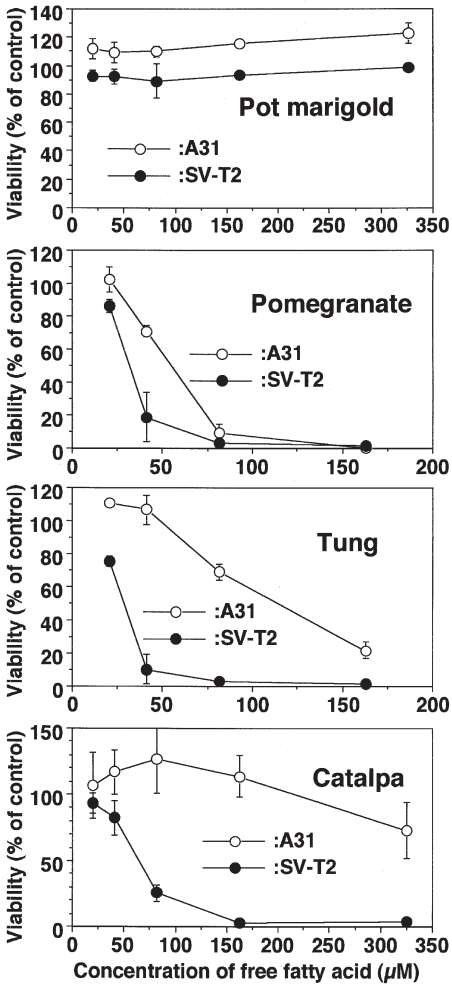


**FIG. 2.** Cytotoxic effect of fatty acids from four kinds of seed oils on A31 and SV-T2 cells. Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) at 37°C in a humidified $CO_2$ (5%) atmosphere. Viability was assessed spectrophotometrically by using WST-1 reagent. Each point is the mean ± SD of three values obtained from separate cultures. For abbreviation see Figure 1.

tung, pomegranate, and catalpa contained different types of geometrical 9,11,13-CLN isomers, whereas CLN from pot marigold contained only 8,10,12-isomers, suggesting that the cytotoxic effect of 9,11,13-CLN would be strong, but that of 8,10,12-CLN would be relatively weak.

The same result was obtained by the determining the viability of human monocytic leukemia cell (U-937) (Fig. 3). The fatty acids from seed oils of pomegranate, tung, and catalpa showed strong cytotoxic effects on U-937 cells, but those from pot marigold oil had a relatively low cytotoxicity. When the cytotoxic effect of free fatty acids from seed oils of pomegranate, tung, and catalpa were compared, pomegranate and tung oils were more effective than catalpa oil. This difference was considered to be due to the different level of total 9,11,13-CLN isomers of these oils (Table 2).

To elucidate the difference in the cytotoxic effect of each CLN isomer, we separated CLN isomer by HPLC from a fatty acid mixture of seed oil and analyzed its cytotoxicity to tumor cells SV-T2 and U-937 (Fig. 4). As expected, the effects of *c*9,*t*11,*c*13-CLN, *c*9,*t*11,*t*13-CLN, and *t*9,*t*11,*c*13-CLN were stronger than that of *t*8,*t*10,*c*12-CLN. Therefore, it was confirmed that 9,11,13-CLN isomers were more cytotoxic than 8,10,12-CLN isomer on tumor cells. The results in Figure 4 also indicated that the cytotoxic effects of three different 9,11,13-CLN isomers were almost the same, suggesting that the *cis*/*trans* configuration of the 9,11,13-CLN isomers had little effect on their effects. Cornelius *et al.* (15) reported that *c*9,*t*11,*t*13,*c*15-18:4 was toxic to U-937 cells at concentrations of 5 μM or less. The toxicity of 9,11,13-CLN on U-937 cells found in this study was comparable to that of *c*9,*t*11,*t*13,*c*15-18:4. These results suggest that the conjugation at 9,11,13-positions of 18-carbon fatty acids may be important for their expression of cytotoxicity.

The mechanism of the cytotoxicity of conjugated fatty acids is supposed to involve lipid peroxidation because the toxic action can be blocked by the addition of antioxidants (15,18). The cytotoxicity of each 9,11,13-CLN isomer was

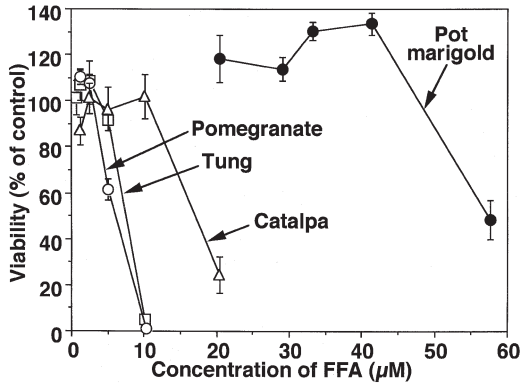


**FIG. 3.** Cytotoxic effect of fatty acids from four kinds of seed oils on U-937 cells. Cells were incubated in RPMI 1640 medium at 37°C in a humidified $CO_2$ (5%) atmosphere. Viability was assessed spectrophotometrically by using WST-1 reagent. Each point is the mean ± SD of three values obtained from separate cultures. For abbreviation see Figure 1.

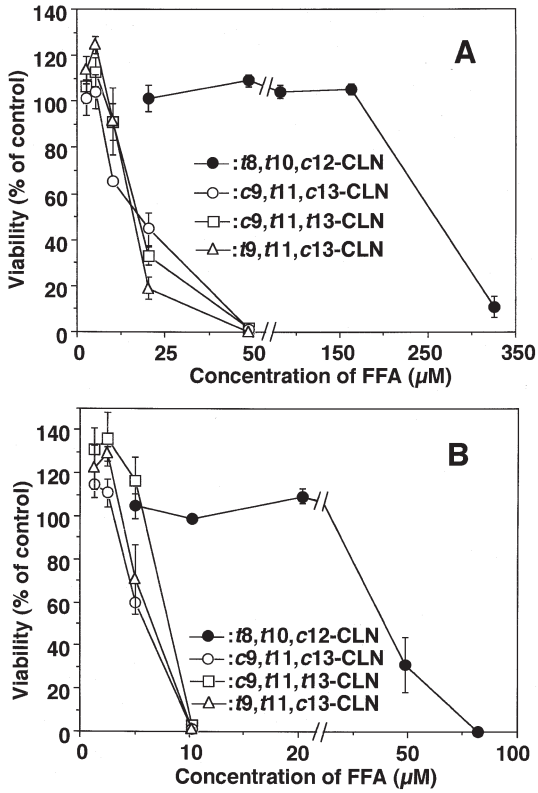


**FIG. 4.** Cytotoxic effect of each conjugated linolenic acid (CLN) isomer on SV-T2 cells (A) and on U-937 cells (B). Cells were incubated in DMEM medium for SV-T2 cells and in RPMI 1640 medium for U-937 cells at 37°C in a humidified $CO_2$ (5%). Viability was assessed spectrophotometrically by using WST-1 reagent. Each point is the mean ± SD of three values obtained from separate cultures. For abbreviation see Figure 2.

also completely inhibited by the addition of quarter molar ratio of BHT as an antioxidant to fatty acid (data not shown), confirming that lipid peroxidation is responsible for the cytotoxicity of conjugated fatty acids.

Furthermore, the possible contribution of lipid peroxidation in the greater cell toxicity exerted by 9,11,13-CLN compared with 8,10,12-CLN may be explained by the different oxidative stability of these CLN isomers. Figure 5 shows the oxidative stability of four kinds of CLN isomers and LN in an aqueous phase. As shown in Figure 5, the stabilities of three kinds of 9,11,13-CLN isomers were the same, but lower than that of *t*8,*t*10,*c*12-CLN isomer, suggesting that the difference in the cytotoxicity of 9,11,13-CLN and 8,10,12-CLN found in Figure 4 would be due to the different susceptibilities of these CLN isomers to peroxidation.

The oxidative stabilities of other kinds of fatty acids used in this study also showed a good correlation between the oxidizability of these fatty acids and their cytotoxic activities. Figure 6A shows that the oxidative stabilities of free fatty acids from pomegranate and tung were almost the same and were lower than that from catalpa, but the stability of pomegranate fatty acids was much greater than for catalpa fatty acids. On the other hand, when the oxidative stabilities of LA, LN, CLA, and IsoLN were compared (Fig. 6B), IsoLN-2 +

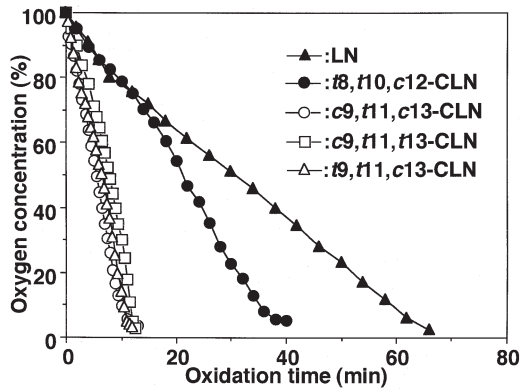


**FIG. 5.** Comparison of oxidative stabilities of CLN isomers and LN in an aqueous dispersion. Fatty acids (1.08 mg/3 mL) were incubated with 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) (1.0 mM) in 3 mL of a phosphate buffer containing 0.1 wt% of Triton X-100. Oxidation was monitored by measuring oxygen uptake during oxidation. For other abbreviations see Figures 1 and 4.

LN was oxidized most rapidly, followed by IsoLN-1 and CLA, LN and LA. These orders in oxidative stabilities (Fig. 6) agreed with those of cytotoxicity on U-937 cells (Figs. 1 and 3).

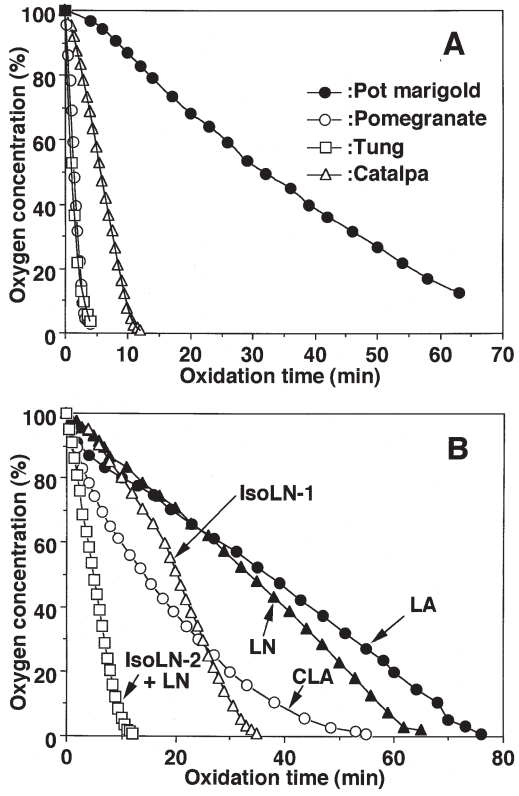


**FIG. 6.** Comparison of oxidative stabilities of free fatty acids from seed oils (A) and conjugated and nonconjugated 18-carbon fatty acids (B) in an aqueous dispersion. Fatty acids (1.08 mg/3 mL) were incubated with AAPH (1.0 mM) in 3 mL of a phosphate buffer containing 0.1 wt% of Triton X-100. Oxidation was monitored by measuring oxygen uptake during oxidation. For abbreviation see Figure 5.

## ACKNOWLEDGMENT

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# Effect of Sesamin on Mitochondrial and Peroxisomal β-Oxidation of Arachidonic and Eicosapentaenoic Acids in Rat Liver

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**ABSTRACT:** The effects of dietary sesamin on the hepatic metabolism of arachidonic (AA) and eicosapentaenoic (EPA) acids, were investigated with respect to their β-oxidation and secretion as triacylglycerol (TG). For 2 wk, rats were fed three types of dietary oils: (i) corn oil (control) group; (ii) EPA group: EPA ethyl esters/rapeseed oil = 2:3; (iii) AA group: AA ethyl esters/palm oil/perilla oil = 2:2:1, with or without 0.5% (w/w) of sesamin. Dietary sesamin significantly increased the activities of hepatic mitochondrial and peroxisomal fatty acid oxidation enzymes (mitochondrial carnitine acyltransferase I, acyl-CoA dehydrogenase, and peroxisomal acyl-CoA oxidase). Dietary EPA increased mitochondrial carnitine acyltransferase I and peroxisomal acyl-CoA oxidase. Dietary AA, however, had an effect on peroxisomal acyl-CoA oxidase only. In whole liver and the TG fraction, EPA and AA concentrations were significantly increased by dietary EPA and AA, respectively, and were decreased by dietary sesamin. In hepatic mitochondria and peroxisomes, EPA concentration was increased by dietary EPA, but AA was not changed by dietary AA. The addition of dietary sesamin to the EPA-supplemented diet significantly decreased the EPA concentration compared to concentrations found with consumption of dietary EPA alone. These results suggest that sesamin increased β-oxidation enzyme activities and reduced hepatic EPA and AA concentrations by degradation. The stimulating effect of sesamin on β-oxidation, however, was more significant in the EPA group than in the AA group. Hepatic AA concentration was altered by the joint effect of sesamin through esterification into TG and the stimulation of β-oxidation.

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Sesamin (a mixture of sesamin and episesamin, 47:53, w/w) is found in sesame seeds and sesame seed oil. It has multiple biological functions such as antioxidative activity (1,2), anticarcinogenicity (2) and antihypertensive effects (3,4) in rats, and alleviation of hepatic injury caused by alcohol or carbon tetrachloride (5) in mice. Sesamin also affects lipid metabolism, inhibits cholesterol absorption from the intestine, reduces 3-hydroxy-3-methyl-glutaryl CoA reductase activity in liver microsomes (6,7), and affects the incorporation of linoleic acid (LA) into lipid subfractions (8) in rats. It also increases the concentration of dihomo-γ-linolenic acid (DGLA) by inhibiting Δ5-desaturase activity in the *Mortierella alpina* fungus and rat liver microsomes (9,10). In human studies, sesamin has been reported to have hypocholesterolemic effects (11).

In our previous studies, we reported that sesamin 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 rats (13). We also found that sesamin inhibits extreme changes of the n-6/n-3 ratio *in vivo* through the reduction of polyunsaturated fatty acid (PUFA) concentration in rat liver (13,14). This regulating effect is especially significant in the presence of high amounts of EPA (13) and AA (14), which are PUFA as well as precursors of eicosanoids. An investigation of the concentrations of serum triacylglycerol (TG) and ketone bodies (14) revealed that the reduction of hepatic PUFA concentration may have occurred because sesamin intervenes in PUFA degradation (β-oxidation) and esterification into TG. Ashakumary *et al.* (15) reported that sesamin increased the activity and gene expression of hepatic fatty acid oxidation enzymes. However, the combined actions of sesamin and different classes of long-chain PUFA (AA, n-6, and EPA, n-3) on the enzyme activities involved with fatty acid oxidation in rats have not yet been investigated.

In this study, we focused on the effects of dietary sesamin on the hepatic metabolism of AA and EPA with respect to their oxidation and secretion as TG. In particular, to investigate the effects of sesamin, AA, and EPA on fatty acid oxidation, we determined the activities of hepatic mitochondrial and peroxisomal fatty acid oxidation enzymes. The fatty acid composition of TG and mitochondrial and peroxisomal fatty acid concentrations were also investigated.

## MATERIALS AND METHODS

*Chemicals.* Sesamin (a mixture of sesamin and episesamin, 47:53,w/w), prepared from refined sesame oil and purified by the method of Fukuda *et al.* (16), was donated by Suntory Ltd. (Osaka, Japan). The sesamin included equivalent amounts of sesamin and episesamin as a result of the deodorization process (16). AA ethyl esters (purity >99%) were also donated by Suntory Ltd. EPA ethyl esters (purity >95%) were donated by Q.P. Co. (Tokyo, Japan). Corn oil, rapeseed oil,

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Abbreviations: AA, arachidonic acid; AIN, American Institute of Nutrition; ALA, α-linolenic acid; ANOVA, analysis of variance; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acid; TG, triacylglycerol.

and palm oil were provided by Nisshin Oil Co. (Tokyo, Japan). Palmitoyl-CoA, linolenoyl-CoA, arachidonoyl-CoA, and L-carnitine were purchased from Sigma Chemical (St. Louis, MO). *N*-Ethylmaleimide, 4-aminoantipyrine, and FAD were purchased from Nakarai Tesque (Kyoto, Japan). Phenazinemethosulfate, horseradish peroxidase, tris(hydroxymethyl) amino methane, EDTA, and Triton X-100 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2,6-Dichlorophenolindophenol and bovine serum albumin fraction V (essential fatty acid-free) were purchased from Sigma Chemical.

*Animals and diets.* All experiments were approved by the Animal Experimentation Ethics Committee of Ochanomizu University (Tokyo, Japan). Five-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 a week. Weighing an average of 198 g, the animals were then divided into six groups of five 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 (20 g/d, 1–7 d; 25 g/d, 8–14 d) and water *ad libitum* for 2 wk. The basal diet prepared under standards given by the American Institute of Nutrition (AIN) (17) was provided by Eisai Co. (Tokyo, Japan) and contained the following percentage of ingredients (weight basis): casein, 20; glucose, 25; sucrose, 25; cornstarch, 15; filter paper, 5; AIN mineral mixture, 3.5; AIN vitamin mixture, 1; choline bitartrate, 0.2; DL-methionine, 0.3. The test diets were prepared by mixing 5% (w/w) experimental oil mixtures with the basal diet. Three kinds of oil mixtures were prepared: (i) control group: corn oil only; (ii) EPA group: EPA ethyl esters/rapeseed oil = 2:3; (iii) AA group: AA ethyl esters/palm oil/perilla oil = 2:2:1. Each diet was then split into two types: with or without 0.5% (w/w) of sesamin. The dietary oils of EPA and AA groups are the same as those in our previous studies (13,14). The concentration of EPA or AA ethyl esters was about 40% (w/w) in each dietary oil, respectively. The fatty acid compositions of these oils are shown in Table 1.

**TABLE 1**
**Fatty Acid Composition of Dietary Oils[a]**

| | Control | EPA | AA |
|---|---|---|---|
| 16:0 | 11.4 | 3.1 | 18.1 |
| 18:0 | 1.8 | 1.5 | 2.5 |
| 18:1n-9 | 31.4 | 39.3 | 19.9 |
| 18:2n-6 | 55.4 | 13.1 | 7.7 |
| 18:3n-3 | — | 5.5 | 14.7 |
| 20:4n-6 | — | — | 37.1 |
| 20:5n-3 | — | 37.4 | — |

[a]Values are expressed as weight percentages. EPA, eicosapentaenoic acid; AA, arachidonic acid.

*Enzyme assays.* At the termination of the experimental period, livers of rats were excised under nembutal anesthesia. Three grams of each liver were homogenized with 7 vol of 0.25 M sucrose and centrifuged at 500 × *g* for 10 min. The supernatant was recentrifuged at 9,000 × *g* for 10 min. The supernatant was centrifuged twice at 39,000 × *g* for 10 min to isolate the fraction including peroxisomes which were then finally suspended in 0.25 M sucrose containing 1 mM EDTA and 10 mM Tris-HCl (pH 7.3). The precipitate suspended in 0.25 M sucrose containing 1 mM EDTA and 10 mM Tris-HCl (pH 7.3) was centrifuged twice at 9,000 × *g* for 10 min to isolate the mitochondrial fraction. It was washed twice with 0.25 M sucrose containing 1 mM EDTA and 10 mM Tris-HCl (pH 7.3) and then resuspended in the same medium. The activities of succinate dehydrogenase, the marker enzyme of mitochondria, in the two fractions were measured. The enzyme activity was found in the mitochondrial fraction, but not in the fraction including peroxisomes (data not shown). Therefore, mitochondria were not contaminated in the fraction including peroxisomes. Carnitine palmitoyltransferase I activities in mitochondrial fraction were measured by the method of Markwell *et al.* (18), using palmitoyl-CoA as a substrate. Acyl-CoA dehydrogenase activities in the mitochondrial fraction were measured by the method of Dommes and Kunau (19) by using palmitoyl-CoA, linolenoyl-CoA, and arachidonoyl-CoA as substrates. Acyl-CoA oxidase activities in peroxisomal fraction were measured by the method of Hashimoto *et al.* (20), using palmitoyl-CoA, linolenoyl-CoA, and arachidonoyl-CoA as substrates. Protein was measured by the method of Lowry *et al.* (21).

*Lipid analyses.* Lipids were extracted from 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). As an internal standard, margaric acid (17:0) was added to the lipid extracts, which were then methylated by using HCl-methanol as described 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 (PerkinElmer AutoSystem GC, 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).

*Statistical analysis.* All results are shown as means ± SD. The significance of the differences in mean values was evaluated by a Student's *t*-test and an analysis of variance (ANOVA). After ANOVA, a Bonferroni-Donn *post-hoc* test was used. Analyses were performed by using a Stat View (System 4.02) computer package (Abacus Concepts, Inc., Berkeley, CA).

## RESULTS

*Growth parameters and tissue weights.* No significant differences in food intake and body weight gain were observed among all groups. We concluded that the administration of sesamin and the three kinds of dietary oils had no influence on the growth of rats. Dietary sesamin significantly increased the liver weight of rats (control + sesamin, 4.06 ± 0.26; EPA + sesamin, 4.57 ± 0.20; AA + sesamin, 4.42 ± 0.19 g/100 g body weight vs. control, 3.20 ± 0.06; EPA, 3.02 ± 0.12; and AA, 3.15 ± 0.14 g/100 g body weight, respectively). The difference between the with and without-sesamin groups was statistically significant ($P < 0.05$).

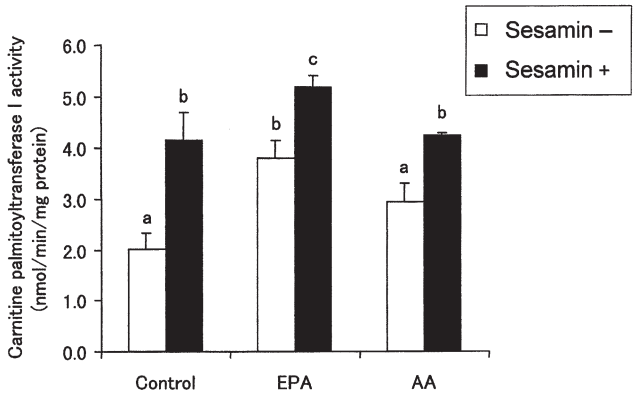


**FIG. 1.** Effect of dietary sesamin, eicosapentaenoic acid (EPA), and arachidonic acid (AA) on mitochondrial carnitine palmitoyltransferase I activity in rat liver. Values are means ± SD of five determinations made on separate animals. The significance of differences between the dietary treatments was analyzed by an analysis of variance. Bars with different superscript letters are significantly different ($P < 0.05$).

*Activities of fatty acid oxidation enzymes in rat liver.* Mitochondrial carnitine palmitoyltransferase I activities in rat liver are shown in Figure 1. Dietary sesamin significantly increased the enzyme activities in all dietary oil groups. Dietary EPA also increased enzyme activity, but dietary AA had no significant effect. The highest enzyme activity was observed in the combination of EPA with sesamin.

Mitochondrial acyl-CoA dehydrogenase activities in rat liver are shown in Figure 2. Dietary sesamin significantly increased the enzyme activities, using every substrate (palmitoyl-CoA; 16:0-CoA, linolenoyl-CoA; 18:3-CoA, and arachidonoyl-CoA; 20:4-CoA) in all dietary oil groups. When a 16:0-CoA (saturated fatty acid-CoA) was used as a substrate, dietary sesamin increased the activities less than twofold compared with the no-sesamin group. But when a 18:3-CoA (n-3 PUFA-CoA) was used, dietary sesamin increased the activities 4.4-fold in the control group, 2.4-fold in the EPA group, and 3.4-fold in the AA group. When a 20:4-CoA (n-6 PUFA-CoA) was used, dietary sesamin increased the activities 4.1-fold in the control group, 4.2-fold in the EPA group, and 4.6-fold in the AA group. One may compare the enzyme activity levels of 16:0-CoA, 18:3-CoA, and 20:4-CoA; a conclusion would be that either chain length, or degree of unsaturation, or both, could relate to their increased suitability as substrates. Dietary EPA and AA had no effect on the enzyme activity.

Peroxisomal acyl-CoA oxidase activities in rat liver are shown in Figure 3. When a 16:0-CoA was used as a substrate, dietary sesamin increased the enzyme activities about fourfold in all dietary oil groups. Furthermore, EPA and AA also significantly increased the activities. The effect of sesamin on increasing the activity was much greater than for EPA or AA. When 18:3-CoA was used, dietary sesamin increased the activities 3.2-fold in the control group, 4.5-fold in the EPA group, and 2.9-fold in the AA group, compared with the no-sesamin group. When 20:4-CoA was used, the activities in the EPA + sesamin and AA + sesamin groups were significantly higher than in the control group. The effectiveness of enzyme activity is in the order of 16:0-CoA > 18:3-CoA > 20:4-CoA, similar to mitochondrial acyl-CoA dehydrogenase.

*Liver fatty acid levels.* The fatty acid concentrations of liver are shown in Table 2. In the control group, dietary sesamin tended to decrease LA (18:2n-6) and α-linolenic acid (ALA; 18:3n-3) but significantly increased DGLA (20:3n-6). Dietary EPA significantly increased EPA (20:5) and docosapentaenoic acid (DPA, 22:5n-3) but decreased AA (20:4) compared with the control group. In the EPA group, dietary sesamin significantly decreased n-3 PUFA: ALA, EPA, and DPA. On the contrary, dietary sesamin increased the n-6 PUFA, DGLA, and AA. Dietary AA significantly increased the AA compared with the

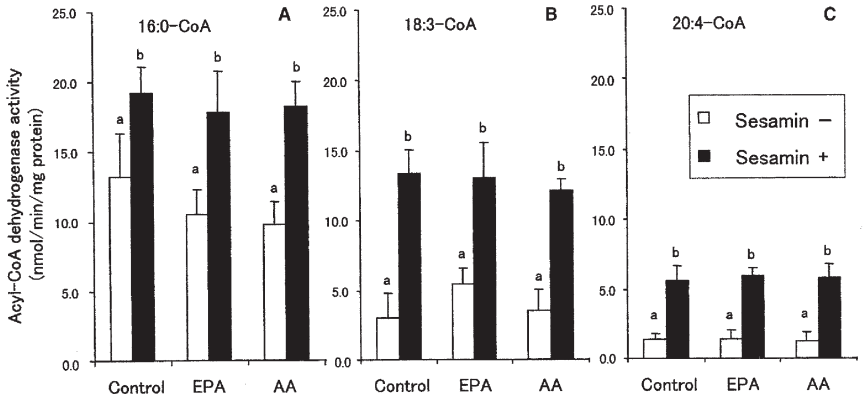


**FIG. 2.** Effect of dietary sesamin, EPA, and AA on mitochondrial acyl-CoA dehydrogenase activity in rat liver. Values are means ± SD of five determinations made on separate animals. The significance of differences between the dietary treatments using the same substrate (16:0-CoA, 18:3-CoA, or 20:4-CoA) was analyzed by analysis of variance. Bars with different superscript letters are significantly different ($P < 0.05$). (A) 16:0-CoA, palmitoyl-CoA; (B) 18:3-CoA, linolenoyl-CoA; (C) 20:4-CoA, arachidonoyl-CoA. See Figure 1 for abbreviations.

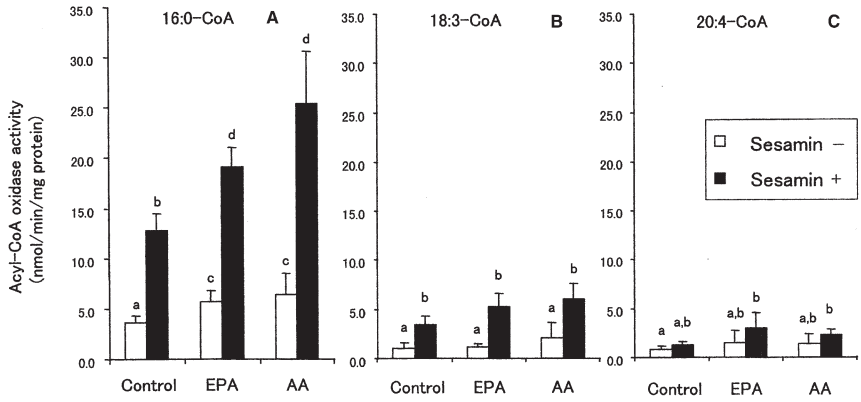


**FIG. 3.** Effect of dietary sesamin, EPA, and AA on peroxisomal acyl-CoA oxidase activity in rat liver. Values are means ± SD of five determinations made on separate animals. The significance of differences between the dietary treatments using the same substrate (16:0-CoA, 18:3-CoA, or 20:4-CoA) was analyzed by an analysis of variance. Bars with different superscript letters are significantly different ($P < 0.05$). (A) 16:0-CoA, palmitoyl-CoA; (B) 18:3-CoA, linolenoyl-CoA; (C) 20:4-CoA, arachidonoyl-CoA. See Figures 1 and 2 for abbreviations.

control group. In the AA group, dietary sesamin significantly decreased AA to the control group level. Furthermore, dietary sesamin also decreased n-3 PUFA: ALA, EPA, and DPA. EPA concentration in the EPA group was reduced to about one-fifth and AA concentration in the AA group to about one-half by the administration of sesamin.

The fatty acid composition of liver TG is shown in Table 3. In the control group, dietary sesamin significantly increased AA but decreased ALA. Dietary EPA significantly increased EPA, DPA, and docosahexaenoic acid (DHA, 22:6n-3), but decreased AA, compared with the control group. In the EPA group, dietary sesamin decreased EPA and DHA and increased AA. Dietary AA significantly increased the n-6 PUFA: AA and DPA (n-6) compared with the control group. In the AA group, dietary sesamin decreased γ-linolenic acid (18:3n-6), AA, and 22:4n-6. In the AA group, dietary sesamin had no effect on n-3 PUFA compositions. EPA composition in the EPA group was reduced to about one-fifth and AA composition in the AA group to about one-half by the administration of sesamin.

The mitochondrial fatty acid concentration in liver is shown in Table 4. In the control group, dietary sesamin had no effect on mitochondrial fatty acid concentration. Dietary EPA significantly increased EPA, but decreased AA. In the EPA group, dietary sesamin significantly decreased EPA, but increased AA. Dietary AA had no effect on mitochondrial AA concentration.

The peroxisomal fatty acid concentration in liver is shown in Table 5. In the control group, dietary sesamin had no effect on peroxisomal fatty acid concentration. Dietary EPA significantly increased EPA, but decreased AA. In the EPA group, dietary sesamin significantly decreased EPA, but increased AA. Dietary AA had no effect on peroxisomal AA concentration.

## DISCUSSION

In our previous studies, sesamin inhibited the extreme change of n-6/n-3 PUFA ratio *in vivo* (13,14). This regulating effect was remarkable when an excessive amount of EPA (13) or

**TABLE 2**
**Effects of Dietary Sesamin, EPA, and AA on Fatty Acid Concentrations in Rat Liver[a]**

| | Control | | EPA | | AA | |
|---|---|---|---|---|---|---|
| Sesamin | – | + | – | + | – | + |
| 18:2n-6 | 47.2 ± 26.9 | 17.8 ± 3.34 | 16.6 ± 1.42 | 12.6 ± 1.78* | 14.7 ± 4.53 | 6.64 ± 1.85* |
| 18:3n-6 | 1.54 ± 1.07 | 0.23 ± 0.06 | ND | ND | 0.56 ± 0.25 | 0.14 ± 0.07* |
| 20:3n-6 | ND | 0.79 ± 0.19 | 0.37 ± 0.21 | 1.40 ± 0.69* | ND | 0.32 ± 0.19 |
| 20:4n-6 | 38.0 ± 14.9 | 39.6 ± 5.08 | 14.1 ± 1.07** | 19.0 ± 2.03* | 89.4 ± 13.7** | 43.6 ± 4.04* |
| 18:3n-3 | 0.81 ± 0.51 | 0.16 ± 0.06 | 1.12 ± 0.14 | 0.20 ± 0.10* | 4.42 ± 1.68** | 0.57 ± 0.18* |
| 20:5n-3 | ND | ND | 21.1 ± 3.53 | 4.67 ± 0.52* | 1.31 ± 0.40 | ND |
| 22:5n-3 | ND | ND | 5.57 ± 0.93 | 3.26 ± 0.55* | 2.38 ± 0.58 | 1.59 ± 0.25* |
| 22:6n-3 | 9.85 ± 5.56 | 17.0 ± 14.0 | 11.9 ± 2.55 | 7.10 ± 4.33 | 9.20 ± 3.11 | 9.57 ± 1.11 |

[a]Results are expressed as μmol/g tissues and are means ± SD ($n = 5$). Values with asterisks in sesamin (+) groups are significantly different from sesamin (–) groups of the same dietary oil group ($P < 0.05$). Values with double asterisk in sesamin (–) groups of EPA and AA groups are significantly different from sesamin (–) groups of the Control groups ($P < 0.05$). See Table 1 for other abbreviations; ND, not detected.

**TABLE 3**
**Effect of Dietary Sesamin, EPA, and AA on Fatty Acid Composition in Rat Liver Triacylglycerol[a]**

| | Control | | EPA | | AA | |
|---|---|---|---|---|---|---|
| Sesamin | – | + | – | + | – | + |
| 18:2n-6 | 22.8 ± 2.86 | 19.7 ± 3.04 | 13.4 ± 2.54** | 10.8 ± 1.56 | 9.19 ± 1.43** | 10.3 ± 0.90 |
| 18:3n-6 | 0.31 ± 0.07 | 0.24 ± 0.08 | 1.44 ± 0.20** | 0.57 ± 0.15* | 2.65 ± 0.30** | 1.51 ± 0.05* |
| 20:4n-6 | 3.45 ± 0.53 | 6.13 ± 0.98* | 1.46 ± 0.14** | 2.04 ± 0.43* | 38.5 ± 2.24** | 20.6 ± 1.61* |
| 22:4n-6 | ND | ND | ND | ND | 0.87 ± 0.23 | 0.26 ± 0.07* |
| 18:3n-3 | 0.70 ± 0.07 | 0.28 ± 0.03* | 0.09 ± 0.02 | 0.08 ± 0.03 | 0.36 ± 0.13** | 0.42 ± 0.29 |
| 20:5n-3 | ND | ND | 14.9 ± 2.70 | 3.22 ± 0.87* | ND | ND |
| 22:5n-3 | 0.37 ± 0.18 | 0.52 ± 0.17 | 2.97 ± 0.29** | 3.76 ± 0.81 | 1.03 ± 0.20** | 1.10 ± 0.27 |
| 22:6n-3 | 0.58 ± 0.16 | 0.90 ± 0.35 | 3.68 ± 1.28** | 1.49 ± 0.67* | 2.66 ± 0.36** | 2.12 ± 0.79 |

[a]Results are expressed as percentages and are means ± SD ($n$ = 5). Values with asterisks in sesamin (+) groups are significantly different from sesamin (–) groups of the same dietary oil group ($P < 0.05$). Values with double asterisk in sesamin (–) groups of EPA and AA groups are significantly different from sesamin (–) groups of the Control groups ($P < 0.05$). See Table 1 and 2 for other abbreviations.

**TABLE 4**
**Effect of Dietary Sesamin, EPA, and AA on Mitochondrial Fatty Acid Concentrations in Rat Liver[a]**

| | Control | | EPA | | AA | |
|---|---|---|---|---|---|---|
| Sesamin | – | + | – | + | – | + |
| 16:0 | 120.4 ± 18.5 | 88.5 ± 12.2 | 96.3 ± 12.5 | 94.5 ± 8.94 | 88.2 ± 16.6** | 95.3 ± 8.71 |
| 18:0 | 137.8 ± 30.5 | 105.6 ± 10.5 | 98.7 ± 6.11 | 98.5 ± 5.80 | 107.5 ± 23.8 | 100.0 ± 12.3 |
| 18:1n-9 | 43.5 ± 12.3 | 35.3 ± 9.85 | 49.5 ± 8.72 | 48.9 ± 6.56 | 40.6 ± 9.73 | 43.3 ± 4.96 |
| 18:2n-6 | 45.7 ± 7.59 | 40.4 ± 5.13 | 29.7 ± 8.19** | 46.1 ± 4.50* | 13.2 ± 3.87** | 16.2 ± 2.16 |
| 20:4n-6 | 85.7 ± 15.0 | 87.5 ± 19.7 | 36.5 ± 8.13** | 63.4 ± 6.04* | 91.5 ± 21.6 | 102.3 ± 17.1 |
| 20:5n-3 | ND | ND | 18.0 ± 4.99 | 9.80 ± 0.69* | ND | ND |

[a]Results are expressed as nmol/mg protein and are means ± SD ($n$ = 5). Values with asterisks in sesamin (+) groups are significantly different from sesamin (–) groups of the same dietary oil group ($P < 0.05$). Values with double asterisk in sesamin (–) groups of EPA and AA groups are significantly different from sesamin (–) groups of the Control groups ($P < 0.05$). See Table 1 and 2 for other abbreviations.

AA (14), was given, and the reduction of hepatic PUFA concentration may have occurred because sesamin acts at some point between PUFA degradation (oxidation) and esterification (14). To reveal the mechanism of sesamin's effect, we examined changes in the hepatic fatty acid β-oxidation enzyme activities, the fatty acid composition of TG, and mitochondrial and peroxisomal fatty acid concentrations. This study was an extension of our previous studies on the effect of sesamin on fatty acid metabolism; therefore, the fatty acid compositions of experimental oils were the same as those in our previous studies (13,14).

Fatty acids are incorporated into cells and degraded *via* the β-oxidation pathway in mitochondria and peroxisomes. Acyl-CoA can be directly incorporated into peroxisomes, but in mitochondria, acyl-CoA is first changed to acylcarnitine by carnitine acyltransferase I before passage through the mitochondrial inner membrane. Acyl-CoA dehydrogenase is the first enzyme in mitochondrial β-oxidation, and that of peroxisomal β-oxidation is acyl-CoA oxidase. To investigate the capacity for fatty acid oxidation in each organelle (mitochondria and peroxisomes), we determined the activities of these three enzymes.

Dietary sesamin increased all three enzyme activities (Figs. 1–3). The increasing levels of mitochondrial enzymes

**TABLE 5**
**Effect of Dietary Sesamin, EPA, and AA on Peroxisomal Fatty Acid Concentrations in Rat Liver[a]**

| | Control | | EPA | | AA | |
|---|---|---|---|---|---|---|
| Sesamin | – | + | – | + | – | + |
| 16:0 | 2.90 ± 0.74 | 2.97 ± 0.33 | 2.06 ± 0.32 | 3.48 ± 0.44* | 2.54 ± 0.18 | 2.72 ± 0.68 |
| 18:0 | 3.07 ± 0.80 | 2.88 ± 0.29 | 2.35 ± 0.47 | 3.13 ± 0.44 | 3.14 ± 0.78 | 2.53 ± 0.48 |
| 18:1n-9 | 1.07 ± 0.20 | 1.05 ± 0.22 | 0.98 ± 0.30 | 1.47 ± 0.99 | 0.63 ± 0.17 | 0.82 ± 0.14 |
| 18:2n-6 | 0.96 ± 0.22 | 0.61 ± 0.07 | 0.52 ± 0.14** | 0.80 ± 0.07* | 0.22 ± 0.05** | 0.31 ± 0.01* |
| 20:4n-6 | 2.17 ± 0.57 | 1.62 ± 0.22 | 0.96 ± 0.19** | 1.30 ± 0.09* | 2.68 ± 0.65 | 1.62 ± 0.38 |
| 20:5n-3 | ND | ND | 0.37 ± 0.25 | ND | ND | ND |

[a]Results are expressed as nmol/mg protein and are means ± SD ($n$ = 5). Values with asterisks in sesamin (+) groups are significantly different from sesamin (–) groups of the same dietary oil group ($P < 0.05$). Values with double asterisk in sesamin (–) groups of EPA and AA groups are significantly different from sesamin (–) groups of the Control groups ($P < 0.05$). See Table 1 and 2 for other abbreviations.

(carnitine acyltransferase I and acyl-CoA dehydrogenase) by sesamin were less than twofold, but those of peroxisomal enzyme (acyl-CoA oxidase) were about fourfold, using 16:0-CoA as a substrate. Several studies have demonstrated that fibric acid derivatives such as clofibrate and bezafibrate enhance peroxisomal β-oxidation (25–27). These compounds are peroxisome proliferators and hypolipidemic drugs, and they affect lipid metabolism and decrease TG and cholesterol levels in serum (28,29). In this study, we demonstrated that sesamin significantly increased peroxisomal β-oxidation enzyme activity; therefore, sesamin may have a function similar to fibrate derivatives. Although this study had not investigated the dose-related effect of sesamin on the enzyme activities, Ashakumary *et al.* (15) reported that sesamin increased hepatic fatty acid oxidation enzyme activity dose-dependently [from 0.1 to 0.5% (w/w) of sesamin in the diet]. The amount of sesamin consumed by the rats in our present study was approximately equal to ingestion of 20 g/d sesamin by an adult human.

The activities of carnitine acyltransferase I, which take place when fatty acids are incorporated into mitochondria, were increased by dietary EPA, but not by dietary AA (Fig. 1). A difference was found between EPA and AA in incorporating into mitochondria. In mitochondrial fatty acid content (Table 4), EPA concentration was increased by dietary EPA, but the AA was not changed by dietary AA. The addition of dietary sesamin to the EPA-supplemented diet significantly decreased the EPA concentration compared to concentrations found with consumption of dietary EPA alone. The activities of peroxisomal acyl-CoA oxidase were significantly increased by dietary EPA or AA, and no difference was noted in either increasing ratio (Fig. 3). In peroxisomal fatty acid concentration (Table 5), dietary EPA increased EPA concentration, and dietary sesamin inhibited it. Dietary AA, however, had no effect on peroxisomal AA concentration. In fatty acid concentration in whole liver (Table 2), EPA and AA concentrations were significantly increased by dietary EPA and AA, respectively, and dietary sesamin decreased them. In liver TG (Table 3), the same results were demonstrated as in the whole liver. However, dietary sesamin reduced AA concentration to about half the amount, and EPA concentration to one-fifth. These results suggested that dietary EPA stimulated mitochondrial and peroxisomal β-oxidation enzyme activities in the liver; furthermore, dietary sesamin promoted β-oxidation of EPA. On the contrary, dietary AA had no effect on mitochondrial and peroxisomal AA concentration, despite the significant increase of AA in liver TG. These results suggested that AA may at first have been incorporated into TG as an AA pool, and excess AA was subsequently degraded *via* β-oxidation mainly in peroxisomes when an excessive amount of AA was administered. Sesamin increased mitochondrial and peroxisomal β-oxidation enzyme activities and reduced fatty acid concentrations in the whole liver, but there were differences between the reduction processes of EPA and AA. In the stimulation of fatty acid β-oxidation by sesamin, EPA may be degraded *via* the β-oxidation pathway instead of esterified into TG and phospholipids. On the other hand, AA concentration in the liver can be maintained by a balance between esterification of AA into TG and peroxisomal β-oxidation.

In conclusion, the present study demonstrated that sesamin increased β-oxidation enzyme activities and reduced hepatic EPA and AA concentrations by the degradation of extra EPA and AA. That is why sesamin regulates the extreme change of n-6/n-3 PUFA ratio. The stimulating effect of sesamin on β-oxidation, however, was more notable with the high intakes of EPA than of AA, and the reduction rate of EPA was higher than AA. In the excessive intake of AA, AA concentration was regulated by the joint effect of sesamin through the esterification into TG and a stimulation of β-oxidation by sesamin. It was demonstrated that sesamin stimulated mitochondrial and peroxisomal fatty acid degradation, and the reduction process of AA was different from EPA in liver.

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# Correlation of Fatty Acid Unsaturation of the Major Liver Mitochondrial Phospholipid Classes in Mammals to Their Maximum Life Span Potential

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**ABSTRACT:** Free radical damage is considered a determinant factor in the rate of aging. Unsaturated fatty acids are the tissue macromolecules that are most sensitive to oxidative damage. Therefore, the presence of low proportions of fatty acid unsaturation is expected in the tissues of long-lived animals. Accordingly, the fatty acid compositions of the major liver mitochondrial phospholipid classes from eight mammals, ranging in maximum life span potential (MLSP) from 3.5 to 46 yr, show that the total number of double bonds is inversely correlated with MLSP in both phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) ($r = 0.757$, $P < 0.03$, and $r = 0.862$, $P < 0.006$, respectively), but not in cardiolipin ($P = 0.323$). This is due not to a low content of unsaturated fatty acids in long-lived animals, but mainly to a redistribution between kinds of fatty acids on PtdCho and PtdEtn, shifting from arachidonic ($r = 0.911$, $P < 0.002$, and $r = 0.681$, $P = 0.05$, respectively), docosahexaenoic ($r = 0.931$ and $r = 0.965$, $P < 0.0001$, respectively) and palmitic ($r = 0.944$ and $r = 0.974$, $P < 0.0001$, respectively) acids to linoleic acid ($r = 0.942$, $P < 0.0001$, for PtdCho; and $r = 0.957$, $P < 0.0001$, for PtdEtn). For cardiolipin, only arachidonic acid showed a significantly inverse correlation with MLSP ($r = 0.904$, $P < 0.002$). This pattern strongly suggests the presence of a species-specific desaturation pathway and deacylation-reacylation cycle in determining the mitochondrial membrane composition, maintaining a low degree of fatty acid unsaturation in long-lived animals.

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Several lines of evidence indicate that mitochondria and oxidative damage can be implicated both in pathological responses and in the aging process (1). The available comparative studies indicate that maximum life span potential (MLSP) is inversely related to mitochondrial free radical production (2,3) and mitochondrial DNA oxidative damage (4,5). Although these very important characteristics are consistent with free radical–oxidative stress theories of aging (6,7), additional factors related to other macromolecules also can lead to a low level of oxidative damage in long- *vs.* short-lived animal species.

Among cellular macromolecules, polyunsaturated fatty acids (PUFA) exhibit the highest sensitivity to oxidative damage. It is accepted that their sensitivity increases as a power function of the number of double bonds per fatty acid molecule. Since both oxygen consumption and reactive oxygen species formation occur predominantly in mitochondrial membranes, a low degree of fatty acid unsaturation in these membranes may be advantageous, in oxidative stress terms, by decreasing their sensitivity to lipid peroxidation. This would also protect other molecules against lipoxidation-derived damage. In line with this, it has been suggested that in long-lived species a low degree of total tissue and mitochondrial fatty acid unsaturation (low double-bond content) is accompanied by a low sensitivity to lipid peroxidation and a low concentration of the lipoxidation-derived adducts malondialdehyde-lysine and $N^{\varepsilon}$-(carboxymethyl)lysine in several tissues and mitochondrial proteins (8–12). Independent experiments have also demonstrated a negative correlation between sensitivity to lipid autoxidation and MLSP in brain and kidney homogenates from different mammalian species (13).

Mitochondria from different mammalian tissues are similar with respect to their phospholipid distribution (14). The major phospholipids, phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn), together account for about 70–80% of total phospholipids; cardiolipin constitutes 10–20% of total mitochondrial phospholipids.

Phospholipids play multiple roles in mitochondria including establishing a permeability barrier, providing the matrix for the assembly and function of a wide variety of catalytic processes, acting as donors in the synthesis of macromolecules, and actively influencing the functional properties of membrane-associated processes. The wide range of processes in which specific involvement of phospholipids has been documented explains the need for diversity in phospholipid structure and fatty acid composition (15).

In this work, the fatty acid compositions of the phospho-

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Abbreviations: AA, arachidonic acid; ACL, average chain length; DBI, double bond index; DHA, docosahexaenoic acid; GC–MS, gas chromatography–mass spectrometry; LA, linoleic acid; MLSP, maximum life span potential; MSE buffer, mannitol, sucrose, and EGTA at pH 7.4; MUFA, monounsaturated fatty acids; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acids; TLC, thin-layer chromatography; UFA, unsaturated fatty acids.

lipids PtdCho, PtdEtn, and cardiolipin from liver mitochondria of eight mammalian species ranging in MLSP from 3.5 to 46 yr were analyzed. The results obtained show that the degree of fatty acid unsaturation is inversely correlated with MLSP in PtdCho and PtdEtn, while there is no relationship between cardiolipin composition and MLSP.

## MATERIALS AND METHODS

*Chemicals.* Phospholipid standards were obtained from Avanti Polar Lipids (Alabaster, AL); fatty acid methyl ester standards, from Sigma (St. Louis, MO); gas chromatography columns, from Teknokroma (Teknokroma SCCL, Barcelona, Spain); silica gel thin-layer chromatography (TLC) plates, from Whatman (Fisher, Cincinnati, OH); other reagents were purchased from Sigma unless otherwise specified. All chemicals were of analytical grade.

*Animals and diets.* All animals, namely, mouse (*Mus musculus, n* = 6), hamster (*Critecus critecus, n* = 4), guinea pig (*Cavia porcellus, n* = 4), rabbit (*Oryctolagus cuniculus*, *n* = 6), sheep (*Ovis aries, n* = 6), pig (*Sus scrofa, n* = 5), cow (*Bos taurus, n* = 4), and horse (*Equus caballus, n* = 5), whose MLSP vary from 3.5 to 46 yr, were adult specimens with an age at 15–30% of their MLSP (equivalent to an age ranging from 0.5 to 6 yr). The recorded values of MLSP (in yr) were: mouse, 3.5; hamster, 4; guinea pig, 8; rabbit, 13; sheep, 20; pig, 27; cow, 30; and horse, 46 (16). The animal care protocols were approved by the University of Lleida Animal Experimentation Ethics Committee. Mice, hamsters, guinea pigs, and rabbits were killed by decapitation. Sheep, pigs, cows, and horses (farm animals) were sacrificed at the abattoir. Samples of diet administered during the adult life of the animals were obtained at the sacrifice.

*Mitochondrial isolation.* Tissue samples were taken from the main hepatic lobe and were immediately processed. Mitochondrial fractions were isolated by standard methods of homogenization and differential centrifugation as previously described (8). Liver samples (2–3 g) were briefly and gently homogenized with a loose fitting pestle hand-operated glass-glass homogenizer in 10 mL of MSE buffer (225 mM mannitol, 75 mM sucrose and 1 mM EGTA, pH 7.4) containing 5 mg of nagarse, a bacterial proteinase (EC 3.4.21.14) from Fluka (product no. 82518, Sigma Co.), and 25 mg of albumin. After standing for 1 min, 25 mL of additional MSE buffer containing 25 mg of albumin was added, and homogenization was gently performed again with a tighter-fitting pestle. The homogenates were centrifuged for 3 min at 1,500 × *g* (5°C) in a RC5C Sorvall centrifuge. The supernatants were centrifuged 10 min at 9,800 × *g,* the pellets were resuspended, and the procedure was repeated two more times. All procedures were performed at 0–4°C.

The purity of the mitochondrial fractions was tested by determining 5′-nucleotidase (as marker for plasma membrane), peroxidase (for peroxisomes), glucose-6-phosphatase (for endoplasmic reticulum), acid phosphatase (for lysosomes), and cytochrome c oxidase (for mitochondria) activities according to published methods (17). Based on the specific activities of the different markers, our mitochondrial preparations contains approximately 1% of nonmitochondrial subcellular membranes.

*Lipid extraction and phospholipid classes separation.* Lipids from mitochondria were extracted into chloroform/methanol (2:1 vol/vol) by the method of Folch *et al.* (18) in the presence of 0.01% butylated hydroxytoluene. The phospholipid classes were separated by TLC on silica gel plates. Development with *n*-hexane/1,2-dichloroethane/methanol/formic acid (16:14:4:1 by vol) was performed, followed by 1,2-dichloroethane solvent system (19). Fractions were made visible by spraying with 0.02% 8-anilino-1-naphthalenesulfonic acid in ethanol. The bands on the plates corresponding to major mitochondrial phospholipids (PtdCho, PtdEtn, and cardiolipin), identified by comparison with authentic standards, were scraped, transferred to screw-capped tubes, and transesterified.

To quantify the percentages of major phospholipid classes, lipid samples were separated on Silica gel 60A LK6D TLC plates (Whatman, Clifton, NJ), by using the solvent system described above. Separated lipid fractions were detected using a 10% cupric sulfate in 8% phosphoric acid solution, followed by charring at 160°C for 20 min, and were quantified by scanning densitometry using a Shimadzu CS-9001PC dual-wavelength flying spot scanner (Shimadzu Europe GmbH, Duisburg, Germany).

*Fatty acid analysis.* Mitochondrial phospholipid fatty acids were transesterified in 2.5 mL of 5% methanolic HCl at 75°C for 90 min. The resulting methyl esters were extracted by adding 2.5 mL *n*-pentane and 1 mL saturated NaCl. The *n*-pentane phase was separated and evaporated under $N_2$, and the fatty acid methyl esters were redissolved in 100 μL of carbon disulfide. One microliter was submitted to gas chromatography–mass spectrometry (GC–MS) analysis. GC separation was performed on an SP2330 capillary column (30 m × 0.25 mm × 0.20 μm) in a Hewlett-Packard 6890 Series II gas chromatograph (Hewlett-Packard, S.A., Barcelona, Spain). A Hewlett-Packard 5973 mass spectrometer was used as detector in the electron-impact mode. The injection port was maintained at 220°C, and the detector at 250°C; the temperature program was 2 min at 100°C, then 10°C/min to 200°C, then 5°C/min to 240°C, and finally hold at 240°C for 10 min. Identification of methyl esters was made by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol%.

*Calculations and statistics.* The average chain length is calculated as ACL= $[(\Sigma\ \%\text{Total}_{16} \times 16) + \cdots + (\Sigma\ \%\text{Total}_{n} \times n)]/100$ (*n* = carbon atom number); the double bond index as DBI = (Σ %Monoenoic × 1) + (Σ %Dienoic × 2) + (Σ %Trienoic × 3) + (Σ %Tetraenoic × 4) + (Σ %Pentaenoic × 5) + (Σ %Hexaenoic × 6); saturated fatty acids as SFA = Σ % (16:0 + 18:0); unsaturated fatty acids as UFA = Σ % (16:1 + 18:1 + 18:2 + 18:3 + 20:3 + 20:4 + 22:6); monounsaturated fatty acids as MUFA = Σ % (16:1 + 18:1); polyunsaturated fatty acids as PUFA = Σ % (18:2 + 18:3 + 20:3 + 20:4 + 22:6); n-3 polyunsaturated fatty acids as PUFAn-3 = Σ % (18:3 + 22:6); and finally, n-6 polyunsaturated fatty acids as PUFAn-6 = Σ% (18:2 + 20:3 + 20:4).

Regression equations were obtained by nonlinear regression analyses with the curve estimation statistic by SPSS/PC software for Windows (SPSS, Chicago, IL). These regressions were determined and tested for significance using the mean values for each species. The 0.05 level was selected as the point of minimal statistical significance. Values in tables and figures are expressed as mean ± SEM.

## RESULTS

The fatty acid composition of the diets of the different animal species showed that the DBI of the dietary fats was not correlated with MLSP (Table 1). From analytical TLC, mitochondrial phospholipid distribution was calculated. In all the mammalian species studied in this work, the major phospholipid was PtdCho, in the range of 40–50%, followed by the PtdEtn fraction in the range of 23–35%. Cardiolipin was present in the range of 14–21%. Phospholipid distribution among species was not correlated with MLSP (data not shown). The complete fatty acid composition and indexes related to the degree of unsaturation, the chain length, or to the main fatty acid types or series of the major liver mitochondrial phospholipids, namely, PtdCho, PtdEtn, and cardiolipin, are shown in Tables 2, 3, and 4. Summaries of correlations between MLSP and fatty acid composition or fatty acid indexes of different phospholipid classes from liver mitochondria in the mammalian species are included in Tables 2, 3 and 4.

Neither the ACL nor the percentage of total MUFA shows statistically significant correlation with MLSP in any of the phospholipid classes analyzed. In contrast, total SFA and UFA contents show highly significant correlations with MLSP, in a negative and positive way, respectively, for PtdCho and PtdEtn, whereas no relationship was observed for the cardiolipin fraction. The decrease in the percentage of SFA with increases in MLSP is due to the negative correlation of palmitic acid (16:0) with MLSP in both PtdCho and PtdEtn (Figs. 1A, 1B); in contrast, no significant correlations were observed with cardiolipin. In the PtdCho and PtdEtn fractions docosahexaenoic acid (22:6n-3, DHA) was negatively correlated with maximum longevity, whereas the contrary was true

**TABLE 1**
**Fatty Acid Composition (mol%) and Double Bond Index (DBI) of the Dietary Fats (*n* = 3)**

| | Mouse | Hamster | Guinea pig | Rabbit | Sheep | Pig | Cow | Horse |
|---|---|---|---|---|---|---|---|---|
| 16:0 | 21 | 26 | 19 | 18 | 22 | 31 | 21 | 21 |
| 16:1n-7 | 0.5 | 4 | 0.5 | 0.5 | 2 | 3.5 | 3 | 2 |
| 18:0 | 9 | 9 | 3 | 3 | 8 | 9 | 7 | 8 |
| 18:1n-9 | 29 | 22 | 24 | 23.5 | 29 | 35 | 33 | 26 |
| 18:2n-6 | 39 | 36 | 49 | 50 | 32 | 25 | 35 | 41 |
| 18:3n-3 | 1.5 | 3 | 4.5 | 5 | 7 | 6 | 1 | 2 |
| DBI[a] | 112 | 107 | 136 | 139 | 116 | 107.5 | 109 | 116 |

[a]DBI = (Σ % Monoenoic × 1) + (Σ % Dienoic × 2) + (Σ % Trienoic × 3).

**TABLE 2**
**Fatty Acid Composition (mol%) and General Indexes Related to Membrane Fatty Acid Composition of Liver Mitochondrial Phosphatidylcholine from Mammalian Species[a]**

| | Mouse | Hamster | Guinea pig | Rabbit | Sheep | Pig | Cow | Horse | |
|---|---|---|---|---|---|---|---|---|---|
| | 3.5 | 4 | 8 | 13 | 20 | 27 | 30 | 46 | $r^b$ |
| 16:0 | 33.97 ± 1.03 | 33.11 ± 0.62 | 26.90 ± 1.00 | 24.59 ± 0.81 | 18.49 ± 0.47 | 20.10 ± 1.13 | 19.52 ± 0.85 | 13.39 ± 0.50 | 0.944[d,g] |
| 16:1n-7 | 0.21 ± 0.01 | 0.21 ± 0.001 | 0.59 ± 0.06 | 0.75 ± 0.12 | 1.84 ± 0.54 | 1.40 ± 0.27 | 0.45 ± 0.007 | 0.47 ± 0.12 | 0.225 |
| 18:0 | 7.92 ± 0.53 | 7.81 ± 0.42 | 18.22 ± 1.03 | 11.25 ± 0.67 | 18.04 ± 0.25 | 19.22 ± 0.46 | 17.30 ± 0.41 | 17.62 ± 1.08 | –0.850[d,f] |
| 18:1n-9 | 15.46 ± 0.71 | 19.07 ± 1.31 | 16.57 ± 0.97 | 15.27 ± 0.81 | 18.69 ± 0.38 | 14.96 ± 0.62 | 15.72 ± 0.26 | 14.31 ± 0.67 | –0.518 |
| 18:2n-6 | 17.93 ± 0.45 | 21.72 ± 2.15 | 27.67 ± 0.59 | 36.18 ± 2.28 | 32.90 ± 0.48 | 32.77 ± 0.48 | 41.18 ± 1.20 | 52.58 ± 1.24 | 0.942[e,g] |
| 18:3n-3 | 0.17 ± 0.01 | 0.14 ± 0.006 | 0.47 ± 0.46 | 0.58 ± 0.10 | 0.56 ± 0.01 | 0.48 ± 0.01 | 0.08 ± 0.001 | 0.14 ± 0.03 | –0.187 |
| 20:3n-6 | 2.12 ± 0.03 | 2.24 ± 0.11 | 0.25 ± 0.02 | 0.46 ± 0.11 | 0.31 ± 0.006 | 1.22 ± 0.30 | 0.07 ± 0.001 | 0.12 ± 0.02 | 0.852[d,f] |
| 20:4n-6 | 14.38 ± 0.81 | 11.13 ± 1.43 | 8.71 ± 0.89 | 10.58 ± 0.61 | 8.62 ± 0.28 | 8.93 ± 0.74 | 5.45 ± 0.09 | 1.20 ± 0.04 | –0.911[c,f] |
| 22:6n-3 | 7.79 ± 0.81 | 4.54 ± 0.86 | 0.57 ± 0.11 | 0.30 ± 0.02 | 0.50 ± 0.01 | 0.85 ± 0.03 | 0.19 ± 0.003 | 0.12 ± 0.02 | 0.931[d,g] |
| ACL | 17.95 ± 0.06 | 17.78 ± 0.05 | 17.65 ± 0.003 | 17.72 ± 0.01 | 17.79 ± 0.007 | 17.80 ± 0.03 | 17.71 ± 0.01 | 17.75 ± 0.01 | –0.246 |
| SFA | 41.89 ± 0.50 | 40.93 ± 1.05 | 45.13 ± 0.33 | 35.85 ± 1.39 | 36.54 ± 0.40 | 39.33 ± 1.58 | 36.82 ± 1.25 | 31.02 ± 0.60 | –0.804 |
| UFA | 58.10 ± 0.50 | 59.06 ± 1.05 | 54.86 ± 0.33 | 64.14 ± 1.39 | 63.45 ± 0.40 | 60.66 ± 1.58 | 63.17 ± 1.25 | 68.97 ± 0.60 | 0.804 |
| MUFA | 15.68 ± 0.69 | 19.28 ± 1.31 | 17.17 ± 0.92 | 16.02 ± 0.79 | 20.53 ± 0.54 | 16.37 ± 0.64 | 16.17 ± 0.27 | 14.79 ± 0.60 | –0.397 |
| PUFA | 42.41 ± 1.19 | 39.78 ± 0.26 | 37.69 ± 1.05 | 48.12 ± 2.02 | 42.92 ± 0.29 | 44.28 ± 1.40 | 46.99 ± 1.21 | 54.18 ± 1.18 | 0.820 |
| PUFAn-3 | 7.97 ± 0.82 | 4.68 ± 0.87 | 1.05 ± 0.32 | 0.88 ± 0.11 | 1.07 ± 0.02 | 1.34 ± 0.05 | 0.28 ± 0.004 | 0.27 ± 0.05 | 0.933[d,g] |
| PUFAn-6 | 34.44 ± 0.43 | 35.09 ± 0.61 | 36.64 ± 0.78 | 47.23 ± 1.93 | 41.85 ± 0.28 | 42.93 ± 1.44 | 46.71 ± 1.21 | 53.91 ± 1.20 | 0.900[e,f] |

[a]The maximum life span potential (MLSP, in years) of each mammal is indicated under the species name in the column headings.

[b]For all variables, statistical significance intergroups was $P < 0.0001$, $r$, nonlinear regression coefficient vs. MLSP (for each regression equation the degrees of freedom were 6), (c) $y = a + bx$; (d) $y = a + b/x$; (e) $y = a \cdot x^b$; (f) $P < 0.01$; (g) $P < 0.001$. Abbreviations: ACL, average chain length; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PUFAn-3, polyunsaturated fatty acids n-3 series; PUFAn-6, polyunsaturated fatty acids n-6 series. For more information, see the Materials and Methods section.

**TABLE 3**
**Fatty Acid Composition (mol%) and General Indexes Related to Membrane Fatty Acid Composition of Liver Mitochondrial Phosphatidylethanolamine from Mammalian Species[a]**

| | Mouse 3.5 | Hamster 4 | Guinea pig 8 | Rabbit 13 | Sheep 20 | Pig 27 | Cow 30 | Horse 46 | *r*[b] |
|---|---|---|---|---|---|---|---|---|---|
| 16:0 | 22.80 ± 0.51 | 18.09 ± 1.09 | 11.08 ± 0.96 | 10.88 ± 0.13 | 8.11 ± 0.20 | 7.05 ± 0.10 | 8.08 ± 0.55 | 3.89 ± 0.45 | 0.974[d,f] |
| 16:1n-7 | 0.27 ± 0.01 | 2.35 ± 0.49 | 0.37 ± 0.05 | 0.17 ± 0.01 | 1.03 ± 0.03 | 0.84 ± 0.01 | 0.33 ± 0.01 | 0.30 ± 0.02 | –0.327 |
| 18:0 | 14.81 ± 0.43 | 17.29 ± 1.28 | 26.51 ± 0.44 | 23.99 ± 0.75 | 20.23 ± 0.53 | 24.21 ± 0.64 | 24.26 ± 0.13 | 18.90 ± 0.49 | 0.181 |
| 18:1n-9 | 12.47 ± 0.75 | 14.93 ± 1.09 | 23.62 ± 0.47 | 17.11 ± 0.17 | 18.20 ± 0.35 | 20.64 ± 0.78 | 17.85 ± 0.65 | 22.02 ± 0.35 | 0.533 |
| 18:2n-6 | 8.52 ± 0.17 | 11.45 ± 0.63 | 21.25 ± 0.51 | 30.52 ± 0.15 | 24.96 ± 0.77 | 32.01 ± 0.63 | 39.54 ± 0.65 | 49.57 ± 1.32 | 0.957[e,f] |
| 18:3n-3 | 0.24 ± 0.12 | 0.49 ± 0.22 | 0.33 ± 0.03 | 1.05 ± 0.01 | 0.22 ± 0.03 | 0.69 ± 0.03 | 0.14 ± 0.004 | 0.02 ± 0.005 | –0.382 |
| 20:3n-6 | 0.95 ± 0.01 | 1.22 ± 0.07 | 0.86 ± 0.25 | 0.17 ± 0.01 | 0.26 ± 0.04 | 0.91 ± 0.02 | 0.16 ± 0.02 | 0.02 ± 0.005 | 0.747 |
| 20:4n-6 | 20.60 ± 0.50 | 20.11 ± 0.55 | 14.02 ± 0.99 | 15.02 ± 0.80 | 26.06 ± 0.89 | 12.73 ± 0.71 | 9.42 ± 0.63 | 5.18 ± 0.10 | –0.681 |
| 22:6n-3 | 19.30 ± 0.74 | 14.01 ± 0.64 | 1.93 ± 0.31 | 1.04 ± 0.10 | 0.88 ± 0.04 | 0.88 ± 0.01 | 0.18 ± 0.01 | 0.07 ± 0.01 | 0.965[d,f] |
| ACL | 18.74 ± 0.04 | 18.57 ± 0.005 | 18.14 ± 0.01 | 18.12 ± 0.02 | 18.37 ± 0.02 | 18.15 ± 0.01 | 18.03 ± 0.02 | 18.02 ± 0.007 | –0.714 |
| SFA | 37.61 ± 0.53 | 35.39 ± 0.19 | 37.59 ± 0.54 | 34.88 ± 0.88 | 28.35 ± 0.62 | 31.26 ± 0.59 | 32.35 ± 0.55 | 22.80 ± 0.90 | –0.897 |
| UFA | 62.38 ± 0.53 | 64.60 ± 0.19 | 62.40 ± 0.54 | 65.11 ± 0.88 | 71.64 ± 0.62 | 68.73 ± 0.59 | 67.64 ± 0.55 | 77.19 ± 0.90 | 0.897 |
| MUFA | 12.75 ± 0.74 | 17.29 ± 0.60 | 24.00 ± 0.52 | 17.29 ± 0.15 | 19.24 ± 0.38 | 21.48 ± 0.78 | 18.18 ± 0.67 | 22.32 ± 0.37 | 0.485 |
| PUFA | 49.63 ± 1.15 | 47.31 ± 0.79 | 38.40 ± 0.26 | 47.85 ± 0.72 | 52.40 ± 0.89 | 47.24 ± 1.09 | 49.45 ± 0.89 | 54.86 ± 1.26 | 0.582 |
| PUFAn-3 | 19.54 ± 0.61 | 14.51 ± 0.86 | 2.27 ± 0.32 | 2.10 ± 0.08 | 1.11 ± 0.06 | 1.57 ± 0.03 | 0.32 ± 0.02 | 0.09 ± 0.01 | 0.967[d,f] |
| PUFAn-6 | 30.08 ± 0.66 | 32.80 ± 0.06 | 36.13 ± 0.26 | 45.72 ± 0.64 | 51.29 ± 0.90 | 45.66 ± 1.11 | 49.13 ± 0.90 | 54.77 ± 1.25 | 0.956[e,f] |

[a]For footnotes and abbreviations see Table 2.

**TABLE 4**
**Fatty Acid Composition (mol%) and General Indexes Related to Membrane Fatty Acid Composition of Liver Mitochondrial Cardiolipin from Mammalian Species[a]**

| | Mouse 3.5 | Hamster 4 | Guinea pig 8 | Rabbit 13 | Sheep 20 | Pig 27 | Cow 30 | Horse 46 | *r*[b] |
|---|---|---|---|---|---|---|---|---|---|
| 16:0 | 9.68 ± 0.63 | 10.90 ± 0.29 | 11.39 ± 0.63 | 6.59 ± 0.50 | 15.53 ± 1.35 | 6.04 ± 0.27 | 14.32 ± 1.76 | 18.22 ± 0.32 | 0.544 |
| 16:1n-7 | 5.12 ± 0.64 | 3.36 ± 0.04 | 1.89 ± 0.05 | 1.16 ± 0.22 | 2.87 ± 0.25 | 1.18 ± 0.05 | 0.48 ± 0.08 | 0.61 ± 0.11 | –0.876[d,f] |
| 18:0 | 5.32 ± 0.38 | 4.68 ± 0.49 | 8.06 ± 0.22 | 4.21 ± 0.43 | 8.68 ± 0.94 | 4.31 ± 0.40 | 7.13 ± 0.96 | 2.08 ± 0.13 | –0.372 |
| 18:1n-9 | 19.66 ± 0.37 | 17.58 ± 0.51 | 14.67 ± 0.41 | 8.84 ± 0.39 | 11.92 ± 0.89 | 16.86 ± 0.75 | 11.96 ± 1.38 | 22.97 ± 0.56 | 0.256 |
| 18:2n-6 | 52.60 ± 2.17 | 57.25 ± 0.45 | 59.27 ± 0.45 | 68.45 ± 1.91 | 52.94 ± 2.54 | 62.16 ± 0.75 | 59.50 ± 2.90 | 55.84 ± 0.49 | –0.000 |
| 18:3n-3 | 3.04 ± 0.53 | 3.61 ± 0.13 | 2.29 ± 1.11 | 9.01 ± 0.39 | 6.26 ± 1.08 | 9.16 ± 1.35 | 5.73 ± 0.88 | 0.15 ± 0.004 | –0.000 |
| 20:3n-6 | 0.95 ± 0.01 | 1.22 ± 0.07 | 0.86 ± 0.25 | 0.17 ± 0.01 | 0.26 ± 0.04 | 0.91 ± 0.02 | 0.16 ± 0.02 | 0.02 ± 0.005 | 0.747 |
| 20:4n-6 | 4.54 ± 0.46 | 2.59 ± 0.16 | 1.68 ± 0.04 | 1.71 ± 0.05 | 1.77 ± 0.21 | 0.26 ± 0.02 | 0.85 ± 0.1 | 0.10 ± 0.008 | 0.904[d,f] |
| ACL | 17.79 ± 0.07 | 17.76 ± 0.01 | 17.76 ± 0.01 | 17.87 ± 0.01 | 17.66 ± 0.03 | 17.86 ± 0.01 | 17.72 ± 0.03 | 17.62 ± 0.01 | –0.509 |
| SFA | 15.01 ± 0.94 | 15.58 ± 0.78 | 19.45 ± 0.71 | 10.80 ± 0.94 | 24.21 ± 2.27 | 10.36 ± 0.36 | 21.45 ± 1.39 | 20.30 ± 0.18 | 0.296 |
| UFA | 84.98 ± 0.94 | 84.41 ± 0.78 | 80.54 ± 0.71 | 89.19 ± 0.94 | 75.78 ± 2.27 | 89.63 ± 0.36 | 78.54 ± 1.39 | 79.69 ± 0.18 | –0.296 |
| MUFA | 24.78 ± 0.90 | 20.94 ± 0.49 | 16.57 ± 0.46 | 10.01 ± 0.60 | 14.79 ± 0.67 | 18.04 ± 0.80 | 12.45 ± 1.43 | 23.58 ± 0.66 | 0.000 |
| PUFA | 60.20 ± 1.77 | 63.46 ± 0.60 | 63.96 ± 1.02 | 79.18 ± 1.54 | 60.98 ± 2.34 | 71.59 ± 0.80 | 66.09 ± 2.44 | 56.10 ± 0.49 | –0.202 |
| PUFAn-3 | 3.04 ± 0.53 | 3.61 ± 0.13 | 2.29 ± 0.06 | 9.01 ± 0.39 | 6.26 ± 1.08 | 9.16 ± 1.35 | 5.73 ± 0.88 | 0.15 ± 0.01 | –0.094 |
| PUFAn-6 | 57.15 ± 2.15 | 59.85 ± 0.52 | 61.66 ± 1.07 | 70.16 ± 1.92 | 54.71 ± 2.49 | 62.43 ± 0.73 | 60.35 ± 3.00 | 55.94 ± 0.49 | –0.246 |

[a]For footnotes and abbreviations see Table 2.

for linoleic acid (18:2n-6, LA) (Figs. 1A, 1B). Cardiolipin also showed a significant negative correlation with MLSP in the arachidonic acid (20:4n-6, AA) content, while LA was not affected. As result of this fatty acid redistribution, the total content of double bonds showed significant negative correlations with MLSP in PtdCho and PtdEtn, and was not affected in cardiolipin (Fig. 2).

## DISCUSSION

In agreement with previous comparative studies of mitochondria in mammals and birds (8–12) we found in this investigation that the number of fatty acid double bonds of the major phospholipid classes from liver mitochondria is negatively correlated with MLSP, i.e., the fatty acids of mitochondrial PtdCho and PtdEtn of long-lived mammals have a lower degree of unsaturation than those of short-lived ones. This is due to the redistribution between components of the polyunsaturated n-3 and n-6 fatty acid series, shifting from the highly unsaturated DHA and AA in short-lived animals to the less unsaturated LA in long-lived ones. This leads to a low DBI in the mitochondrial PtdCho and PtdEtn of long-lived animals. Further, since ACL may be seriously altered by the redistribution between DHA/AA and LA fatty acids, the decline in 16:0 and rise in 18:0 with increases in MLSP may be considered as an adaptation to maintain this parameter. In contrast, the cardiolipin fatty acid composition and fatty acid indexes show that only AA and palmitoleic acid (16:1) shows a signifi-

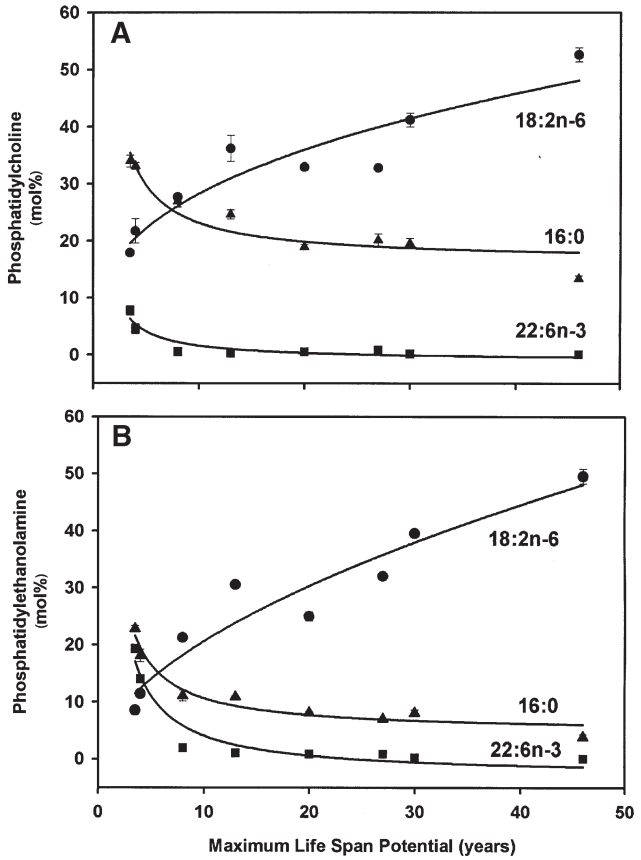


**FIG. 1.** Relationship between maximum life span (MLSP) and mol% of linoleic acid (18:2n-6), mol% of docosahexaenoic acid (22:6n-3), and mol% of palmitic acid (16:0), in liver mitochondrial phosphatidylcholine (A) and phosphatidylethanolamine (B). Regression equations: In A, for phosphatidylcholine, (i) 18:2n-6 (mol%) = 12.65 · MLSP $^{0.348}$, $r = 0.93$; $P < 0.0008$; (ii) 22:6n-3 (mol%) = –0.931 + (25.35/MLSP), $r = 0.93$, $P < 0.0008$; (iii) 16:0 (mol%) = 16.53 + (66.17/MLSP), $r = 0.94$, $P < 0.0004$. In B, for phosphatidylethanolamine, (i) 18:2n-6 (mol%) = 5.72 · MLSP$^{0.555}$, $r = 0.96$; $P < 0.0002$; (ii) 22:6n-3 (mol%) = –2.91 + (70.05/MLSP), $r = 0.96$, $P < 0.0001$; (iii) 16:0 (mol%) = 4.76 + (58.93/MLSP), $r = 0.97$, $P < 0.0001$. Values are means ± SEM.

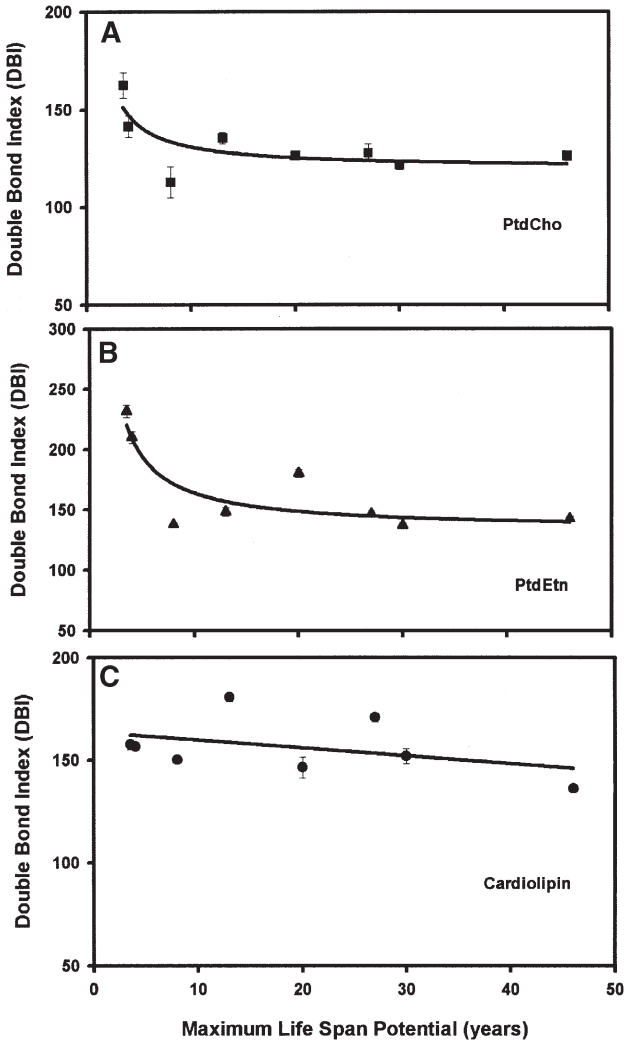


**FIG. 2.** Relationship between maximum life span potential (MLSP) and double bond index (DBI) of liver mitochondrial phosphatidylcholine (PtdCho, A), phosphatidylethanolamine (PtdEtn, B), and cardiolipin (C) in mammalian species. Regression equations: (A) $DBI_{PtdCho} = 119.81 + (110.81/MLSP)$, $r = 0.75$, $P < 0.03$; (B) $DBI_{PtdEtn} = 133.25 + (304.95/MLSP)$, $r = 0.86$, $P < 0.006$; (C) $DBI_{Cardiolipin} = 163.53 - 0.38 \cdot MLSP$, $r = 0.40$, $P = 0.323$. Values are means ± SEM.

cant negative correlation with MLSP. Similarly, previous studies in different tissues, subcellular fractions, or species usually showed that the low degree of fatty acid unsaturation of long-lived animals is obtained by analogous redistributions between types of PUFA without decreasing the total PUFA or UFA content (8–12). These results were later confirmed by another independent laboratory (20). While this had never been described as a function of MLSP, two previous comparative reports exist in mammals in relation to body size (21,22). In the first one (21), DHA decreased sharply as body size increased in the order mouse–rat–rabbit–man–whale, which is also an order of increasing MLSP although the authors did not comment on this. In the second report (22), DBI was found to correlate negatively with body size in the heart, skeletal muscle, and kidney cortex of five species, mouse–rat–rabbit–sheep–cattle, whereas in the liver the negative trend did not reach statistical significance and in the brain a low DBI was observed only in cattle. The fatty acids mainly responsible for these differences were again DHA and AA, which decreased as body size increased, and LA, which showed progressively larger levels in animals of larger size.

These results suggest that cellular and/or subcellular mechanisms exist to bring about the observed distinctive distribution of acyl groups in the mitochondrial membrane phospholipids among different mammals. Two mechanisms may be implied in determining the fatty acid profile observed in the major liver mitochondrial phospholipid classes: (i) the fatty acid desaturation pathway and (ii) the deacylation–reacylation cycle. With respect to (i), these results might be explained by the metabolic characteristics of the recently postulated mitochondrial desaturation pathways (23), which would make the n-6 and n-3 fatty acids available *in situ* to phospholipid acyltransferases in order to remodel the phospholipid

acyl groups and lead to the postulation of a constitutively decreased species-specific desaturase activity in the long-lived animals. Furthermore, since fatty acid composition differs between the zwitterionic (PtdCho and PtdEtn) and anionic (cardiolipin) phospholipids, perhaps there is differential remodeling activity among mammals. With respect to (ii), in order to maintain the appropriate molecular species composition of the different phospholipids, the deacylation–reacylation of mitochondrial phospholipids based on the presence of both phospholipase $A_2$ and acyl-CoA:lysophospholipid acyltransferase activities may be species-specific and rate-limiting for the molecular remodeling of PtdCho, PtdEtn, and cardiolipin in liver mitochondria. The conservation among mammals of the cardiolipin DBI probably reflects its importance as modulator of the activity of a number of mitochondrial membrane enzymes involved in the oxidative generation of ATP (24). Furthermore, special attention must be addressed to the decreased AA content in cardiolipin with MLSP. A recent report demonstrates that AA interaction with the mitochondrial electron transport chain promotes generation of reactive oxygen species (25). The lower AA content in long-lived species is consistent with the lower rate of free radical production in these animal species (26).

The presence of constitutively whole membrane remodeling activities in long-lived animals can explain why feeding corn oil (rich in LA) to primates (marmoset monkeys, *Callithrix jacchus*) increases mainly LA (to 30% of total fatty acids) instead of AA (to only 10% of total) in their tissues (27), whereas in short-lived rodents dietary LA leads to strong increases in AA. Similarly (28), members of human monastic communities that chronically consume only corn oil as the main dietary fat source (67% rich in LA) have lipid profiles with around 30% LA but only 9% AA in their high density lipoproteins (29). Moreover, the diets of all the animals studied contain LA and linolenic acid (18:3n-3), the precursors in the n-3 and n-6 series, but do not contain AA and/or DHA. Nevertheless, the DHA levels reached 8 and 20% in mice, but were only 0.12 and 0.07% in horses, for PtdCho and PtdEtn, respectively. In any case, the double-bond content of the diets was not correlated to MLSP.

Concerning the physiological meaning of the decrease in the degree of unsaturation in long-lived animals, there are various possibilities. Other authors have proposed (22) that mammals of large body size have a low DBI to decrease their metabolic rates, because the lower the DBI of a membrane, the lower is its permeability to ions (ion pumping is one of the main determinants of metabolic rate). The permeability to $Na^+$ and $K^+$ in liver hepatocytes (30) and to $H^+$ in inner mitochondrial membranes (31) also correlates negatively with body size. Although this possibility may be true for mammals of different sizes, it cannot explain the low DBI from birds because they have a metabolic rate similar to or higher than that of mammals of similar size. But the studied birds and the mammals of large body size share a common trait; they have a long life span. We thus hypothesized that the low phospholipid DBI of long-lived homeotherms (mammals or birds) could have been selected during evolution to decrease membrane lipid peroxidation and its peroxidative consequences to other cellular macromolecules including proteins (32–34) and DNA (35). In agreement with this, a low degree of total tissue and mitochondrial fatty acid unsaturation, accompanied by a low sensitivity to lipid peroxidation and a low concentration of the lipoxidation-derived adducts malondialdehydelysine and $N^{\varepsilon}$-carboxymethyllysine in tissue and mitochondrial proteins have been described (8–12). A negative correlation between sensitivity to lipid autoxidation and MLSP in brain and kidney homogenates from different mammalian species has also been described (13). Furthermore, during aging, a modification of fatty acid unsaturation and oxidative damage in membranes occurs, which is prevented by food restriction (36–41). Thus, the low fatty acid unsaturation of long-lived mammals of large body size would protect their tissues against oxidative damage while at the same time it could contribute to lower their metabolic rates. But the more general relationship in all homeotherms is that between DBI and MLSP, not between DBI and metabolic rate, because the low DBI of birds does not fit with their very high metabolic rates. Undoubtedly, other factors must be responsible for the high metabolic activity of these last animals.

The influence of fatty acid unsaturation on the transition temperature, and hence in the membrane fluidity, is well known (42). Whereas strong increases in lipid fluidity are observed after introduction of the first double bonds to a saturated fatty acid, progressively smaller effects are observed after the introduction of additional double bounds. Thus, the change in PUFA composition from the highly unsaturated AA and DHA to the less unsaturated LA found in the present work in PtdCho and PtdEtn from long-lived animals may allow them to decrease their double bond content without greatly changing their membrane fluidity. The membrane fluidity is a parameter needed for a proper function of mitochondrial membrane proteins such as enzymes, ion pumps, or electron carriers (43,44).

Thus, it may be proposed that, during evolution, a low degree of fatty acid unsaturation in liver mitochondria may have been selected for in long-lived mammals in order to protect their tissues against oxidative damage while maintaining an appropriate environment for membrane function.

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# Cloning and Expression of Group IB Phospholipase $A_2$ Isoforms in the Red Sea Bream, *Pagrus major*[1]

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**ABSTRACT:** Two cDNA encoding red sea bream DE-1 and DE-2 phospholipases $A_2$ ($PLA_2$) were cloned from the hepatopancreas of red sea bream, *Pagrus (Chrysophrys) major*. The cDNA of DE-1 $PLA_2$ encoded a mature protein of 125 amino acid residues with an apparent signal peptide of 20 residues and propeptide of 5 residues, and that of DE-2 $PLA_2$, a mature protein of 126 amino acid residues with an apparent signal peptide of 17 residues and propeptide of 6 residues. Comparison of the predicted amino acid sequences for mature DE-1 and DE-2 $PLA_2$ showed that both proteins contain 14 cysteines including Cys 11 and 77 and a pancreatic loop, which are commonly conserved in group IB $PLA_2$; however, the identity in amino acid sequence between DE-1 and DE-2 $PLA_2$ was low (47%). A previous report concerning the cDNA cloning of red sea bream gill G-3 $PLA_2$ and the present results represent the first cloning and sequencing of three distinct isoforms of group IB $PLA_2$ in a single fish species, red sea bream. Reverse transcription-polymerase chain reaction analysis showed that DE-1 $PLA_2$ mRNA was expressed in the hepatopancreas, pyloric ceca, intestine, spleen, gonad, stomach, and kidney, whereas gill G-3 $PLA_2$ mRNA was expressed only in the gills and gonad. The expression of DE-2 $PLA_2$ mRNA was detected in all of the tissues analyzed. These results indicate that three distinct isoforms of group IB $PLA_2$, DE-1 and DE-2 $PLA_2$ in hepatopanceas and gill G-3 $PLA_2$, are expressed in a tissue-specific manner in red sea bream.

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Phospholipase $A_2$ (phosphatide 2-acyl hydrolase, EC 3.1.1.4) ($PLA_2$) comprises a diverse family of enzymes that catalyze the hydrolysis of a fatty-acyl ester bond at the *sn*-2 position of glycerophospholipids to liberate free fatty acids and lysophospholipids. $PLA_2$ plays a central role in cellular processes as diverse as phospholipid digestion and metabolism, host defense, and signal transduction (1–3). Secretory $PLA_2$ ($sPLA_2$) are $Ca^{2+}$-dependent, low molecular mass enzymes (13–18 kDa) with five to eight disulfide bridges and a broad specificity for the phospholipid headgroup and fatty acids (3). At present, they are classified into six groups, I, II, III, V, IX and X, depending on the primary structure characterized by the number and positions of cysteine residues (2). Group IB $PLA_2$ has been referred to as pancreatic-type $PLA_2$ because it is abundant in the pancreatic juice in many mammals (4,5). However, group IB $PLA_2$ mRNA and protein have been recently found in nondigestive organs, including the spleen, lung, kidney, and ovary (6–16). The discovery of specific receptors for group IB $PLA_2$ from various mammalian tissues and cells has led to the notion that group IB $PLA_2$ evokes various biological responses by binding to the receptor in addition to its digestive function (17–19). This implies a physiological role for group IB $PLA_2$ in nondigestive tissues and cells, in addition to the function of digestive lipolysis in the digestive system.

Compared with the amount of information available on mammalian group IB $PLA_2$, little is known about the primary structure and enzymology of fish $PLA_2$. $PLA_2$ has been partially purified or purified from rainbow trout *(Salmo gairdneri)* liver (20,21) and cod *(Gadus morhua)* muscle (22,23), but its primary structure has not yet been determined. Zambonino Infante and Cahu (24) recently obtained a cDNA clone encoding group IB $PLA_2$ from seabass *(Dicentrarchus labrax)* and found that the mRNA level of $PLA_2$ in seabass larvae increased in culture with diets containing higher lipid levels. Previously, we detected $PLA_2$ in the pancreatic acinar cells and secretory materials of certain epithelial cells in the pyloric ceca of red sea bream, by immunohistochemical analysis using an antiserum against *Naja naja* venom $PLA_2$ (25). We have further purified six low molecular weight $Ca^{2+}$-dependent $PLA_2$ from the pyloric ceca (26), hepatopancreas (27,28) and gills (29), and classified hepatopancreas DE-1 and DE-2 $PLA_2$ and three gill G-1, G-2 and G-3 $PLA_2$ as group I $PLA_2$, based on the analysis of N-terminal amino acid sequence and enzyme properties. In addition, we have very recently cloned a cDNA encoding red sea bream gill G-3 $PLA_2$ and classified gill G-3 $PLA_2$ as a group IB $PLA_2$, based on the amino acid sequence deduced from nucleotide sequence of the cDNA (29). On the other hand, the primary structure of red sea bream hepatopancreas DE-1 and DE-2 $PLA_2$ remains to be established. In the present paper, we describe the cloning and sequencing of the cDNA for red sea bream hepatopancreas DE-1 and DE-2 $PLA_2$ as well as the three distinct isoforms that exist in a single fish species, red sea bream. We further investigated the distribution of mRNA for hepatopancreas DE-1, DE-2 $PLA_2$, and gill G-3 $PLA_2$ in various tissues by reverse transcription-polymerase chain reaction (RT-PCR) to better understand the structure–function relationship of the three isoforms.

[1]The sequences of DE-1 and DE-2 $PLA_2$ and β-actin presented in this article have been submitted to DDBJ/EMBL/GenBank with accession numbers AB050632, AB009286, and AB050670, respectively.

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Abbreviations: bp, base pair(s); dNTP, deoxynucleoside triphosphate; DTT, dithiothreitol; kb, kilobase(s); KOD Dash, *Pyrococcus kodakaraensis* KOD1; MMLV, Moloney murine leukemia virus; nt, nucleotides; PCR, polymerase chain reaction; $PLA_2$, phospholipase(s) $A_2$; RACE, rapid amplification of cDNA ends; RAV, Rous-associated virus; RT, reverse transcription.

## MATERIALS AND METHODS

*Extraction of poly (A)$^+$ RNA.* The hepatopancreas and other tissues were removed immediately from freshly killed red sea bream and were stored in liquid nitrogen. Total RNA was extracted from the hepatopancreas using Isogen (Nippon Gene, Tokyo, Japan), and poly (A)$^+$ RNA was isolated using Oligotex-dT30 Super (Roche Japan, Tokyo, Japan), according to the manufacturer's protocol.

*cDNA amplification of DE-2 PLA$_2$.* Primers P-1 (5′-GC(A/G/C/T)TT(A/G/C/T)AATCAGTTTTG(A/G/C/T)CA-GATGAT-3′) and P-2 (5′-CGGTCGCAGTTGCAGATGAA-3′) were derived from possible cDNA sequences corresponding to a part of the amino acid sequence of red sea bream hepatopancreas DE-2 PLA$_2$ (28), ALNQFRQM (1st–8th in Fig. 1), and that of the conserved amino acid sequences of mammalian pancreatic PLA$_2$ (30), FICNCD (96th–101th in Fig. 3), respectively. Hepatopancreas total RNA (1 μg) was reverse-transcribed using P-2 primer, 1 mM deoxynucleoside triphosphate (dNTP) and 0.25 units of Rous-associated virus (RAV)-2 reverse transcriptase (Takara, Tokyo, Japan) in a reaction buffer [50 mM Tris-HCl, 75 mM KCl, 8 mM $MgCl_2$, 10 mM dithiothreitol (DTT), pH 8.3] at 50°C for 15 min, and then denatured at 99°C for 5 min. After the addition of P-1 primer and 2.5 units of Taq DNA polymerase (Takara) to the reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), PCR was carried out for 28 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C, and 90 s extension at 72°C. Then the PCR product was purified with 2% agarose gel and sequenced. From the nucleotide sequence of the internal cDNA, the new primers P-3 [5′-CTAG-GATCCGCAATAGCAGCCATAGTCAGC-3′, complementary to nucleotides (nt) 136–159 in Fig. 1], P-4 (5′-CATCT-GTCCAGATCATCCACGGGTGTG-3′, complementary to nt 174–200 in Fig. 1), and P-5 (5′-CTGTCTAGATGCTGC-CAAGTGCACGA-3′, identical to nt 196–215 in Fig. 1) were designed for 5′- and 3′-end amplifications. The 5′RACE (rapid amplification of cDNA ends) method was performed using 5′-AmpliFINDER™ RACE Kit (Clontech, Palo Alto, CA). For 5′-end amplification of PLA$_2$ cDNA, a single-stranded cDNA was synthesized with 2 μg of red sea bream hepatopancreas poly (A)$^+$ RNA, 25 units of avian myeloblastosis virus (AMV) reverse transcriptase, and primer P-4. The resulting 5′-end PLA$_2$ cDNA was ligated with an AmpliFINDER anchor using T4 RNA ligase and was then amplified with 10 mM dNTP, P-3 primer, AmpliFINDER anchor primer, and 2.5 units of AmpliTaq DNA polymerase in a reaction buffer (10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.1% (wt/vol) gelatin, pH 8.3). For PCR, 28 cycles of 45 s denaturation at 94°C, 45 s annealing at 60°C, and 2 min extension at 72°C were carried out. Then the PCR product was separated on 1% agarose gel, and the DNA of expected size was isolated and sequenced. For 3′-end amplification of PLA$_2$ cDNA, red sea bream hepatopancreas total RNA (1 μg) was used to prepare first-stranded cDNA with an RNA PCR kit (Takara), employing 10 mM dNTP, Oligo $(dT)_{20}$ adaptor primer (Takara), and 20 units of Moloney murine leukemia virus (MMLV)reverse transcriptase. The resulting dT primed single-stranded cDNA was amplified with 2.5 units of Taq polymerase (Takara), Oligo $(dT)_{20}$ adaptor primer, and P-1 primer as described above. After the first PCR, the products were used as a template for the second PCR. The primers used in the second PCR were P-5 primer, Oligo $(dT)_{20}$ adaptor primer, and 0.5 units of Ex Taq polymerase (Takara), and the PCR conditions were the same as those used in the first PCR. Then the PCR product was separated and sequenced.

*cDNA amplification of DE-1 PLA$_2$.* Primers P11, 5′-TGG-CA(A/G)TT(C/T)GGIAA(C/T)ATGATICA-3′, and P2a, 5′-GC(A/G)GCCTT(C/T)CTGTCGCACTC(A/G)CA-3′,were derived from possible cDNA sequences corresponding to a part of the amino acid sequence of red sea bream hepatopancreas DE-1 PLA$_2$ (28), WQFGNMIQ (3rd–10th), and that of conserved amino acid sequences of mammalian pancreatic PLA$_2$ (30) and red sea bream DE-2 PLA$_2$, CECDRKAA (98th–105th in Fig. 1), respectively. The cDNA amplification of DE-1 PLA$_2$ by PCR was performed essentially as described previously (29). One microgram of red sea bream hepatopancreas poly (A)$^+$ RNA was used to prepare first-stranded cDNA with an RNA PCR kit (Takara) employing 20 units of MMLV reverse transcriptase. An internal cDNA fragment encoding PLA$_2$ was generated by PCR from first-stranded cDNA of red sea bream hepatopancreas using primers P11 and P2a, 0.5 mM dNTP, and 1.25 units of *Pyrococcus kodakaraensis* KOD1 (KOD Dash) (Toyobo, Tokyo, Japan) in a reaction buffer (20 mM Tris-HCl, 7.5 mM DTT,1.8 mM $MgCl_2$, pH 8.3). After an initial denaturation for 160 s at 94°C, 30 cycles of amplification were carried out with 30 s denaturation at 94°C, 10 s annealing at 58°C, and 30 s extension at 74°C. Then the PCR product was separated on 2% agarose gel, and the DNA of an expected size was isolated and sequenced. From the determined nucleotide sequence of the internal cDNA, new primers AP11 (5′-GGAAGGTCAGCGACAGCCGTGCATCCAGG-3′, complementary to nt 247–275 in Fig. 2) and P15 (5′-GACGTG-GATGCGTGCTGTAAGG-3′, identical to nt 190–211 in Fig. 2) were designed for 5′- and 3′-end amplifications, respectively. First-stranded and second-stranded cDNA were synthesized with 1 μg of red sea bream hepatopancreas poly (A)$^+$ RNA and Marathon cDNA Amplification kit (Clontech) according to the manufacturer's instruction. The resulting second-stranded cDNA was precipitated and ligated to a Marathon cDNA adaptor using T4 DNA Ligase for 40 min at room temperature. The 5′-end amplification of PLA$_2$ cDNA was carried by PCR with adaptor-ligated double-stranded cDNA, adaptor primer (5′-CCA TCCTAA TAC GAC TCA CTA TAG GGC-3′), AP11 primer, and 1.25 units of KOD Dash (Toyobo) in the above reaction buffer. PCR conditions were: an initial denaturation for 160 s at 94°C, followed by 30 cycles of amplification, with 30 s denaturation at 94°C, 10 s annealing at 58°C, and 30 s extension at 74°C. The resulting PCR products were subcloned into pGEM-T vector (Promega, Madison, WI) and transformed into JM109 cells,

and positive clones were selected on LB/ampicillin/IPTG/X-Gal plates according to the manufacturer's protocol. Plasmid DNA was purified from positive clones with QIAprep Spin Miniprep Kit (Qiagen, Tokyo, Japan) and sequenced. For 3′-end amplification of $PLA_2$ cDNA, PCR was carried out with adaptor-ligated double-stranded cDNA, adaptor primer, P15 primer, and 1.25 units of KOD Dash, and the resulting PCR products were subcloned into pGEM-T vector as described above and sequenced.

*Sequencing of PCR products.* The sequences of DNA fragments were determined with an Applied Biosystems 373A DNA sequencer using the Dye terminator cycle sequencing kit (PerkinElmer, Norwalk, CT), according to the manufacturer's protocol.

*RT-PCR.* Total RNA was isolated from the hepatopancreas, pyloric ceca, intestine, spleen, gill, gonad, heart, brain, stomach, kidney, and muscle of three fishes (200 g in body weight) using Isogen. Three micrograms of total RNA was used as the template to synthesize the first-stranded cDNA using oligo $(dT)_{20}$-M4 adaptor primer (5′-GTTTTCCCAGTCACGACTTTTTTTTTTTTTTTTTTTT-3′) and MMLV reverse transcriptase, RNase H Minus (Point mutant) (Promega). The cDNA fragments containing coding and noncoding regions of DE-1, DE-2, and gill G-3 $PLA_2$ cDNA were amplified by PCR from the first-stranded cDNA. The primers used were: DE-1 $PLA_2$, 5′-GCCTTATGGCAGTTTGGGAACA-3′ (identical to nt 76–97 in Fig. 2) and 5′-CTCTAACTTCAACAAATCAGG-3′ (complementary to nt 540–560 in Fig. 2); DE-2 $PLA_2$, 5′-GCACTCAACCAGTTCAGACAG-3′ (identical to nt 70–92 in Fig. 1) and 5′-TAGTAGGGAATGATGGATGGC-3′ (complementary to nt 572–592 in Fig. 1); gill G-3 $PLA_2$ (29), 5′-GCTATATGGCAGTTTGGGGACA-3′ (identical to nt 73–94) and 5′-ATGCTGAACTGATTGGACACA-3′ (complementary to nt 1009–1029). A pair of primers (5′-CGGGATCCACTACCTCATGAAGATCCTG-3′ and 5′-CCGCTCGAGTTGCTGATCCACATCTGCTG-3′) specific for red sea bream heart β-actin gene (DDBJ, accession number AB050670) was used for amplifying a 478 base pair (bp) fragment of red sea bream β-actin as an internal control. PCR conditions were: an initial denaturation for 2 min at 94°C, followed by 35 cycles of amplification, with 30 s denaturation at 94°C, 5 s annealing at 56°C for DE-1 and DE-2 $PLA_2$ and at 60°C for gill G-3 $PLA_2$, and 10 s extension at 74°C. The reaction products were electrophoresed on a 1% agarose gel.

## RESULTS

*Isolation and characterization of cDNA clones for DE-1 and DE-2 $PLA_2$.* Total RNA and poly $(A)^+$ RNA were prepared from the hepatopancreas of red sea bream. Primers for PCR were designed according to the amino-terminal amino acid sequence of purified hepatopancreas DE-2 $PLA_2$ and the highly conserved amino acid sequences among mammalian pancreatic $PLA_2$. A full-length cDNA clone was isolated by RT-PCR and RACE methods. The nucleotide sequence of the DE-2 $PLA_2$ cDNA included a 447 bp open reading frame that encoded a prepropeptide of 23 amino acids, followed by a mature protein of 126 amino acids (Fig. 1). The calculated molecular mass and isoelectric point of the mature protein were 14,422 Da and 4.16, respectively. The sequence AATAAA, which is the conserved sequence of a polyadenylation signal, was found 23 bp upstream of the poly A tail. The nucleotide sequence of the DE-1 $PLA_2$ cDNA included a 450 bp open reading frame that encoded a putative prepropeptide and mature protein of 25 and 125 amino acids, respectively (Fig. 2). The calculated molecular mass and isoelectric point of the mature protein were 13,634 Da and 5.63, respectively. The 3′-noncoding region contained two putative polyadenylation signals located 17 and 34 bp upstream of the poly A tail. The nucleotide sequences of DE-1 and DE-2 $PLA_2$ encoded no putative sites of N-glycosylation (Asn-X-Ser/Thr, where X is any amino acid) (Figs. 1 and 2).

The alignment of amino acid sequences for mature protein

```
agagactctgtgactagccctctgacacacagg ATG AAT ACC CTC   12
                                    M   N   T   L   -20
CAG ACT CTG CTT CTC GTG GCT GCA AGC CTC   42
Q   T   L   L   L   V   A   A   S   L   -10
TGT GTT GCC CAG TCT TTG GAC AAC AAG GCA   72
C   V   A   Q   S   L   D   N   K   A     1
CTC AAC CAG TTC AGA CAG ATG ATC CTG TGC  102
L   N   Q   F   R   Q   M   I   L   C    11
GTG ATG CCT GAC AGC TGG CCA ATT TTT GAT  132
V   M   P   D   S   W   P   I   F   D    21
TAC GCT GAC TAT GGC TGC TAT TGC GGA AAA  162
Y   A   D   Y   G   C   Y   C   G   K    31
GGA GGC TCG GGC ACA CCC GTG GAT GAT CTG  192
G   G   S   G   T   P   V   D   D   L    41
GAC AGA TGC TGC CAA GTG CAC GAC GCG TGT  222
D   R   C   C   Q   V   H   D   A   C    51
TAC ACT GAT GCC ATG CAG CAC CCC GAG TGC  252
Y   T   D   A   M   Q   H   P   E   C    61
TGG GCC ATC CTG GAC AAT CCT TAC ACC GAG  282
W   A   I   L   D   N   P   Y   T   E    71
TTC TAC GCC TAC AAC TGT GAC GAG CAG AGC  312
F   Y   A   Y   N   C   D   E   Q   S    81
AAG AAG GTC ACC TGT GGC AAC AAC AAT GAC  342
K   K   V   T   C   G   N   N   N   D    91
GAA TGC GAG ATG TTC ATC TGT GAG TGC GAC  372
E   C   E   M   F   I   C   E   C   D   101
AGG AAG GCT GCC GAG TGC TTT GCC AGA TCA  402
R   K   A   A   E   C   F   A   R   S   111
CCC TGG ATC CCC GAG CAC GAG CAC CTG CCC  432
P   W   I   P   E   H   E   H   L   P   121
AGC GAC AAA TGT CAA TAA agagcgaacaaccgtctcaaat  472
S   D   K   C   Q   *                          126
caggaatttatgcttcaactcaaatatcagttttttagttttcaaaagtgacaatttgctcaagat
ccatggatgaattgtcattttggtgacctatagccatccatcattccctactagtactagttacac
ttgctgtgtttgtaattgtgaagtaattctgacaataaacacagacgtgaacacacaaaaaaa
aaaaaaaaaaa                                                    679
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**FIG. 1.** Nucleotide and deduced amino acid sequences of red sea bream hepatopancreas DE-2 phospholipase $A_2$ (DE-2 $PLA_2$). The predicted preprosegment is boxed and a possible initiator methionine is shown in **bold**. The putative polyadenylation signal is underlined and shown in **bold**. An asterisk shows the termination codon.

```
cc  ATG AAT GTG TCA GGT CCT CTG CTG CTG   27
     M   N   V   S   G   P   L   L   L   -17

CTG CTG CTC ACT GCA GCC TGT GTG GTC AGC   57
 L   L   L   T   A   A   C   V   V   S    -7

GGT GCC ATG CTG CCA AAA GCC TTA TGG CAG   87
 G   A   M   L   P   K   A   L   W   Q     4

TTT GGG AAC ATG ATC CAG TGT GCT CAG CCT  117
 F   G   N   M   I   Q   C   A   Q   P    14

GGT GTT AAC CCC TTT TTG TAC AAC GAC TAC  147
 G   V   N   P   F   L   Y   N   D   Y    24

GGC TGC TGG TGC GGC TTC GGG GGG AAG GGA  177
 G   C   W   C   G   F   G   G   K   G    34

GCC CCT CTG GAT GAC GTG GAT GCG TGC TGT  207
 A   P   L   D   D   V   D   A   C   C    44

AAG GTT CAT GAC AAC TGC TAC AAA GCG AGC  237
 K   V   H   D   N   C   Y   K   A   S    54

AGA TTG GCT CCT GGA TGC ACG GCT GTC GCT  267
 R   L   A   P   G   C   T   A   V   A    64

GAC CTT CCT TAC GTT CTT GTT TAT GAT CAC  297
 D   L   P   Y   V   L   V   Y   D   H    74

ACC TGT TCC AAT CAG CAG GTG ACC TGC TCA  327
 T   C   S   N   Q   Q   V   T   C   S    84

GCG ACC AAC AAT AAG TGC CAG GCT GCT GTG  357
 A   T   N   N   K   C   Q   A   A   V    94

TGT GAG TGT GAT CGG GTG GCG GCT CAC TGC  387
 C   E   C   D   R   V   A   A   H   C   104

TTC GCT CAG ACC CAG TAC AAC CCC GAC AAC  417
 F   A   Q   T   Q   Y   N   P   D   N   114

AAG AAC GTG GAT CAG AAA GTC CAT TGT GTC  447
 K   N   V   D   Q   K   V   H   C   V   124

AAC TGA  cttcaccaacagactaaacaaaacacacatcagagaacactggatttcttt  504
 N   *

tcaatgtttaatttctttataatttgtaaatcaatcctgatttgttgaagttagagtttgtcaagaaataaa
tgtttcattaaaataaaaactagccgtcaaaaaaaaaaaaaaaaaaaaaaaaaaaa                632
```

**FIG. 2.** Nucleotide and deduced amino acid sequences of red sea bream hepatopancreas DE-1 $PLA_2$ (DE-1 $PLA_2$). The predicted prepro-segment is boxed and a possible initiator methionine is shown in **bold**. The putative polyadenylation signals are underlined and shown in **bold**. An asterisk shows the termination codon.

and prepropeptide of red sea bream hepatopancreas DE-1 and DE-2 $PLA_2$ with the sequences of red sea bream gill G-3 $PLA_2$, seabass *(D. labrax)* $PLA_2$, human and porcine group IB $PLA_2$, and *N. naja atra* group IA $PLA_2$ are presented in Figures 3 and 4, respectively (6,24,29,31,32). In amino acid sequence, DE-1 and DE-2 $PLA_2$ have characteristics in common with mammalian pancreatic type, group IB $PLA_2$, including the presence of Cys11 and 77 and the alignment of other Cys residues; residues of N-terminal helix Gln4, Phe5, and Ile9, and the presence of the absolutely conserved active-site His48, Tyr52, Tyr73, and Asp101; the "pancreatic loop" of residues 63–67 that are conserved in group IB $PLA_2$; and the conserved sequence of the calcium-binding segment Tyr25-Gly35, except for the substitution of Trp for Tyr 28 in DE-1 $PLA_2$ (Fig. 3). Insertion of lysine doublet at positions 82 and 83 was found in DE-2 and seabass $PLA_2$. The degree of identity for amino acid sequence between red sea bream hepatopancreas DE-1 and DE-2 $PLA_2$ was low (47%) (Table 1). DE-2 PLA2 shows high identity to gill G-3 $PLA_2$ (65%), and DE-1 $PLA_2$ shares higher identity to seabass $PLA_2$ (87%). SignalP computer analysis (33) for the potential cleavage positions in the signal sequence suggested that DE-1 and DE-2 $PLA_2$ contain five and six residues of propeptide preceding the mature enzyme, respectively (Fig. 4). In addition, signal peptide sequences of DE-1 and DE-2 $PLA_2$ were homologous to those of gill G-3 and seabass $PLA_2$, respectively. A phylogenetic tree was derived from an alignment of known protein sequences between fish $PLA_2$ and secretory $PLA_2$, using the CLUSTAL W program (34) and Tree view (35). The sequences of mouse group IIA, IIC, IID, IIE, IIF and V $PLA_2$, and rat group X $PLA_2$ were obtained from the DNA Data Bank of Japan. The others are the following: porcine (31), cow (36), dog and rat (37), human (38), guinea pig (39), horse (40), and rabbit (41) pancreatic group IB $PLA_2$, *Oxyuranus scuttus scutatus* OS 1 group IB″ $PLA_2$ and OS 2 group IA $PLA_2$ (42), *Notechis scutatus scutatus* II-1 and II-5 (43,44), *Pseudochis australis* (45), *Pseudonaja texistilis* (46), *Haemachatus hemachatus* (47), *N. melanoleuca* DE II and DE III (48,49), *N. naja naja* (50), *N. naja atra* (32) and *N. naja kaouthia* (51) group IA $PLA_2$, and *Bitis gabonica* group IIB $PLA_2$ (52). Subdivision of snake venom and mammalian group I and group II $PLA_2$ shown in Figure 5 are based on the proposals of Danse *et al.* (42) and Valentin *et al.* (15). This tree shows that fish $PLA_2$ were placed in the branch of mammalian group IB $PLA_2$ but were separated into two subgroups, red sea bream DE-1 and gill G-3 $PLA_2$, and DE-2 and seabass $PLA_2$, respectively. DE-2 and seabass $PLA_2$ are more distantly related to mammalian group IB $PLA_2$ than DE-1 and gill G-3 $PLA_2$.

*RT-PCR.* The mRNA expressions of hepatopancreas DE-1 and DE-2 $PLA_2$ and gill G-3 $PLA_2$ were compared among various tissues of red sea bream by RT-PCR. As shown in Figure 6, amplification of β-actin mRNA produced a band of 478 bp, providing a positive control. The expected cDNA of DE-1 $PLA_2$ (483 bp) was amplified in the hepatopancreas, pyloric ceca, intestine, gonad, spleen, stomach, and kidney. The DE-2 $PLA_2$ cDNA (522 bp) was detected in the hepatopancreas, pyloric ceca, intestine, spleen, gill, gonad, heart, brain, stomach, kidney, and muscle, suggesting that this $PLA_2$ isoform is ubiquitously expressed. On the other hand, gill G-3 $PLA_2$ (957 bp) was expressed only in the gills and gonad.

**TABLE 1**
**Identity (%) of Amino Acid Sequence in Mature Protein Among Fish, Mammalian, and Snake Venom Group I Phospholipase $A_2$ ($PLA_2$)[a]**

| Group I $PLA_2$ | DE-2 | G-3 | Seabass | pGIB | nGIA |
|---|---|---|---|---|---|
| DE-1 | 46.7 | 64.5 | 47.5 | 48.4 | 48.4 |
| DE-2 | | 54.5 | 87.3 | 54.0 | 48.8 |
| G-3 | | | 54.5 | 53.2 | 48.7 |
| Seabass | | | | 57.3 | 47.2 |
| pGIB | | | | | 52.8 |

[a]DE-1, red sea bream hepatopancreas DE-1 $PLA_2$; DE-2, red sea bream hepatopancreas DE-2 $PLA_2$; G-3, red sea brim gill G-3 $PLA_2$; seabass, seabass group 1B $PLA_2$; pGIB, porcine pancreatic group IB $PLA_2$; nGIA, *Naja naja naja* venom group 1A $PLA_2$.

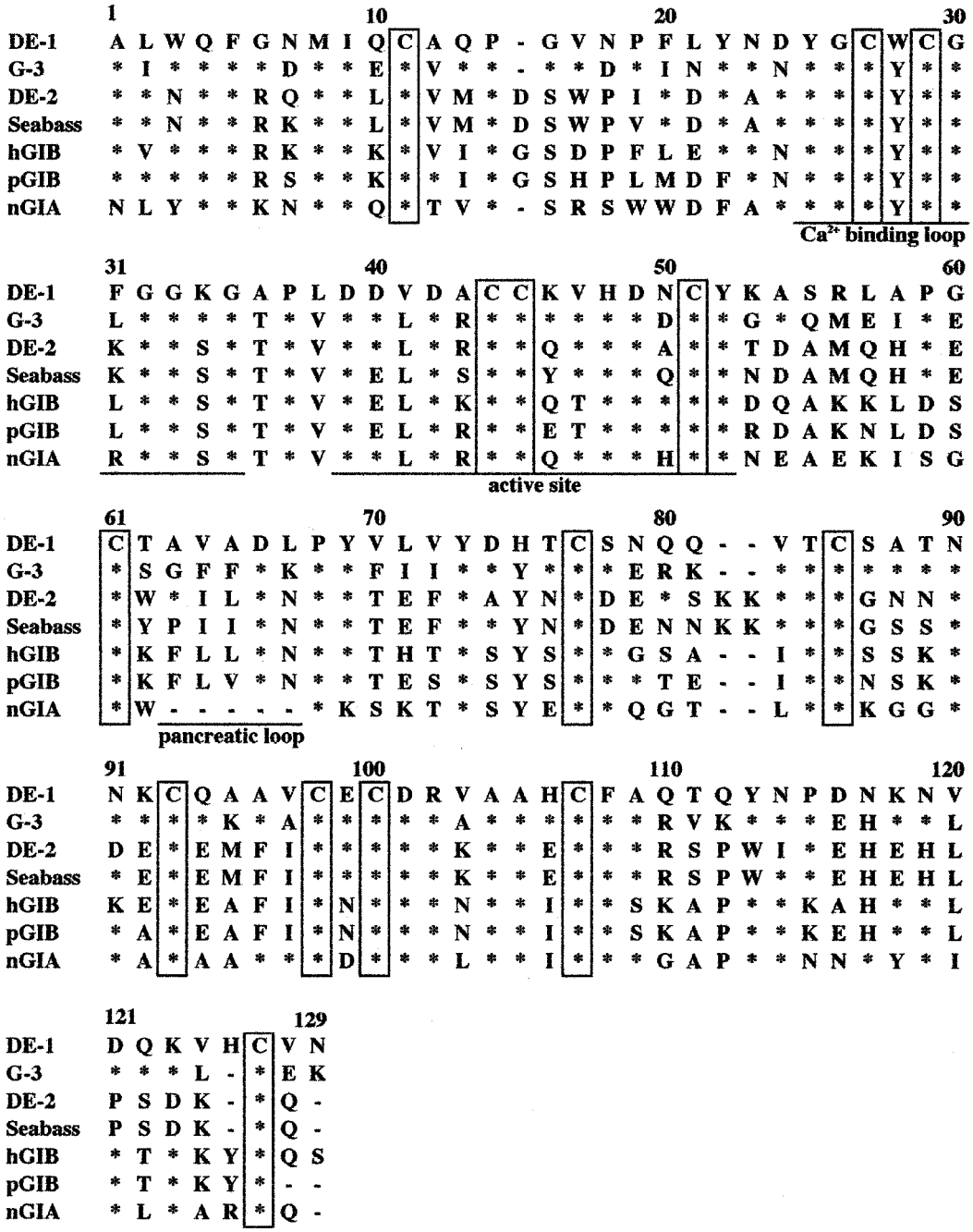


**FIG. 3.** Alignment of the amino acid sequences of red sea beam hepatopancreas DE-1 and DE-2 $PLA_2$, gill G-3 $PLA_2$ and seabass $PLA_2$ with mammalian and snake venom group I $PLA_2$. Sequences of mature $PLA_2$ proteins are shown: DE-1, red sea bream hepatopancreas DE-1 $PLA_2$; DE-2, red sea bream hepatopancreas DE-2 $PLA_2$; G-3, red sea bream gill G-3 $PLA_2$; seabass, seabass group IB $PLA_2$; hGIB, human pancreatic group IB $PLA_2$; pGIB, porcine pancreatic group IB $PLA_2$; nGIA, *Naja naja atra* venom group IA $PLA_2$. Asterisks indicate the amino acid residues identical to those of red sea bream hepatopancreas DE-1 $PLA_2$. Cysteines that are conserved among all $PLA_2$ are indicated in boxes.

Thus, the three distinct isoforms of red sea bream group IB $PLA_2$, DE-1, DE-2 and G-3 $PLA_2$, were expressed in a tissue-specific manner.

## DISCUSSION

Group I $PLA_2$ include mammalian pancreatic $PLA_2$ and those forms from elapid and hydrophid snake venoms. This group possesses 14 cysteines including a disulfide bridge formed by Cys 11 and 77 and can be further subdivided to group IA and group IB $PLA_2$, based on the presence or absence of the pancreatic loop (30,42). In a previous report, we showed that red sea bream hepatopancreas contains two enzymatically distinct group I $PLA_2$ isoforms, DE-1 and DE-2 $PLA_2$ (28). In the present study, we reported the complete amino acid sequences of red sea bream hepatopancreas DE-1 and DE-2 $PLA_2$, as deduced from their cDNA sequences. From the comparison of amino acid sequences of mature mammalian and snake

| Source | Signal peptide | | | | | | | | | | | | | | | | | | | | | Propeptide | | | | | | Mature | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DE-1 | M | N | V | S | G | - | P | L | L | L | L | L | T | A | A | C | V | V | S | G | ? | A | M | L | P | K | A | L | N |
| G-3 | M | N | V | S | G | - | P | L | L | M | L | L | L | T | A | - | C | T | V | S | G | ? | E | R | R | A | R | A | I | W |
| DE-2 | | | | M | N | T | L | Q | T | L | L | L | V | A | A | S | L | C | V | A | ? | Q | S | L | D | N | K | A | L | N |
| Seabass | | | | M | N | T | L | Q | T | L | C | L | L | A | A | S | L | S | V | A | ? | Q | S | L | D | Y | K | A | L | N |
| rGIB | | | | M | K | - | L | L | L | L | A | A | L | L | T | A | G | V | T | A | | H | S | I | S | T | R | A | V | W |
| pGIB | | | | M | K | - | F | L | V | L | A | V | L | L | T | V | G | A | A | | | Q | E | G | I | S | P | R | A | V | W |
| hGIB | | | | M | K | - | L | L | V | L | A | V | L | L | T | V | A | A | A | | | D | S | G | I | S | P | R | A | V | W |
| nGIA | M | T | P | A | H | - | L | L | I | L | A | A | V | C | V | S | P | L | G | A | S | ? | S | N | R | P | M | P | L | N | L | Y |

**FIG. 4.** Alignment of amino acid sequences in signal peptides and propeptides of red sea beam hepatopancreas DE-1 and DE-2 $PLA_2$, gill G-3 $PLA_2$, and seabass $PLA_2$ with mammalian and snake venom group I $PLA_2$. The potential cleavage site of the signal peptidase is indicated with a question mark. For abbreviations see Figure 3.

venom group I $PLA_2$, DE-1 and DE-2 $PLA_2$ were found to contain 14 cysteines including Cys 11 and Cys 77 and a pancreatic loop of residues 63–67, which are commonly conserved in group IB $PLA_2$ (Fig. 3). In addition, both DE-1 and DE-2 $PLA_2$ are predicted to have five and six propeptides preceding the mature protein, respectively (Fig. 4). These results indicate that red sea bream hepatopancreas DE-1 and DE-2 $PLA_2$ belong to the mammalian pancreatic type, group IB $PLA_2$, similar to red sea bream gill G-3 $PLA_2$ (1). This work and a previous report (1) represent the first cloning and sequencing of three distinct isoforms of group IB $PLA_2$ in a single fish species.

From the results of phylogenetic tree, fish $PLA_2$ were classified into the branch containing mammalian group IB $PLA_2$; however, they were further divided into two subgroups, DE-1 and gill G-3 $PLA_2$, and DE-2 and seabass $PLA_2$, respectively. In addition, signal peptide sequences of DE-1 $PLA_2$ and DE-2 $PLA_2$ are also homologous to those of gill G-3 $PLA_2$ and seabass $PLA_2$, respectively. As DE-2 and seabass $PLA_2$ are more distantly related to mammalian group IB $PLA_2$ than DE-1 and gill G-3 $PLA_2$, a duplication event would have occurred to generate DE-1 and gill G-3 $PLA_2$ and another subgroup, DE-2 and seabass $PLA_2$, similar to mammalian group IB $PLA_2$.

From the result of RT-PCR analysis, both DE-1 and DE-2 $PLA_2$ were expressed in digestive tissues such as the hepatopancreas, pyloric ceca, and intestine. In mammals, pancreatic type group IB $PLA_2$ were synthesized as the prepro-$PLA_2$ and were processed with signal peptidase in the rough

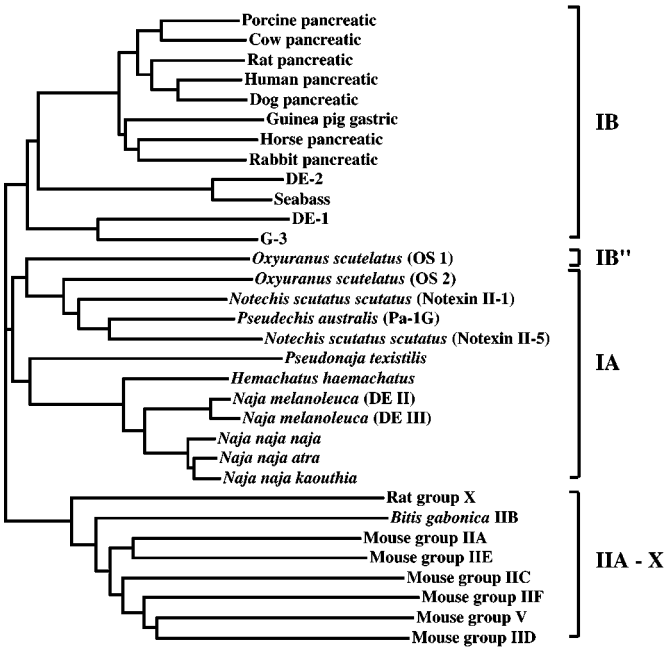


**FIG. 5.** Phylogenetic tree of fish group IB $PLA_2$, and mammalian and snake venom secretory $PLA_2$. $PLA_2$ belonging to the same group are denoted as IB, IB″, IA, and IIA-X, as described in the text.

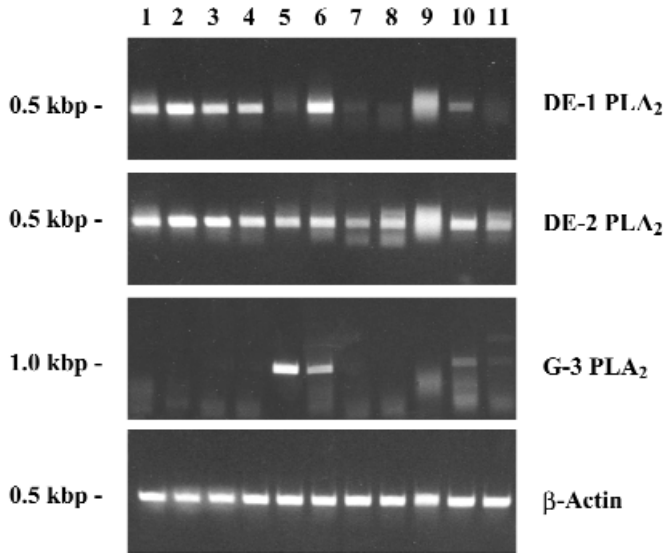


**FIG. 6.** Reverse transcription-polymerase chain reaction (RT-PCR) analysis for the tissue distribution of hepatopancreas DE-1 and DE-2 $PLA_2$ and gill G-3 $PLA_2$. Total RNA from various tissues of red sea bream were used as templates for RT-PCR. DE-1, DE-2 and G-3 $PLA_2$ were amplified with a single round of PCR as described in the Materials and Methods section. β-Actin was used as an internal standard. The calculated size of the transcript detected is indicated at the left. 1, Hepatopancreas; 2, pyloric ceca; 3, intestine; 4, spleen; 5, gill; 6, gonad; 7, heart; 8, brain; 9, stomach; 10, kidney; 11, muscle.

endoplasmic reticulum of pancreatic acinar cells (5). They were then stored as an inactive proPLA$_2$ in the zymogen granules. After being secreted in the intestinal lumen, proPLA$_2$ was activated by limited tryptic proteolysis and digest dietary phospholipids. As described above, red sea bream hepatopancreas DE-1 and DE-2 PLA$_2$ have prepro sequences; both preproPLA$_2$ may be processed and secreted in the digestive tract from the hepatopancreas, pyloric ceca, intestine, and stomach, similar to mammalian pancreatic group IB PLA$_2$. Red sea bream hepatopancreas DE-1 and DE-2 PLA$_2$ were expressed also in the nondigestive tissues; DE-1 PLA$_2$ was expressed in the spleen and gonad, and DE-2 PLA$_2$ in spleen, gill, gonad, heart, brain, kidney, and muscle. These results may indicate that DE-1 and DE-2 PLA$_2$ have different physiological functions in the nondigestive tissues and cells of red sea bream, except for the digestion of dietary phospholipids. Mammalian group IB PLA$_2$ are expressed in spleen, lung, and gonad of human (14), rat (8), and mouse (15,16). At present, two main types of PLA$_2$ receptor, M-type and N-type, have been identified in mammals. Group IB PLA$_2$ elicits a variety of biological responses, including cell proliferation, cell migration, hormone release, and eicosanoid production by interacting with the M-type receptor in various tissues and cells (17,53). In addition, neurotoxic snake venom PLA$_2$ and bee venom PLA$_2$ are said to bind to the N-type receptor, which is abundantly distributed in the brain (19). From the above results, it is reasonable to consider that the PLA$_2$ receptor, reacted specifically to group IB PLA$_2$, exists also in red sea bream. However, it still remains unclear whether DE-1 and DE-2 PLA$_2$ express as a funtional enzyme in the above tissues. Although PLA$_2$ activity was detected in the spleen, gonad, heart, kidney, and muscle (29), we do not yet analyze the distribution of both enzymes in these tissues. In order to demonstrate the above question, it is necessary to investigate the distribution of DE-1 and DE-2 PLA$_2$ by immunoblotting and immunohistochemistry. We are now trying to make recombinant DE-1 and DE-2 PLA$_2$, for preparing monoclonal antibodies and for the analysis of PLA$_2$ receptor.

Gill G-3 PLA$_2$ was expressed only in the nondigestive tissues, gills, and gonad, as shown by RT-PCR analysis (Fig. 6). However, we had found previously that PLA$_2$ activity in the gills was extremely high compared with that in the gonad (29). In addition, gill PLA$_2$ was detected only in the gills, especially the mucous cells and pavement cells located on the surface of gill epithelia, by immunoblotting and immunohistochemistry using monoclonal antibody raised against gill PLA$_2$ (Uchiyama, S., Fujikawa, Y., Uematsu, K., Matsuda, H., Aida, S., and Iijima, N., unpublished data). These aspects indicate that gill PLA$_2$ is mainly expressed in the gills and the expression of gill PLA$_2$ is extremely low in the gonad.

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# Inhibitory Effects of Triterpenoids and Sterols on Human Immunodeficiency Virus-1 Reverse Transcriptase

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**ABSTRACT:** Fifty-five triterpenoids consisting of 19 tetracyclic, 32 pentacyclic, and 4 incompletely cyclized triterpenoids, and 2 sterols, mostly isolated from various plant and fungal materials, were examined for their inhibitory effects on a purified human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. Twenty triterpenoids and one sterol showed inhibitory effects with 50% inhibition concentration ($IC_{50}$) values less than 5.0 μM. Among these cycloartenol ferulate ($IC_{50}$ = 2.2 μM), 24-methylenecycloartanol ferulate (1.9 μM), lupenone (2.1 μM), betulin diacetate (1.4 μM), and karounidiol 29-benzoate (2.2 μM) inhibited most effectively. Some of the triterpenoids and sterols may be potential new lead compounds to find still more potent HIV-1 reverse transcriptase inhibitors.

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Reverse transcriptase (RT) is a key enzyme of the human immunodeficiency virus (HIV), catalyzing the RNA-dependent and DNA-dependent synthesis of double-stranded proviral DNA. Since the replicative cycle of HIV is interrupted by RT inhibitors, the inhibition of HIV RT is considered as a useful approach in the prophylaxis of acquired immunodeficiency syndrome (AIDS). Several nucleoside HIV RT inhibitors, including AZT (3′-azido-2′,3′-dideoxythymidine), DDC (2′,3′-dideoxycytidine), and DDI (2′,3′-dideoxyinosine), have been developed and are clinically used in AIDS patients (1). Furthermore, a number of natural (flavonoids, tannins, and alkaloids) and synthetic {tetrahydro-5-methylimidazo-[4,5,1-jk][1,4]benzodiazepin-2(1*H*)-one (TIBO) and piperazine derivatives} nonnucleoside-type compounds with diverse molecular structures have been reported as being HIV RT inhibitory (2–4). However, the efficiency of both nucleoside and nonnucleoside RT inhibitors is limited by the high rate of the virus mutation, which rapidly leads to the emergence of drug-resistant viral strains (5). Compounds possessing potent anti-HIV activity with novel structures and modes of action are urgently needed to add to the existing anti-HIV agents.

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Abbreviations: AIDS, acquired immunodeficiency syndrome; DMSO, dimethylsulfoxide; DTT, dithiothreitol; HIV, human immunodeficiency virus; methyl-dTTP, methyl thymidine 5′-triphosphate; RT, reverse transcriptase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Triterpene alcohols (3-monohydroxy triterpenoids) and sterols occur in all major groups of organisms, from fungi to humans, accompanied by their oxygenated derivatives (6,7). These compounds constitute minor but ubiquitous components of our diet including edible fats and oils. In the course of our research on the pharmacological aspects of triterpenoids and sterols, we have demonstrated that various compounds of these classes possess considerable activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammatory ear edema in mice and tumor promotion in mouse skin (8–13). In continuing our study, we were especially interested to evaluate the inhibitory effects on HIV-1 RT of various types of triterpenoids and sterols, since we recently found that oleanolic acid (**32**), an olanane-type triterpenoid, isolated from the methanol extract of *Salvia officinalis* (Labiatae) has HIV-1 RT inhibitory effect (14). In this paper, we report the inhibitory effect against HIV-1 RT of 55 triterpenoids with various structural types and two stigmastane-type sterols.

## MATERIALS AND METHODS

*Chemicals and materials.* HIV-1 RT was purchased from Seikagaku Co. (Osaka, Japan), methyl-[$^3$H] thymidine 5′-triphosphate (methyl-dTTP, specific activity: 1.63 TBq/mmol; radioactive concentration: 37 MBq/mL) from Amersham Pharmacia Biotech (Tokyo, Japan) and scintillation fluid Aquasol-2 from NEN Research Products (Boston, MA). Poly(rA), primer$(dT)_{15}$, and unlabeled dTTP were obtained from Sigma Aldrich Japan (Tokyo, Japan), and DEAE-cellulose paper discs (DE81, 2.3 cm) from Whatman International, Ltd. (Maidstone, England). Sources of the 55 triterpenoid and 2 sterol samples used in this study for the HIV-1 RT assay are shown in Table 1.

*Assay of HIV-1 RT activity.* Inhibitory activity was measured in a way similar to that described previously by Ono *et al.* (15). The reaction mixture consisted of 2.0 μL of 1 M Tris-HCl (pH 8.3), 0.4 μL of 1 M dithiothreitol (DTT), 1.5 μL of 2 M KCl, 0.3 μL of 1 M $MgCl_2$, 0.2 μL of 5 mg/mL poly(rA) · p$(dT)_{15}$, 1.0 μL of 3.7 MBq/mL methyl-[$^3$H]dTTP, 0.5 μL of 10 mM dTTP, 2.5 μL of glycerol, 1.6 μL of $H_2O$, and 10 μL of 5 U/mL HIV-1 RT. A test sample was dissolved in 0.2 mM dimethylsulfoxide (DMSO), and 20 μL of this solution was added to the reaction mixture in a final volume of

**TABLE 1**
**Inhibitory Effect of Triterpenoids and Sterols on HIV-1 Reverse Transcriptase[a]**

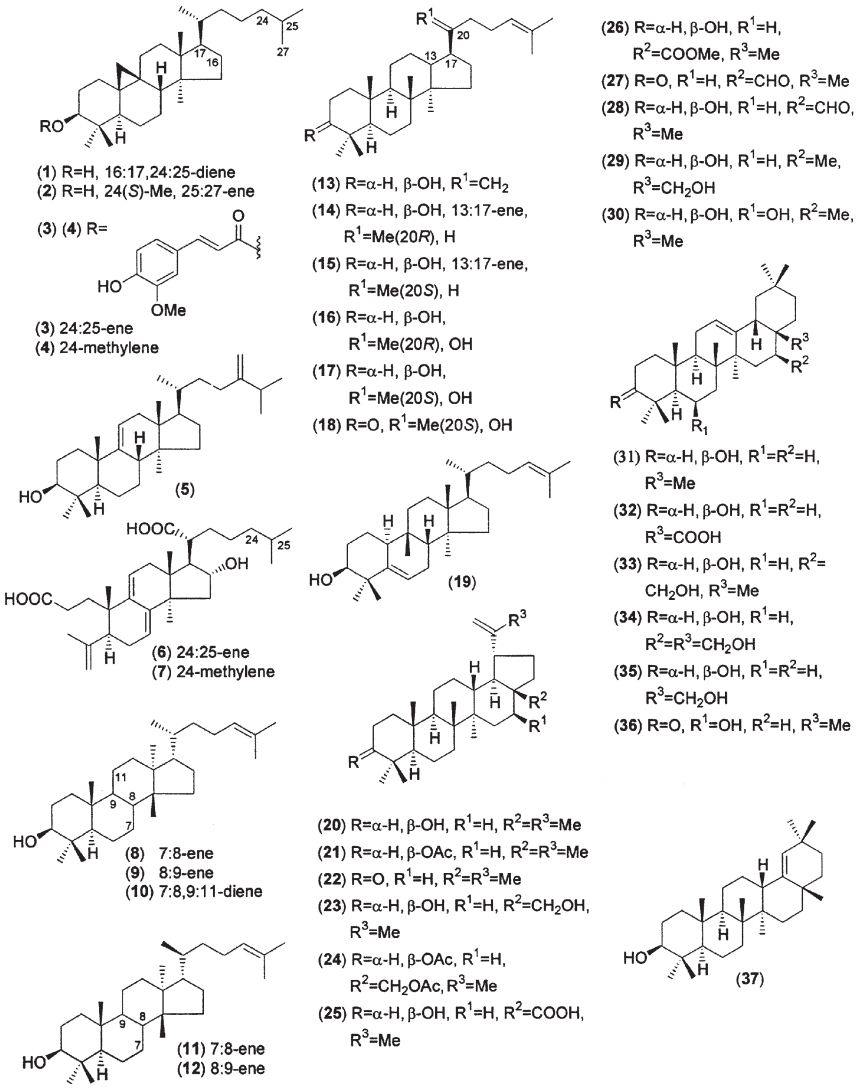
| Code | Compound | Inhibition % (4 mg/mL) | Inhibition $IC_{50}$ (μM) | Source | Ref. |
|---|---|---|---|---|---|
| Cycloartane | | | | | |
| **1** | Cimicifugenol | 72 | 4.2 | *Cimicifuga simplex* roots | 30 |
| **2** | Cyclolaudenol | NE | — | Cucurbitaceae seed oils | 31 |
| **3** | Cycloartenol ferulate | 94 | 2.2 | Rice bran | 32 |
| **4** | 24-Methylenecycloartanol ferulate | 89 | 1.9 | Rice bran | 32 |
| Lanostane | | | | | |
| **5** | 24-Methylene-24(25)-dihydroparkeol | 3 | >45 | Cucurbitaceae and Theaceae seed oils | 31 |
| **6** | Poricoic acid A | 21 | >45 | *Poria cocos* | 33 |
| **7** | Poricoic acid B | NE | — | *Poria cocos* | 33 |
| Euphane | | | | | |
| **8** | Butyrospermol | 96 | 3.1 | Camellia and sasanqua oils | 11 |
| **9** | Euphol | 20 | 14 | Camellia and sasanqua oils | 11 |
| **10** | Eupha-7,9(11)-dien-3β-ol | 90 | 3.3 | *Euphorbia antiquorum* latex[b] | |
| Tirucallane | | | | | |
| **11** | $\Delta^7$-Tirucallol | 51 | 9.2 | Camellia and sasanqua oils | 11 |
| **12** | Tirucallol | 61 | 3.8 | Camellia and sasanqua oils | 11 |
| Dammarane | | | | | |
| **13** | Dammaradienol | 87 | 3.5 | Camellia and sasanqua oils | 11 |
| **14** | Isoeuphol | 75 | 4.5 | Camellia and sasanqua oils | 11 |
| **15** | Isotirucallol | 90 | 3.5 | Camellia and sasanqua oils | 11 |
| **16** | Dammarenediol I | 5 | >45 | Dammar resin[b] | |
| **17** | Dammarenediol II | 10 | >45 | Dammar resin[b] | |
| **18** | Hydroxydammarenone II | NE | — | *Betula platyphylla* leaves[b] | |
| Cucurbitane | | | | | |
| **19** | 10α-Cucurbitadienol | 13 | 28 | Cucurbitaceae seed oils | 31 |
| Lupane | | | | | |
| **20** | Lupeol | 92 | 3.8 | Camellia and sasanqua oils | 11 |
| **21** | Lupeol acetate | 59 | 6.4 | Derived from lupeol (**20**) | 34 |
| **22** | Lupenone | 93 | 2.1 | Derived from lupeol (**20**) | 34 |
| **23** | Betulin | 38 | 14 | *Betula platyphylla* outer bark[b] | |
| **24** | Betulin diacetate | 96 | 1.3 | Derived from betulin (**23**)[b] | |
| **25** | Betulinic acid | 55 | 7.9 | Derived from betulin (**23**)[b] | |
| **26** | Betulinic acid methyl ester | 41 | 11 | Derived from betulin (**23**)[b] | |
| **27** | Betulone aldehyde | 88 | 3.4 | Derived from betulin (**23**) | 34 |
| **28** | Betulin aldehyde | 18 | >45 | Derived from betulin (**23**) | 34 |
| **29** | 30-Hydroxylupeol | 72 | 4.5 | Derived from lupeol (**20**) | 34 |
| **30** | Calenduladiol | 63 | 5.4 | Compositae flowers | 35 |
| Oleanane | | | | | |
| **31** | β-Amyrin | 70 | 4.7 | Camellia and sasanqua oils | 11 |
| **32** | Oleanolic acid | 86 | 3.1 | *Salvia officinalis* | 14 |
| **33** | Maniladiol | 27 | 23 | Compositae flowers | 35 |
| **34** | Longispinogenin | NE | — | Compositae flowers | 35 |
| **35** | Erythrodiol | 60 | 5.0 | Olive oil | 36 |
| **36** | Daturaolone | NE | — | *Datura stramonium* seed oil | 37 |
| **37** | Germanicol | 1 | 45 | Camellia and sasanqua oils | 11 |
| Ursane | | | | | |
| **38** | α-Amyrin | 77 | 3.3 | Camellia and sasanqua oils | 11 |
| **39** | α-Amyrenone | 94 | 3.3 | *Lingnania chungii* | 38 |
| **40** | Uvaol | 49 | 9.5 | Olive oil | 36 |
| **41** | Ursolic acid | 63 | 6.4 | *Perilla frutescens* leaves[b] | |
| **42** | Ursolic acid methyl ester | 66 | 5.5 | Derived from ursolic acid (**41**)[b] | |
| Multiflorane | | | | | |
| **43** | Karounidiol | 48 | 11 | *Trichosanthes kirilowii* seed oil | 39 |
| **44** | Karounidiol 29-benzoate | 97 | 2.2 | Derived from karounidiol (**43**)[b] | |
| **45** | 7-Oxodihydrokarounidiol | 31 | 33 | *Trichosanthes kirilowii* seed oil | 40 |
| **46** | Bryonolic acid | 60 | 5.3 | *Trichosanthes kirilowii* seed oil | 40 |
| **47** | 25-Nor-5(10)-dehydroisokarounidiol | 29 | 43 | Derived from isokarounidiol | 41 |
| **48** | 25-Nor-5(10)-dehydroisokarounidiol diacetate | 85 | 3.3 | Derived from isokarounidiol | 41 |
| Taraxastane | | | | | |
| **49** | Arnidiol | 40 | 20 | Compositae flowers | 34 |
| **50** | Faradiol | 2 | >45 | Compositae flowers | 34 |
| **51** | Heliantriol C | 12 | >45 | Compositae flowers | 34 |

*(Continued)*

**TABLE 1** ***(continued)***

| Code | Compound | Inhibition % (4 mg/mL) | Inhibition $IC_{50}$ (μM) | Source | Ref. |
|---|---|---|---|---|---|
| Incompletely cyclized triterpenoid | | | | | |
| **52** | Camelliol A | 5 | >47 | Sasanqua oil | 42 |
| **53** | Camelliol B | 5 | >47 | Sasanqua oil | 42 |
| **54** | Camelliol C | 3 | >47 | Sasanqua oil | 42 |
| **55** | Achilleol A | NE | — | Sasanqua oil | 42 |
| Stigmastane | | | | | |
| **56** | Stigmastanol | 82 | 3.9 | Rice bran | 43 |
| **57** | Sitosterol | NE | — | Rice bran | 43 |

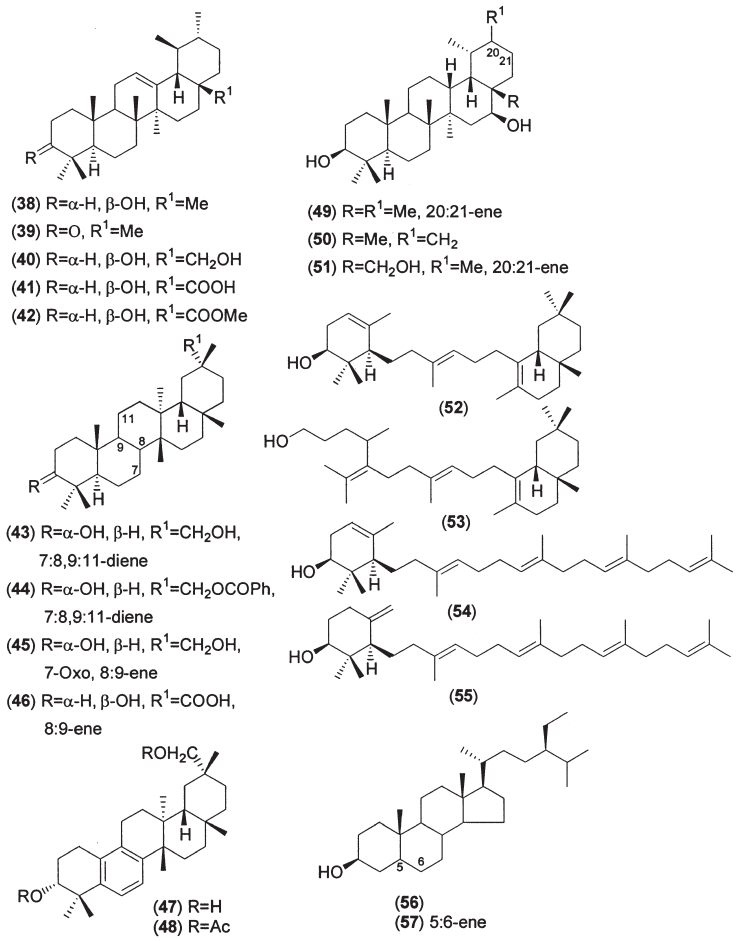
[a]$IC_{50}$, the test sample concentration inhibiting human immunodeficiency virus (HIV)-1 reverse transcriptase by 50%. NE, Not effective.
[b]Unpublished results (T. Akihisa).




**SCHEME 1**

40 μL. The enzyme was added immediately prior to incubation. The reaction was allowed to proceed for 30 min at 37°C and terminated by immersion in ice and addition of 20 μL of 0.2 M EDTA·2Na. A portion (50 μL) of each assay was applied to DEAE-cellulose discs, kept at room temperature for 10 min, and washed batchwise with 5% $Na_2HPO_4$ (eight times), $H_2O$ (two times), 99% ethanol (two times), and diethyl ether (two times). The discs were dried, and their radioactivities were counted in a scintillation fluid.

The HIV-1 RT inhibition was measured as the inhibition




**SCHEME 1 (continued)**

of the incorporation of [$^3$H]-labeled substrate (dTTP) into a polymer fraction in the presence of a tested compound as follows:

$$\text{inhibition (\%)} = [1 - (\text{cpm test/cpm control})] \times 100 \qquad [1]$$

The control assay was performed by adding 0.2 mM DMSO containing no test sample, and its count was *ca.* 19,000 cpm. The assay was undertaken at the concentrations of 20, 10, 4, and 0.8 μg/mL for each sample, and the inhibition was the mean value from the duplicated experiments. The 50% inhibitory concentration ($IC_{50}$) values were calculated by interpolation from the linear regression curve.

## RESULTS AND DISCUSSION

Table 1 summarized the inhibitory effect against HIV-1 RT of 55 triterpenoids consisting of four cycloartanes (**1**–**4**), three lanostanes (**5**–**7**), three euphanes (**8**–**10**), two tirucallanes (**11**,**12**), six dammaranes (**13**–**18**), one cucurbitane (**19**), eleven lupanes (**20**–**30**), seven oleananes (**31**–**37**), five ursanes (**38**–**42**), six multifloranes (**43**–**48**), three taraxastanes (**49**–**51**), four incompletely cyclized triterpenoids (**52**–**55**), and two stigmastane-type sterols (**56**,**57**). Chemical structures of these triterpenoids and sterols are given in Scheme 1. Although seven (**2**, **7**, **18**, **34**, **36**, **55**, **57**) and eleven (**5**, **6**, **16**, **17**, **28**, **37**, **50**–**54**) compounds showed no or weak inhibitory effect [$IC_{50} \geq 45$ μM (20 μg/mL)], respectively, 20 triterpenoids, including oleanolic acid (**32**), and a sterol, stigmastanol (**56**), exhibited significant inhibitory effects with $IC_{50}$ smaller than 5.0 μM, among which cycloartenol ferulate (**3**) ($IC_{50}$ = 2.2 μM), 24-methylenecycloartanol ferulate (**4**) (1.9 μM), lupenone (**22**) (2.1 μM), betulin diacetate (**24**) (1.4 μM), and karounidiol 29-benzoate (**44**) (2.2 μM) were the most inhibitory compounds.

Whereas betulinic acid (**25**; $IC_{50}$ = 7.9 μM), a lupane-type triterpenoid, oleanolic acid (**32**; 3.1 μM) (14), and ursolic acid (**41**; 6.4 μM) were shown to possess potent inhibitory effect against HIV-1 RT in this and in our recent studies (14), some synthetic derivatives of **25** (4,16–21), **32** (19), and **41** (22) have been reported to show strong anti-HIV-1 activity by inhibiting HIV-1 replication in H9 lymphocyte cells (16–19,22) and in CEM 4 and MT-4 cells (20,21) without affecting HIV-1 RT (16–21) and protease activities (20,21). Mechanistic

studies have revealed that betulinic acid derivatives interfere with HIV-1 entry in the cells at a postbinding step (20,21). Several other triterpenoids have been described as antiviral compounds. Glycyrrhizin, an oleanane-type triterpenoid saponin, displays some limited activity against a whole range of viruses including HIV-1 (4,23). Salaspermic acid, a friedelane-type triterpenoid, and related compounds (24) and suberol, a $C_{31}$ lanostane-type triterpenoid (25), inhibit HIV-1 in H9 cells in the upper micromolar range. Triterpenoids **25**, **32**, and ursolic acid (**41**) (26), and ganoderic acids, which are lanostane-type triterpenoids from *Ganoderma lucidum* (27), inhibit HIV-1 protease activity. Nigranoic acid, an A-ring-seco-cycloartane triterpenoid, is one of the few examples evaluated for its HIV-1 RT inhibitory activity, although it shows low $IC_{50}$ value (74 μg/mL) (28).

Various types of triterpenoids, *viz.*, cycloartanes, euphanes, tirucallanes, dammaranes, and multifloranes, and a stigmastane-type sterol, in addition to triterpenoids of lupane-, oleanane-, and ursane-types, have been demonstrated in this study to possess potent HIV-1 RT inhibitory effect, and these triterpenoids and sterols might be new compounds to find still more potent HIV-1 RT inhibitors and anti-HIV-1 drugs although one should be cautious in extrapolating from cell-free enzymatic assays to the mode of action of these compounds in intact cells (29).

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# A Novel Sphingophosphonolipid Head Group 1-Hydroxy-2-aminoethyl Phosphonate in *Bdellovibrio stolpii*

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**ABSTRACT:** Members of the bacterial genus *Bdellovibrio* include strains that are free-living, whereas others are known to invade and parasitize larger Gram-negative bacteria. The bacterium can synthesize several sphingophospholipid compounds including those with phosphoryl bonds as well as phosphonyl bonds. In the present study, the dominant sphingophosphonolipid component was isolated by column chromatography, and the long-chain bases, fatty acids, and polar head groups were identified by thin-layer and gas–liquid chromatographic procedures. The definitive structural identity of the sphingolipid was established by nuclear magnetic resonance and mass spectrometry of hydrolysis products and the intact compound. The compound was identified as *N*-2′-hydroxypentadecanoyl-2-amino-3,4-dihydroxyheptadecan-1-phosphono-(1-hydroxy-2-aminoethane).

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Members of the genus *Bdellovibrio* are small, comma- or crescent-shaped bacteria, commonly found in soil. Although many species are free-living, most are characterized by their predatory behavior. They can attack other larger bacteria, and proliferate as parasites in the intraperiplasmic space of various Gram-negative species (1–5). Obligate and facultative parasitic strains have been reported. Much of the lipids in parasitically grown organisms are scavenged from the host bacterium, but the parasite synthesizes its own distinct array of lipids (2,3) and like eukaryotic cilia and flagella (6), the bacterial flagellar domain differs in lipid composition compared to the rest of the cell surface membrane (7). The free-living and facultative parasitic strains can be grown in the laboratory under axenic culture conditions, thus biochemical analysis of these organisms eliminates the potential of contamination by host lipids (5).

Sphingolipids have been reported in all groups of eukaryotic organisms thus far analyzed, and they occur in the membranes as phosphosphingolipids, glycosphingolipids, and inositolsphingolipids (8–20). Sphingolipids, their precursors and metabolic products (e.g., sphingosine, sphingosine-1-phosphate, and ceramide) play important roles in transmembrane signaling and appear to be important in mammalian cell proliferation, differentiation, aging, and apoptosis (21–24). There is evidence that sphingolipid metabolism within host cell membranes augments the ability for invasion by pathogens (25,26). It has been suggested that sphingolipids may play a role in the attack and invasion processes of *Bdellovibrio* (5).

Sphingolipids are not widely distributed among the prokaryotes (27,28), and have been mainly studied among organisms in the genus *Sphingomonas* (27). To date, there is a single report of sphingophosphonolipids in *Bdellovibrio*. Steiner *et al*. (5) compared the phospholipids of two strains of *B. stolpii (B. bacteriovorus)* to determine whether phospholipids in the predator cell membrane could play a role in the attack phase. Two strains, UKi2 (a facultative parasite) and UKi1 (a free-living strain derived from the same obligate parasite parent), were both grown axenically under the same culture conditions. Three sphingophospholipids were detected in UKi2 but not in UKi1. The most abundant of the three was designated as compound 9, and the other two, compounds 8 and 11. These components were identified as sphingolipids by their resistance to alkaline hydrolysis, and by several chromatographic properties. Based on reactions to differential hydrolysis, it was tentatively concluded that compound 11 was a sphingolipid with a phosphoryl bond, and this minor lipid component was not further characterized. The two major sphingolipids compounds 8 and 9 were tentatively identified as sphingophosphonolipids. $C_{17}$ dihydrosphingosine and phytosphingosine were identified as the long-chain bases (LCB) of compounds 8 and 9, respectively. Both contained a similar mixture of α-hydroxy fatty acids, dominated by a branched $C_{15}$ hydroxy fatty acid.

Phosphonolipids appear rare as naturally occurring compounds. Most reports of this group of lipids have been from studies of the ethanolamine glycerophosphonolipids and sphingophosphonolipids in the ciliated protozoa *Tetrahymena* and *Paramecium* (29–32). The discovery of sphingophosphonolipids in a bacterium was a novel and significant finding (5), hence it is important that definitive structural characterizations of these *Bdellovibrio* lipids be elucidated. In the

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Abbreviations: 2-AEP, 2-aminoethylphosphonic acid; BSTFA, *N,O*-bis-(trimethylsilyl)trifluoroacetamide; EI, electron impact; FAB, fast atom bombardment; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; HPTLC, high-performance thin-layer chromatography; LCB, long-chain base; LSI, liquid secondary ion; MS, mass spectrometry; NMR, nuclear magnetic resonance; PHG, polar head group; TLC, thin-layer chromatography; TMS, trimethylsilyl.

present study, we obtained the definitive structural identity of the major *B. stolpii* sphingolipid component, compound 9 (5) employing mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy.

## MATERIALS AND METHODS

*Organism. Bdellovibrio stolpii* (ATCC 27052) was the same strain previously analyzed for sphingolipids (5). It was grown axenically in 2-L flasks containing 660 mL of 1% (wt/vol) Bactopeptone and 0.3% (wt/vol) yeast extract (Difco, Becton Dickinson, MD). The cultures were grown at 30°C without shaking until stationary phase was achieved. Cultures were centrifuged at 10,000 × *g* for 5 min, and the cell pellet was washed with physiological saline and recentrifuged.

*Lipid extraction and purification of the major sphingolipid component.* The cell pellet was immediately extracted with chloroform/methanol (2:1, vol/vol) and stored at −35°C. The lipids from 18 L of culture were pooled, water (0.25 vol) was added to the extract, and the lower organic layer was recovered and evaporated using a rotary evaporator. The residue was dissolved in the minimum amount of $CHCl_3$/MeOH (2:1, vol/vol). The sample was placed in an ice bath, and acetone (3 vol) added. After standing overnight at 4°C, it was centrifuged, and the acetone-insoluble pellet containing the phospholipids was dissolved in $CHCl_3$.

Adsorption column chromatography of the phospholipid fraction was performed to isolate the major sphingophosphonolipid. The phospholipid fraction (approximately 1 g) in $CHCl_3$ was loaded onto a 1.5 × 30.0-cm glass column containing Iatrobeads (6RS-8060, Iatron Laboratories, Tokyo, Japan) and eluted with (i) 70 mL $CHCl_3$; (ii) 70 mL of $CHCl_3/CH_3OH$, 95:5; (iii) 70 mL $CHCl_3/CH_3OH$, 4:1; (iv) 140 mL $CHCl_3/CH_3OH$, 3:2; (v) 70 mL $CHCl_3/CH_3OH$, 2:3; (vi) 70 mL $CHCl_3/CH_3OH$, 1:4; (vii) 70 mL $CHCl_3/CH_3OH$, 1:9; and (viii) 70 mL $CH_3OH$. After thin-layer chromatography (TLC) analysis of all the fractions, those containing the major sphingophosphonolipid were pooled and dried.

The sample was resuspended in *n*-propanol/*n*-propylamine/$H_2O$ (80:5:3, by vol), applied to a second Iatrobeads column, and the sphingophosphonolipids were eluted using the same solvent system. After analyzing each fraction by TLC, the fractions containing only the major sphingphosphonolipid were pooled and dried to approximately 0.5 mL. The purified sphingophosphonolipid was recrystallized from 5 mL of ethyl acetate/methanol (3:2, vol/vol). Approximately 200 mg of the purified sphingophosphonolipid were obtained from 54 L of bacterial cultures, representing approximately 2 kg wet wt of cells.

*Analysis of fatty acid, polar head group (PHG), and LCB.* The purified sphingophosphonolipid was hydrolyzed with $CH_3OH/H_2O$/HCl (11:2.6:1, by vol) at 80°C overnight (11–13), and the fatty acid methyl esters (FAME) were extracted into *n*-hexane for analysis by gas–liquid chromatography (GLC) and MS. The remaining material was dried under $N_2$ and dissolved in approximately 2 mL $H_2O$. The pH was then adjusted to 10.0–11.0 (with 1 M NaOH), and the LCB were extracted into $CHCl_3$ and concentrated under $N_2$. The LCB were purified on an Iatrobeads column, eluting with $CHCl_3/CH_3OH/H_2O$/25% $NH_4OH$ (70:17.5:1.5:0.25, by vol). The LCB fractions were identified by TLC, pooled, and dried under $N_2$. The LCB was acetylated in $CH_3OH$/acetic anhydride (4:1, vol/vol) and analyzed by GLC–MS. Alternatively, it was converted to its *O*-trimethylsilyl (TMS) derivative with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Tokyo Kasei Co., Tokyo, Japan) and analyzed. The acetylated TMS LCB derivative was subjected to GLC–MS analysis and compared with derivatized authentic dihydrosphingosine, sphingosine, and phytosphingosine (Sigma, St. Louis, MO).

The remaining water phase was neutralized with 1 M acetic acid, dried, redissolved in approximately 2 mL of $H_2O$; and the polar head group (PHG) was purified by cation exchange chromatography (Amberlite IR-120B). Ethanolamine- and phosphorus-positive fractions were pooled, lyophilized, and an aliquot was derivatized with BSTFA.

The ceramide moiety was obtained by treating the intact sphingophosphonolipid with HF (33). Fast-atom bombardment (FAB)-MS was obtained by the JEOL Company (Tokyo, Japan).

*TLC.* Phospholipids were separated by Silica Gel 60 high-performance TLC (HPTLC; Merck, Darmstadt, Germany) using $CHCl_3/CH_3OH/H_2O$ (60:35:8, by vol). After the solvent reached 5 cm from the top of the plate, it was dried, then re-developed with $CHCl_3/CH_3OH$/acetic acid (90:2:8, by vol). Development in the second dimension was with *n*-propanol/*n*-propylamine/$H_2O$ (80:15:5, by vol). The solvent system for LCB analysis was $CHCl_3/CH_3OH/H_2O$/25% $NH_4OH$ (70:17.5:1.5:0.25, by vol).

The PHG was analyzed by cellulose TLC (Avicel, Asahi-Kasei, Tokyo, Japan) using authentic 2-aminoethylphosphonic acid (2-AEP, Sigma) as standard. After one-dimensional development with either 88% phenol/$H_2O$ (4:1, vol/vol), isobutyric acid/$H_2O$/25% $NH_4OH$ (66:33:1, by vol), or butanol/acetic acid/$H_2O$ (5:3:1, by vol), plates were stained for ethanolamine (ninhydrin) and phosphorus (34).

*GLC–MS, FAB-MS, and liquid secondary ion MS (LSIMS) analysis.* The hydrolysis products were analyzed by GLC–MS as FAME, the PHG TMS, and the LCB N-acetylated TMS (11) using a Hewlett-Packard 5890 Series II GLC equipped with an HP-1 capillary column and interfaced with a 5971 mass selective detector. The initial 2-min column temperature of 100°C was increased at 10°C/min for 15 min and held at 250°C for 10 min. The helium carrier gas flow rate was 1.0 mL/min. The MS electron-impact (EI) source and transfer line temperatures were 280 and 190°C, respectively.

The underivatized ceramide was analyzed by FAB-MS on a JEOL MS700 mass spectrometer. 3-Nitrobenzyl alcohol was the matrix. Spectra were recorded in a positive ion mode at an accelerating voltage of 6.0 kV. The intact sphingophosphonolipid was analyzed by LSIMS on a Kratos MS-890 mass spectrometer (Kratos, Manchester, United Kingdom) in the positive ion mode. The sample on a glycerol matrix was bombarded with cesium ions, transferring a proton to the intact molecule.

*$^{31}P$, $^{13}C$, $^{15}N$, and $^{1}H$ NMR analysis of the PHG.* The PHG dissolved in $D_2O$ was analyzed by NMR spectroscopy (Bruker DMX600 spectrometer) by one- and two-dimensional (1-D and 2-D) analyses. Chemical shifts were estimated from the external TMS for proton and carbon, from 85% $H_3PO_4$ for $^{31}P$, and from 90% formamide (112.4 ppm) for $^{15}N$.

## RESULTS

*The phospholipid composition of* B. stolpii. The major phospholipid components of *B. stolpii* UKi2, identified by 2-D HPTLC, were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and three sphingophospholipids. The total phospholipid profile in organisms analyzed in the present study was similar to that reported in the earlier study of the UKi2 strain. After alkaline hydrolysis, three phosphorus-containing sphingolipids were left intact. The sphingolipid, with similar chromatographic properties as the component designated as compound 9 by Steiner *et al*. (5), was also identified as the dominant sphingolipid in this study.

*Analysis of hydrolysis products: N-linked fatty acids.* The alkali-stable, acid-labile amide-linked fatty acids in the purified sphingophosphonolipid preparation were analyzed as their methyl ester derivatives by GLC and quantified by integration of peak areas obtained by using a total ion current detector. Results were reported as retention time in min (wt%): 8.741 (3.4); 9.755 (3.8); 10.938 (89.6); 11.305 (3.3). By EI-MS, the molecular ion was observed at *m/z* 272. The fragmentation pattern resulting from cleavage between the C-1 and C-2 positions produced the dominant *m/z* 213 ion (Figs. 1,2). This indicated the presence of a hydroxyl group neighboring the carboxyl group. The structure of the major fatty acid in this preparation was shown by MS to be the straight-chained $C_{15}$ α-hydroxy fatty acid. The other FAME components were at too low a concentration for MS analysis. This sphingophosphonolipid, which had previously been isolated by TLC and analyzed for its fatty acid composition, had 68% iso-branched α-hydroxy $C_{15}$ fatty acid and 13% of an unbranched α-hydroxy-$C_{15}$ fatty acid (5). The apparent disparity can be explained by the large-scale column chromatographic procedures used in the present study. Since some fractions containing other sphingolipids were discarded, the isolated population was enriched with the molecular species with the straight-chain $C_{15}$ α-hydroxy fatty acid.

*Analysis of hydrolysis products: LCB.* The N-acetylated TMS-derivatized LCB fraction was analyzed by GLC and found to contain a single major component (not shown). The GLC-EI-MS analysis of the N-acetylated TMS derivative of the LCB ($C_{17}$ phytosphingosine) did not indicate the molecular ion, but an ion at *m/z* 546 corresponding to [$M^{+\cdot}$ – 15] typical of TMS derivatives (loss of a TMS methyl group). Other ions detected were *m/z* 456 (loss of trimethylsilanol), 387 (loss of $C_1$ and $C_2$ with the attached N-acetyl and the TMS), 286 [285 + H] ($C_4$ through $C_{17}$ including the silylated hydroxyl group at $C_4$), 276 ($M^{+\cdot}$ – 285), 218 (loss of the amino acyl group at $C_2$ from the *m/z* 276 ion), 187 (loss of *O*-TMS from *m/z* 276 ion), 174 ($C_1$ and $C_2$ with their derivatives), 132 ($C_1$ with its TMS group and $C_2$ with an $NH_2$ group), and 73 (TMS). The fragmentation pattern and the mass spectrum are typical of the derivatized phytosphingosine (11).

*Analysis of hydrolysis products: PHG.* The TMS derivative of the polar head group was analyzed by cellulose TLC and by GLC–MS. A single TLC component was detected in the sample after staining the plate with ninhydrin. By using three different solvent systems, the polar head group from the *B. stolpii* sphingophosphonolipid migrated at a slower rate than authentic 2-AEP or phosphorylethanolamine, indicating that the former was more polar (Table 1). It eluted earlier than 2-AEP on an amino acid analyzer (data not shown), further demonstrating that they were distinct compounds. The GLC retention time of the TMS derivative of 2-AEP was 8.8 min. In contrast, the TMS derivative of *B. stolpii* sphingophosphonolipid head group eluted at 8.2 min.

Analysis by GLC–MS of the TMS-derivatized head group did not show the molecular ion at *m/z* 429, but the ion representing ($M^{+\cdot}$ – 15, loss of TMS methyl group) was observed at *m/z* 414. The mass of the derivative indicated the presence of four TMS groups, which is only possible if there is a hydroxyl group on C-2. This fragmentation pattern clearly demonstrated that, unlike 2-AEP, a hydroxyl group was present in the head group of the *B. stolpii* sphingophosphonolipid. Other important ions observed included the ions at *m/z* 328 [327 + H] (loss of C-2 and TMS amine group), and 204 (oxygen and *N*-TMS aminoethanol, which further indicates that there must be a hydroxyl group at C-1, and that C-1 is directly linked to the phosphorus).

The $^{1}H$, $^{13}C$, $^{15}N$, and $^{31}P$ NMR spectra of the PHG (dissolved in $D_2O$, pH 4.6) were obtained. Three protons were found at 3.27 (multiplet of eight peaks, $H_a$), 3.08 (multiplet of eight peaks, $H_b$), and 3.87 ppm (multiplet of six peaks because $J_{bc}$ and $J_{cf}$ are equivalent in value) in the $^{1}H$ NMR spectrum (Fig. 3A). However, the multiple splits of each proton were collapsed to a multiplet of four peaks ($H_a$, $J_{ab}$ = 13.2 Hz and $J_{ac}$ = 3.3 Hz), multiplet of four peaks ($H_b$, $J_{ab}$ = 13.2 Hz and $J_{bc}$ = 10.0 Hz), and the multiplet of four peaks ($H_c$, $J_{ac}$ = 3.4 Hz and $J_{bc}$ = 10.0 Hz), respectively, by $^{31}P$ decoupling experiments (data not shown). The protons $H_a$ and $H_b$ are nonequivalent; both protons are bonded to $C_d$ and exhibit a large H-H coupling constant of 13.2 Hz. The $H_c$ proton at 3.87 ppm

**TABLE 1**
**Thin-Layer Chromatographic Migrations of the Hydrolyzed Polar Head Group of the Major *Bdellovibrio stolpii* Sphingophosphonolipid**

| Head group | $R_f$ A[a] | $R_f$ B[b] | $R_f$ C[c] |
|---|---|---|---|
| Authentic phosphorylethanolamine | 0.42 | 0.50 | 0.19 |
| Authentic 2-aminoethylphosphonate | 0.32 | 0.53 | 0.27 |
| Head group of *B. stolpii* sphingophosphonolipid | 0.19 | 0.47 | 0.06 |

[a]88% phenol/water (4:1, vol/vol).
[b]Isobutyric acid/water/25% $NH_4OH$ (66:33:1, by vol).
[c]Butanol/acetic acid/water (5:3:1, by vol).

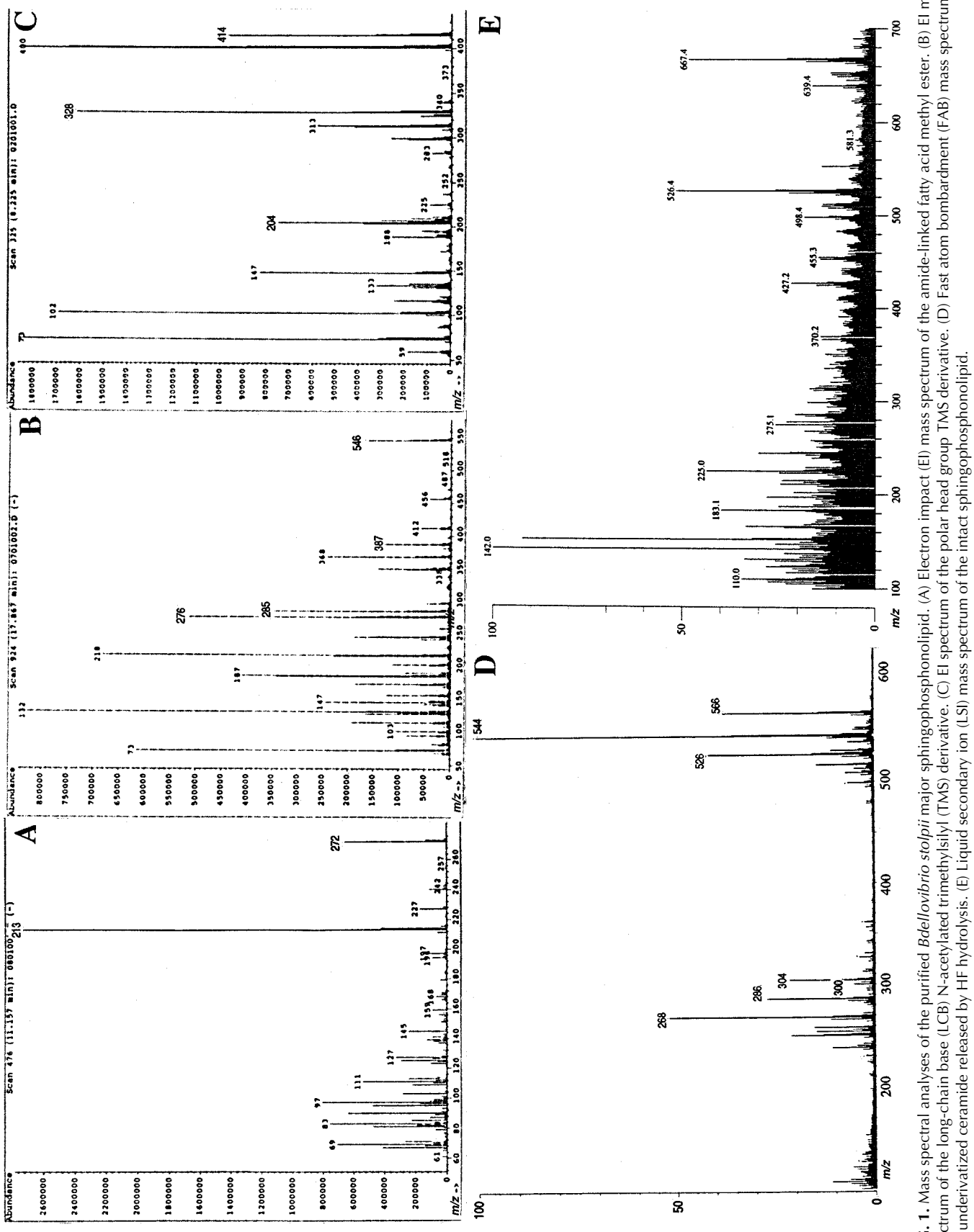



**FIG. 1.** Mass spectral analyses of the purified *Bdellovibrio stolpii* major sphingophosphonolipid. (A) Electron impact (EI) mass spectrum of the amide-linked fatty acid methyl ester. (B) EI mass spectrum of the long-chain base (LCB) N-acetylated trimethylsilyl (TMS) derivative. (C) EI spectrum of the polar head group TMS derivative. (D) Fast atom bombardment (FAB) mass spectrum of the underivatized ceramide released by HF hydrolysis. (E) Liquid secondary ion (LSI) mass spectrum of the intact sphingophosphonolipid.

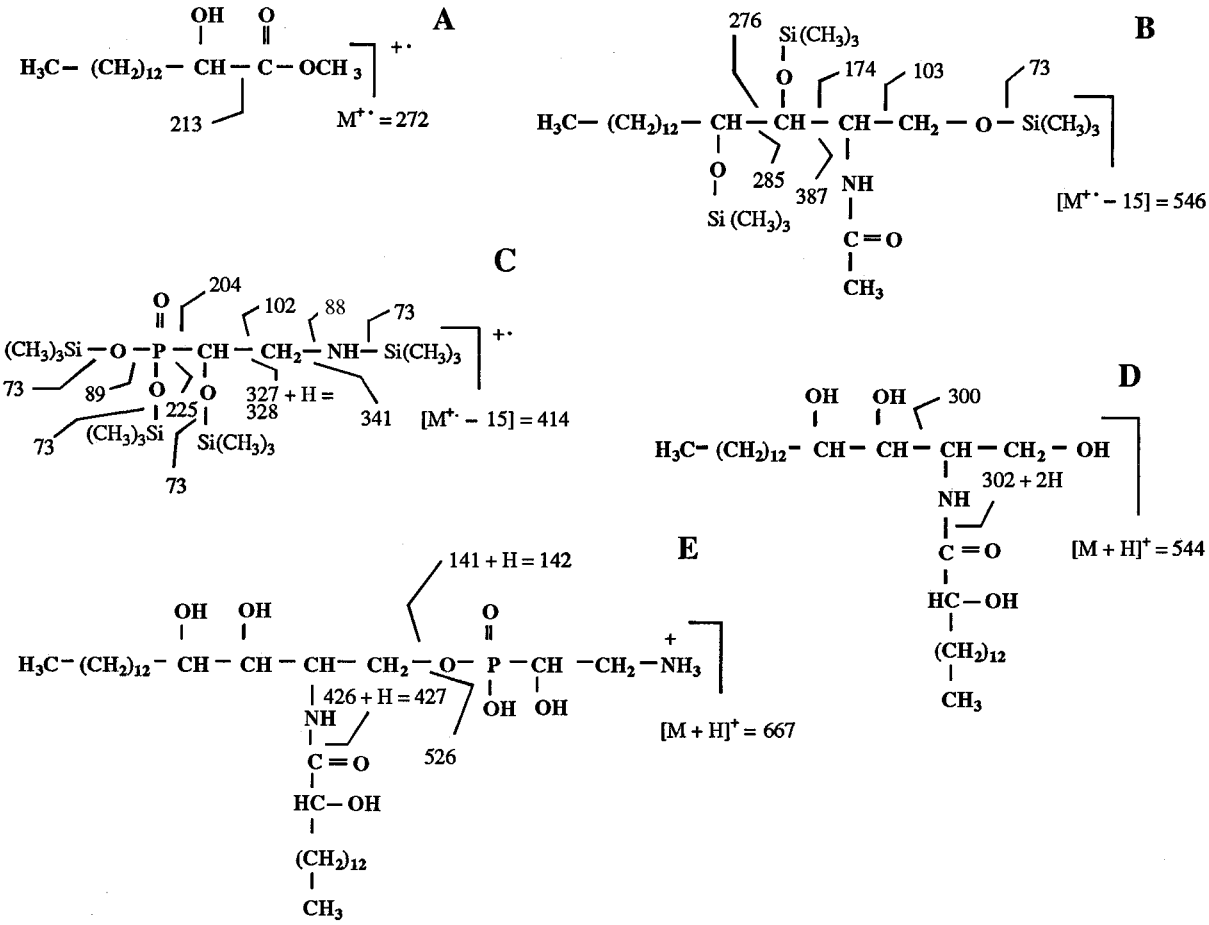



**FIG. 2.** Fragmentation patterns of samples shown in Figure 1. (A) The amide-linked fatty acid methyl esters indicate it is an α-hydroxy 15-carbon straight-chain acid. (B) The N-acetylated LCB TMS derivative was identified as C-17 phytospingosine. (C) The polar head group TMS derivative indicated it was ethanolamine with a direct P–C bond and a hydroxyl group at C-1. (D) FAB mass spectrum of the underivatized ceramide moiety verified the structural assignments of the fatty acid and LCB. (E) LSI mass spectrum of the underivatized intact sphingophosphonolipid verified the structural assignments of the fatty acid ($C_{15}$ α-hydroxy *n*-fatty acid), LCB ($C_{17}$ phytosphingosine), and the polar head group (1-hydroxy-2-aminoethyl phosphonate). For abbreviations see Figure 1.

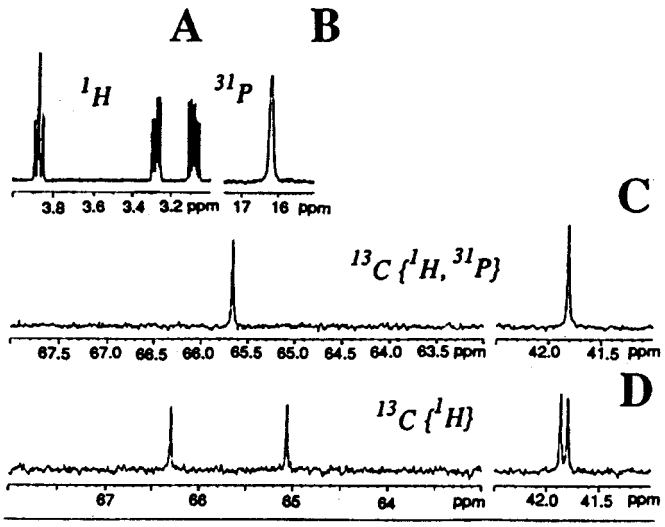
resonated at a lower field, compared to those of $H_a$ and $H_b$. This suggested that $H_c$ is bonded to carbon $C_e$, which is also bonded to the hydroxy group. All other protons in the head group were not observed due to the isotopic exchange with deuterium atoms of the $D_2O$ solvent.

The proton-decoupled $^{13}C$ NMR spectrum showed four signals, with two peaks centered at 65.7 and 41.8 ppm. A double-decoupled experiment (in which both the $^1H$ and $^{31}P$ were decoupled from $^{13}C$) showed that the $C_e$-$P_f$ coupling constants were $J_{c_e}P_f$ = 155.1 Hz and $J_{c_d}P_f$ = 8.8 Hz (Figs. 3C,3D). The large coupling constant of 155.1 Hz indicated that $C_e$ is bonded to $P_f$. The chemical shifts of 65.7 and 41.8 ppm are also suggestive of the presence of $C_e$-OH and $C_d$-N centers, respectively. The $^{31}P$ NMR spectrum showed a single peak with a chemical shift of 16.2 ppm (Fig. 3B) corresponding to the chemical shift of the alkylphosphonic acid reference standard. $^{15}N$ showed a single peak at 26.0 ppm.

The detailed analyses by 2D-NMR including $^1H$-$^{13}C$, $^1H$-$^{15}N$, and $^1H$-$^{31}P$ heteronuclear multiple bond correlation (pulse-field gradient mode) made clear the position of each atom in the PHG, as shown in Figure 3E. The chemical shifts and coupling constants of $^1H$, $^{13}C$, $^{15}N$, and $^{31}P$ are summarized in Figure 3. The NMR data, together with those obtained from the other analyses presented in this paper, unequivocally demonstrated that the PHG of the major *B. stolpii* phosphonosphingolipid is 1-hydroxy-2-aminoethylphosphonic acid.

*Analysis by FAB-MS and LSIMS.* The FAB-MS analysis of underivatized ceramide released by HF digestion showed the $(M + H)^+$ ion at 544 Da. The fragmentation pattern confirmed the assignments of the LCB and the fatty acid moieties. The ion at *m/z* 566 is the result of the addition of a sodium ion to the molecule. The ion at 526 Da is due to the loss of $H_2O$ from the $(M + H)^+$ ion. The ion at 304 Da is due to the protonated LCB phytosphingosine (Figs. 1,2).

The LSIMS analysis of the intact underivatized molecule demonstrated that the ion at *m/z* 667 $(M + H)^+$ was intense. The fragment ion at *m/z* 142 $(141 + H)^+$ corresponds to the polar head group, and the ion at *m/z* 526 (667 − 141) corresponds to ceramide [loss of the polar head group from $(M + H)^+$]. This analysis demonstrated that it is a phosphonolipid,




| Observed Chemical Shifts (ppm) | | Observed Coupling Constants (Hz) |
|---|---|---|
| $H_a$ | 3.27 | $J_{ab} = 13.2, J_{ac} = 3.3, J_{H_aP_f} = 6.5$ |
| $H_b$ | 3.08 | $J_{ab} = 13.2, J_{bc} = 10.0, J_{H_bP_f} = 6.3$ |
| $H_c$ | 3.87 | $J_{ac} = 3.4, J_{bc} = 10.0, J_{H_cP_f} = 10.0$ |
| $C_d$ | 41.8 | $J_{C_dP_f} = 8.8$ |
| $C_e$ | 65.65 | $J_{C_eP_f} = 155.1$ |
| $P_f$ | 16.2 | |
| $^{15}N_g$ | 26 | |

E

Analyses of 2-D NMR

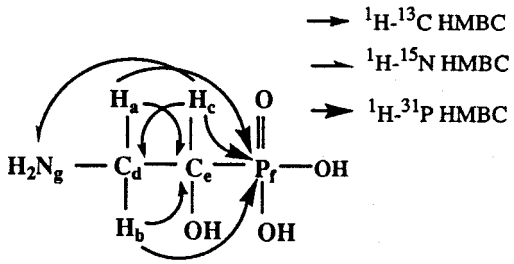



**FIG. 3.** Nuclear magnetic resonance (NMR) spectra of the polar head group. (A) Proton NMR spectrum showing peaks at 0.87 ppm for $H_c$, 3.37 ppm for $H_a$, and 3.08 ppm for $H_b$. (B) $^{31}P$ NMR spectrum. The single peak present was at 16.2 ppm, the chemical shift for a direct P–C bond. (C) The decoupled $^{13}C$ spectrum showing the single peak of $C_e$ (bonded to $P_f$) at 65.65 ppm and the single peak of $C_d$ (bonded to $N_g$) at 41.8 ppm. (D) The coupled $^{13}C$ spectrum showing two peaks at 65.05 ppm and 66.3 ppm from $C_e$ (bonded to $P_f$), and two peaks between 41.8 ppm and 41.9 ppm from $C_d$ (bonded to $N_g$). (E) Summary of analyses by two-dimensional (2-D) NMR showing coupling between atoms in the polar head group. HMBC, heteronuclear multiple bond correlation.

and that the C-1 of the ethanolamine head group is directly linked to phophorus and to a hydroxyl group.

Together, all data obtained from alkaline hydrolysis, TLC, GLC, MS analyses of hydrolyzed products and the intact compound, and NMR analysis of the polar head group, identified its structure as *N*-2′-hydroxypentadecanoyl-2-amino-3,4-dihydroxyheptadecan-1-phosphono-(1-hydroxy-2-aminoethane). It cannot be ruled out that other molecular species were present in the spot or band on TLC plates. This was the structure for the major component that was isolated by the purification procedures used in the present study.

## DISCUSSION

Sphingophosphonolipids have been isolated from various organisms (5,6,8,9,11,12,17,20,29–31), and occur as minor lipid components in mammals (32). In most cases, the PHG is 2-AEP, but 2-*N*-monomethyl-AEP has also been found (11,12). Steiner *et al.* (5) found that the isolated head group from the major sphingophosphonolipid of *Bdellovibrio* did not migrate as far as 2-AEP in the two paper chromatographic systems they used. They also noted that N-methyl derivatives of authentic standards of 2-AEP had $R_f$ values greater than underivatized 2-AEP, thus eliminating N-methyl derivatives as possible structures for the head group of the sphingophosphonolipid in this bacterium. The only previously reported identification of naturally occurring 1-hydroxy-2-AEP was a hydrolysis product of complex polysaccharides of the soil amoeba *Acanthamoeba castellanii* (35). Thus, this is the first report of a lipid with a phosphonoethanolamine head group containing a hydroxyl group. The definitive structure of the intact *B. stolpii* phosphonosphingolipid was here shown to be *N*-2′-hydroxypentadecanoyl-2-amino-3,4-dihydroxyheptadecan-1-phosphono-(1-hydroxy-2-aminoethane).

The lipids of many bacterial lipids can have high proportions of fatty acids with odd-numbered carbons (e.g., $C_{15}$, $C_{17}$); branched (iso, anteiso), or with an α- or 2-hydroxy group (36–39). There is evidence for high α-oxidation activity that could account for the high proportions of α-hydroxy fatty acids (36) in bacteria, including *Cornebacterium* (*Arthrobacter) simplex* grown on 1-octadecene and other hydrocarbons as the only sources of carbon (39). In *C. simplex*, radiolabeled palmitate was mainly incorporated into α-hydroxypalmitate. Furthermore, the fatty acid composition of lipopolysaccharide from intraperiplasmically grown *B. stolpii* (*bacteriovorus*) was found to be highly enriched in β-hydroxy fatty acids (7). The discovery of the hydroxylated ethanolamine head group in this *B. stolpii* sphingophosphonolipid suggests that oxidative reactions in aerobic bacteria may play greater roles in several metabolic pathways than earlier appreciated.

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# New Glycosphingolipid Containing an Unusual Sphingoid Base from the Basidiomycete *Polyporus ellisii*

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**ABSTRACT:** A new 9-methyl-sphinga-4,8-dienine-containing glucocerebroside (**1**), together with two additional known analogs, cerebrosides B and D, was isolated from the chloroform-soluble lipid fraction of the ethanol and chloroform/methanol extract of the fruiting bodies of the basidiomycete *Polyporus ellisii* Berk. and characterized. The structure and relative stereochemistry of the new compound were identified as (2*S*,3*R*,4*E*,8*E*)-1-(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyheptadecanoyl]amino-9-methyl-4,8-octadecadiene by means of spectroscopic ($^1$H, $^{13}$C, and two-dimensional nuclear magnetic resonance; mass spectrometry) and chemical methods.

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Two groups of sphingolipids in higher mushrooms, or basidiomycetes, are distinguished from one another by the relation of their carbohydrate to the ceramide moiety. Classical glycosphingolipids (GSL) of the first group have their sugar portion linked directly to the ceramide by a glycoside link. In the second group, the glyco-inositol-phospho-sphingolipids, carbohydrate is coupled to the lipophilic portion of the molecule *via* an inositol phosphate. Sphingolipids, e.g., ceramides, sphingomyelin, cerebrosides and gangliosides, are important building blocks of the plasma membrane of eukaryotic cells. Their function is to anchor lipid-bound carbohydrates to cell surfaces and to create an epidermal water permeability barrier, as well as to participate in antigen–antibody reactions and transmission of biological information (1,2). Some are also anti-ulcerogenic, ionophoretic, antihepatotoxic, antitumor, immunostimulatory or stimulatory to axon growth (3–7). In the course of our investigation on the sphingolipid composition of higher mushrooms collected from Yunnan Province of the People's Repulic of China, we recently reported the occurrence of two antifungal glucocerebrosides from *Russula ochroleuca* (8), and two ceramides containing $C_{18}$-phytosphingosine from *R. cyanoxantha* (9) and *Armillaria mellea* (10).

In continuing our studies on basidiomycete-derived bioactive secondary metabolites, we investigated the chemical constituents of the mushroom, *Polyporus ellisii* Berk. (Polyporaceae). A new glucocerebroside (**1**) has now been isolated and described.

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Abbreviations: CC, column chromatography; EI, electron impact; EI-MS, electron impact-mass spectrometry; FAB-MS, fast atom bombardment-mass spectrometry; GC–MS, gas chromatography-mass spectrometry; Glc, glucose; GSL glycosphingolipids; HMBC, heteronuclear multiple bond correlation; IR, infrared; LCB, long-chain base; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

## EXPERIMENTAL PROCEDURES

*Chromatographic and instrumental methods.* Melting points were obtained on an XRC-1 apparatus (Sichuan University, Sichuan, People's Republic of China). Optical rotations were taken on a Horiba SEPA-300 automatic polarimeter (Horiba, Tokyo, Japan). The nuclear magnetic resonance (NMR; $^1$H, $^{13}$C, and two-dimensional NMR) spectra were acquired on Bruker AM 400 (Rheinstetten, Germany) and DRX-500 NMR instruments (Karlsruhe, Germany); tetramethylsilane was used as an internal standard, and coupling constants were represented in Hertz. Mass spectra were measured with a VG Autospec3000 mass spectrometer (VG, Manchester, England). Infrared (IR) spectra were obtained in KBr pellets on a Bio-Rad FTS-135 IR spectrophotometer (Bio-Rad, Richmond, CA). Gas chromatography–mass spectrometry (GC–MS) was performed with a Finnigan 4510 GC–MS spectrometer (San Jose, CA) employing the electron impact (EI) mode (ionizing potential 70eV) and a capillary column (30 m × 0.25 mm) packed with 5% phenyl/95% methylsilicone on 5% phenyl-dimethylsilicone (HP-5) (Hewlett-Packard, Palo Alto, CA). Helium was used as carrier gas; column temperature 160–240°C (rate of temperature increase: 5°C/min).

*Materials.* Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China) and Sephadex LH-20 gel (25–100 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Reversed-phase chromatography was carried out on LiChroprep$^R$ RP-8 (40–63 μm) (Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) analysis was carried out on plates precoated with silica gel $F_{254}$ (Qingdao Marine Chemical Ltd.), and detection was achieved by spraying with 10% $H_2SO_4$ followed by heating. All solvents were distilled before use.

Fresh fruiting bodies of *P. ellisii* were collected from Ailao Mountains in Yunnan Province in August 1998 and identified by Professor P.G. Liu, X.H. Wang, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, People's Republic of China, where a voucher specimen (no. HKAS 32905) has been deposited.

*Extraction and isolation.* Dried fruiting bodies (228 g) of *P.*

*ellisii* were first extracted twice with 95% ethanol (1 L × 24 h × 2) for 48 h and then four times with chloroform/methanol (1:1, vol/vol; 0.5 L × 36 h × 4) at room temperature. The combined organic phase was concentrated *in vacuo*. The residue was suspended in water and partitioned with chloroform. The crude chloroform extract (7.6 g) was chromatographed on a reversed-phase (RP)-8 column, eluted with a gradient of methanol in water. The fraction (1.1 g), eluted with 200 mL methanol, was passed through vacuum liquid chromatography with a chloroform/methanol mixture containing increasing amounts of methanol to provide six fractions. Of these, the fraction eluted with chloroform/methanol (5:1, vol/vol) was further purified by chromatography on an RP-8 column by elution with methanol/water (85:15, 90:10, 95:5, vol/vol) and followed by separation on Sephadex LH-20 using methanol to produce compounds **2** (6 mg), **1** (20 mg), and **3** (8 mg).

*(2*S*,3*R*,4*E*,8*E*)-1-(β-*D*-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyheptadecanoyl]amino-9-methyl-4,8-octadecadiene (**1**).* White amorphous powder (methanol); mp 154–156°C; $[\alpha]_D^{26}$ +4.9° (*c* 0.40, MeOH); IR (KBr) $\nu_{max}$ 3393 (OH), 2921, 2852 (C–H), 1650 (HNC=O), 1540 (NH), 1469, 1304, 1079 (C–O), 963 (*trans* C=C), 721 (methylenes) $cm^{-1}$; $^1H$ and $^{13}C$ NMR spectra are given in Table 1; EI–MS (70 eV) *m/z* (relative intensities, %) 724 $[M - OH]^+$ (0.5), 562 $[M - OH - 162]^+$ (4.8), 530 (2.8); negative fast atom bombardment-mass spectrometry (FAB-MS) *m/z*: 740 $[M - 1]^-$, 579 $[M - H - 162]^-$, 561 $[M - H - 179]^-$; negative high resolution FAB-MS *m/z* 740.5659 $[M - 1]^-$ (calcd. for $C_{42}H_{78}NO_9$, 740.5677).

*Acetylation of **1**.* Compound **1** (6.3 mg) was dissolved in pyridine (0.3 mL), and the mixture was treated with acetic anhydride (0.3 mL) and left standing overnight at room temperature. The reaction solution was then diluted with 2 mL of water and extracted with ethyl acetate (3 × 4 mL). The ethyl acetate extract was washed with brine and dried over $Na_2SO_4$, then evaporated to dryness under reduced pressure. The residue obtained was subjected to silica gel CC, with elution by petroleum ether/ethyl acetate (8:2, vol/vol) to give 7 mg of its peracetate derivative **1a**.

*(2*S*,3*R*,4*E*,8*E*)-1-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-3-*O*-acetyl-2-[(*R*)-2′-acetoxyheptadecanoyl]amino-9-methyl-4,8-octadecadiene (**1a**).* White powder solid. $[\alpha]_D^{26}$ +5.6° (*c* 0.36, $CHCl_3$); IR (KBr) $\nu_{max}$ 3369 (OH), 2925, 2856 (C–H), 1745 (C=O of ester), 1675 (C=O of amide), 1533

**TABLE 1**
**$^1H$ and $^{13}C$ Nuclear Magnetic Resonance (NMR) Data for Compound 1 in Pyridine-$d_5$**

| Atom no. | $^1H$ (multiplicity, *J*, in Hz) | $^{13}C$ (multiplicity)[a] | $^1H$-$^1H$ COSY | HMBC |
|---|---|---|---|---|
| Long-chain base | | | | |
| 1a | 4.71 (*dd,* 10.5, 6.0) | 69.9 ($CH_2$) | H-1b, H-2 | H-1″, H-2, H-4 |
| 1b | 4.23 (*dd,* 10.4, 6.8) | | H-1a, H-2 | |
| 2 | 4.81 (*m*) | 54.5 (CH) | H-1a, H-1b, H-3, NH | H-1, H-3, H-4 |
| 3 | 4.75 (*m*) | 72.3 (CH) | H-2, H-4 | H-1, H-2, H-4 |
| 4 | 5.95 (*dt,* 15.3) | 132.2 (CH) | H-3, H-5 | H-3, H-6 |
| 5 | 5.99 (*dd,* 15.3, 5.8) | 131.7 (CH) | H-4, H-6 | H-3, H-7, H-8 |
| 6 | 2.15 (*m*) | 32.9 ($CH_2$) | H-7 | H-4, H-5 |
| 7 | 2.15 (*m*) | 28.2 ($CH_2$) | H-8 | H-5, H-8 |
| 8 | 5.25 (*br*) | 124.0 (CH) | H-7 | H-6, H-7, H-10, H-19 |
| 9 | | 135.6 (C) | | H-7, H-19 |
| 10 | 2.00 (*br t,* 7.5) | 39.8 ($CH_2$) | | H-8, H-19 |
| 11 | 1.38 (*m*) | 28.1 ($CH_2$) | | |
| 12–17 | 1.25 (*br s*) | 22.7–31.9 ($CH_2$) | | |
| 18-$CH_3$ | 0.86 (*t,* 7.0) | 14.0 ($CH_3$) | | |
| 19-$CH_3$ | 1.61 (*s*) | 15.9 ($CH_3$) | | H-8, H-10 |
| NHCO | 8.36 (*d,* 8.6) | | H-2 | |
| N-acyl moiety | | | | |
| 1′ | | 175.5 (C) | | H-2, H-2′, H-3′ |
| 2′ | 4.57 (*dd,* 7.8) | 72.4 (CH) | H-3′ | NH, H-3′ |
| 3′ | 2.00, 2.14 (*m*) | 35.5 ($CH_2$) | H-2′ | H-2′ |
| 4′–16′ | 1.25 (*br s*) | 22.7–31.9 ($CH_2$) | | |
| 17′-$CH_3$ | 0.86 (*t,* 7.0) | 14.0 ($CH_3$) | | |
| Sugar moiety | | | | |
| 1″ | 4.91 (*d,* 7.8) | 105.4 (CH) | H-2″ | H-1, H-2″ |
| 2″ | 4.03 (*m*) | 74.9 (CH) | H-1″, H-3″ | H-1″, H-3″, H-4″ |
| 3″ | 4.25 (*m*) | 78.3 (CH) | H-2″, H-4″ | H-1″, H-2″ |
| 4″ | 4.21 (*m*) | 71.5 (CH) | H-5″, H-3″ | H-3″, H-6″ |
| 5″ | 3.90 (*m*) | 78.3 (CH) | H-6″, H-4″ | H-3″, H-4″, H-6″ |
| 6″ | 4.36 (*dd,* 5.2, 11.8) | 62.6 ($CH_2$) | H-5″ | H-4″ |
| | 4.51 (*dd,* 2.2, 11.9) | | | |

[a]Assignments were made by distortionless enhancement by polarization transfer (DEPT) and heteronuclear multiple quantum coherence (HMQC) analysis. HMBC, heteronuclear multiple bond coherence. COSY, correlation spectroscopy.

(N–H), 1467, 1376, 1234, 1125–1037 (glycosidic C–O), 977 (*trans* C=C), 889, 721 ($nCH_2$) $cm^{-1}$; $^1H$ NMR 500 MHz ($CDCl_3$), δ ppm 0.88 (6H, *t*-like, *J* = 6.7 Hz, 2 × Me), 1.25 (*s*, methylenes), 1.57 (3H, *s*, 19-Me), 2.05 (2H, *m*, H-3′), 1.95 (2H, *br t, J* = 7.8 Hz, H-10), 1.80 (2H, *m*, H-6), 2.06 (2H, *m*, H-7), 3.61 (1H, *dd, J* = 4.5, 10.3 Hz, H-1), 3.69–3.71 (1H, *m*, H-5″), 3.93 (1H, *dd, J* = 4.0, 10.3 Hz, H-1), 4.14 (1H, *dd, J* = 2.1, 12.3 Hz, H-6″), 4.24 (1H, *dd, J* = 4.6, 12.3 Hz, H-6″), 4.31 (1H, *m*, H-2), 4.48 (1H, *d, J* = 7.9 Hz, H-1″), 4.95 (2H, *m*, H-2″, H-3″), 5.08 (1H, *br t, J* = 9.7 Hz, H-8), 5.15 (1H, *dd, J* = 4.7, 7.1 Hz, H-2′), 5.19 (1H, *t, J* = 9.5 Hz, H-4″), 5.32 (1H, *dd, J* = 5.3, 6.8 Hz, H-3), 5.41 (1H, *dd, J* = 15.3, 7.4 Hz, H-4), 5.82 (1H, *dt, J* = 15.3 Hz, H-5), 6.36 (1H, *d, J* = 8.8 Hz, NHCO), 2.00 (*s*, MeCO), 2.02 (*s*, 2 × MeCO), 2.03 (*s*, MeCO), 2.09 (*s*, MeCO), 2.15 (*s*, MeCO); $^{13}C$ NMR 100 MHz ($CDCl_3$) δ ppm 14.0 (2 × Me), 15.9 (19-Me), 2 × 22.6, 24.7, 27.4, 28.0 (C-7), 29.3, 29.6 (all $CH_2$), 31.9 (C-6), 32.5 (C-3′), 39.7 (C-10), 67.2 (C-1), 50.8 (C-2), 74.0 (C-3), 73.2 (C-2′), 100.6 (C-1″), 71.3 (C-2″), 72.0 (C-3″), 68.4 (C-4″), 72.8 (C-5″), 62.2 (C-6″), 123.0 (C-8), 136.6 (C-9), 124.6 (C-4), 136.2 (C-5), 20.5 (4 × COMe), 20.9 (2 × COMe), 2 × 169.3, 3 × 169.6, 170.0, 170.4 (all COMe, NHCO); EI-MS (70 eV) *m/z* (relative intensities, %): 993 $[M]^+$ (2.0), 933 $[M - HOAc]^+$ (3), 874 $[M - HOAc - OAc]^+$ (1.0), 826 (1.5), 700 (0.5), 663 $[M - Glc(OAc)_4 + 1$, where glucose = $Glc]^+$ (0.5), 646 (2.8), 632 (1.2), 586 (2.2), 572 (2.2), 526 (1.0), 512 (0.5), 433 $[AcOCH^+CH_2OGlc(OAc)_4]$ (0.5), 390 $[H_2N^+{=}CHCH_2OGlc(OAc)_4]$ (15), 359 (1.8), 332 (17), 331 $[Glc(OAc)_4]^+$ (100), 276 $[C_{16}H_{28}CH{=}CH(NH_2)CH_2^+]$ (1.2), 271 (9), 229 (4), 211 (5), 170 (11), 169 (95.8), 145 (7.8), 139 (8.0), 127 (10.5), 109 (32.5), 81 (14), 60 (31.5).

*Methanolysis of **1***. Compound **1** (7.3 mg) was refluxed with 2.2 mL 0.9 M HCl in 82% aqueous methanol at 80°C for 18 h. The resultant reaction mixture was extracted with *n*-hexane, and the combined organic layer was dried over $Na_2SO_4$. Concentration of the hexane yielded a fatty acid methyl ester, which was purified by silica gel CC with *n*-hexane/ethyl acetate (9:1–7:3, vol/vol) to give a methyl ester of fatty acid **1b** (2.6 mg) and then analyzed by GC–MS.

*Methyl (2*R*)-2-hydroxyheptanoate (**1b**).* The retention time ($t_R$) of **1b** was 14.3 min; white solid. $[\alpha]_D^{25}$ –4.1° (*c* 0.061, $CHCl_3$) [lit. (11) $[\alpha]_D^{24}$ – 3.6° ($CHCl_3$)]; IR (KBr) $\nu_{max}$ 3400 (OH), 2934, 1740 (C=O), 1465, 1284, 720 (methylenes) $cm^{-1}$; $^1H$ NMR 400 MHz ($CDCl_3$) δ ppm 4.19 (1H, *dd, J* = 4.2, 7.4 Hz, H-2), 3.79 (3H, *s*, $COOCH_3$), 2.74 (1H, *bs*, OH), 1.76 (1H, *m*, H-3), 1.63 (1H, *m*, H-3), 1.25 (*br s*, methylenes), and 0.88 (3H, *t, J* = 7.0 Hz, terminal methyl); EI-MS (70 eV) *m/z* (relative intensities, %) 300 $[M]^+$ (2), 241 $[M - COOMe]^+$ (13.5), 189 (2.4), 149 (12.2), 83 (34), 69 (52.2), 57 (72.6), and 43 (100).

*2-Acetoamino-1,3-diacetoxy-9-methyl-4,8-octadecanediene (**1c**).* The aqueous methanolic layer was neutralized with saturated $NaHCO_3$, concentrated to dryness, and extracted with ether. The ether phase was dried over $Na_2SO_4$, filtered, and then concentrated to yield a long-chain base (LCB), which was heated with acetic anhydride/pyridine (1:1, vol/vol) for 1.5 h at 70°C. The reaction mixture was diluted with water and then extracted three times with ethyl acetate. The residue of the ethyl acetate fraction was chromatographed over silica gel using *n*-hexane/ethyl acetate (8:2, vol/vol) as eluents to furnish a peracetate of the LCB (**1c**, 1.4 mg) as white solid. $^1H$ NMR 500 MHz ($CDCl_3$) δ ppm 5.78 (1H, *m*, H-5), 5.67 (1H, *d, J* = 9.2 Hz, NHAc), 5.42 (1H, *m*, H-4), 5.29 (1H, *m*, H-3), 5.08 (1H, *m*, H-8), 4.42 (1H, *m*, H-2), 4.29 (1H, *dd, J* = 11.6, 6.0 Hz, H-1a), 4.05 (1H, *dd, J* = 11.6, 3.4 Hz, H-1b), 2.05, 2.08 (each 3H, *s*, 2 × OAc), 2.03 (3H, *s*, HNAc), 1.95–2.15 (6H, *m*, H-6, H-7, and H-10), 1.58 (3H, *s*, H-19), 1.21–1.63 (12H, *m*), 0.88 (3H, *t, J* = 6.1 Hz, $CH_3$); EI-MS (70 eV) *m/z* (relative intensities, %) 438 $[M + 1, 2]^+$, 396 $[M + 1 - Ac]^+$ (4), 378 $[M + 1 - HOAc]^+$ (5), 318 $[M + 1 - 2 \times HOAc]^+$ (7), 284 (5.2), 268 (38), 185 (24.5), 144 $[AcOCH_2CHNHAc + H]^+$ (43), 102 $[144 - Ac + 1]^+$ (48), 84 $[144 - HOAc]^+$ (97.5), 69 (68), 55 (100).

*1*-O-*Methyl-*D*-glucopyranoside*. The remaining water layer was evaporated *in vacuo*. The residue was then chromatographed on silica gel using chloroform/methanol/water (7:3:0.5, by vol) to afford methyl glucopyranoside. $[\alpha]_D^{27}$ + 74.2° (*c* 0.01, methanol), [literature (12) $[\alpha]_D^{25}$ + 77.3° (*c* 0.1, methanol)]; negative FAB-MS *m/z* 193 $[M - 1]^-$.

*(2*S*,3*R*,4*E*,8*E*)-1-(β-*D*-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxypalmitoyl]amino-9-methyl-4,8-octadecadiene (= cerebroside B) (**2**)*. White amorphous powder. $[\alpha]_D^{27}$ +5.1° (*c* 0.3, methanol) IR (KBr) $\nu_{max}$: 3380 (OH), 2960, 1650, 1540, 1000–1100, 720 $cm^{-1}$; negative FAB-MS *m/z* 726 $[M - 1]^-$, 564 $[M - 1 - 162]^-$; negative high resolution FAB-MS *m/z* 726.5561 $[M - 1]^-$ ($C_{41}H_{76}NO_9$, calcd. 726.55200). Methanolysis of **2** yielded a methyl 2-hydroxy palmitate (retention time 12.4 min) identified by GC–MS. The NMR (Table 2) and IR spectra of **2** were identical with those reported in the literature (8,18).

*(2*S*,3*R*,4*E*,8*E*)-1-(β-*D*-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyoctadecanoyl]amino-9-methyl-4,8-octadecadiene (= cerebroside D) (**3**)*. White amorphous powder. $[\alpha]_D^{27}$ +4.8° (*c* 0.2, methanol); IR (KBr) $\nu_{max}$: 3385 (OH), 2960, 1650, 1541, 1000–1100, 721 $cm^{-1}$; negative FAB-MS *m/z* 754 $[M - 1]^-$, 592 $[M - 1 - 162]^-$. Methanolysis of **3** yielded a methyl 2-hydroxy stearate (retention time 16.2 min) identified by GC–MS. The NMR (Table 2) and IR spectra of **3** were identical with those reported in the literature (8,18).

## RESULTS AND DISCUSSION

The chloroform-soluble part of the ethanol and chloroform/methanol extract from the fruiting bodies of *P. ellisii* was separated by normal-phase followed by reversed-phase CC to give compounds **1**, **2**, and **3**. The structural elucidation of new compound **1** was as follows.

Compound **1** was obtained as white amorphous powder, $[\alpha]_D^{26}$ +4.9° (*c* 0.40, methanol). The molecular formula of $C_{42}H_{79}NO_9$ for **1** was determined by negative high resolution FAB-MS at *m/z* 740.5659 $[M - H]^-$ (calcd. 740.5677). In the negative FAB-MS, compound **1** exhibited significant frag-

**TABLE 2**
**$^1$H and $^{13}$C NMR Data[a] for Compounds 2 and 3 in Pyridine-$d_5$**

| Atom no. | **2** δ $^1$H (*J* in Hz) | δ $^{13}$C (ppm) | **3** δ $^1$H (*J* in Hz) | δ $^{13}$C (ppm) |
|---|---|---|---|---|
| Long-chain base | | | | |
| 1 | 4.69 (*dd,* 5.4, 10.7) | 70.05 *t* | 4.69 (*dd,* 5.3, 10.8) | 69.83 *t* |
| | 4.20 (*m*) | | 4.20 (*m*) | |
| 2 | 4.75 (*m*) | 54.68 *d* | 4.73 (*m*) | 54.47 *d* |
| 3 | 4.72 (*m*) | 72.55 *d* | 4.68 (*m*) | 72.37 *d* |
| 4 | 5.94 (*dd,* 15.3, 6.8) | 131.85 *d* | 5.93 (*dd,* 15.4, 5.8) | 131.64 *d* |
| 5 | 5.97 (*dt,* 15.3) | 132.35 *d* | 5.97(*dt,* 15.4) | 132.22 *d* |
| 6 | 2.14 (*m*) | 33.04 *t* | 2.15 (*m*) | 32.86 *t* |
| 7 | 2.14 (*m*) | 32.12 *t* | 2.15 (*m*) | 31.93 *t* |
| 8 | 5.25 (*m*) | 124.17 *d* | 5.23 (*m*) | 123.99 *d* |
| 9 | | 135.51 *s* | | 135.64 *s* |
| 10 | 2.00 (*br t,* 7.5) | 39.99 *t* | 1.98 (*br t,* 7.4) | 39.80 *t* |
| 11 | 1.36 (*m*) | 28.35 *t* | 1.35 (*m*) | 28.17 *t* |
| 12–15 | 1.25 (*br s*) | 30.00–29.59 *t* | 1.25 (*br s*) | 29.80-29.41 *t* |
| 16 | | 32.12 *t* | | 31.93 *t* |
| 17 | | 22.91 *t* | | 22.73 *t* |
| 18-$CH_3$ | 0.86 (*t,* 6.9) | 14.24 *q* | 0.84 (*t,* 6.4) | 14.05 *q* |
| 19-$CH_3$ | 1.61 (*s*) | 16.12 *q* | 1.59 (*s*) | 15.93 *q* |
| NH | 8.36 (*d,* 8.7) | | 8.33 (*d,* 8.7) | |
| N-acyl moiety | | | | |
| 1′ | | 175.64 *s* | | 175.52 *s* |
| 2′ | 4.57 (*m*) | 72.40 *d* | 4.55 (*dd,* 3.7, 7.4) | 72.21 *d* |
| 3′ | 1.74 (*m*), 2.14 (*m*) | 35.66 *t* | 1.75 (*m*), 2.15 (*m*) | 35.46 *t* |
| 4′–13′/15′ | 1.25 (*br s*) | 30.00–29.59 *t* | 1.25 (*br s*) | 29.80–29.41 *t* |
| 14′/16′ | | 28.22 *t* | | 28.04 *t* |
| 15′/17′ | | 22.91 *t* | | 22.73 *t* |
| 16′/18′-$CH_3$ | 0.86 (*t,* 6.9) | 14.24 *q* | 0.84 (*t,* 6.4) | 14.05 *q* |
| Sugar moiety | | | | |
| 1″ | 4.90 (*d,* 7.6) | 105.54 *d* | 4.87(*d,* 7.8) | 105.30 *d* |
| 2″ | 4.03 (*m*) | 75.07 *d* | 4.00 (*m*) | 74.87 *d* |
| 3″ | 4.20 (*m*) | 78.44 *d* | 4.20 (*m*) | 78.23 *d* |
| 4″ | 4.19 (*m*) | 71.63 *d* | 4.18 (*m*) | 71.45 *d* |
| 5″ | 3.89 (*m*) | 78.44 *d* | 3.87 (*m*) | 78.23 *d* |
| 6″ | 4.48 (*br d,* 11.8) | 62.75 *t* | 4.47 (*dd,* 2.0, 11.9) | 62.55 *t* |
| | 4.33 (*dd,* 5.0, 11.8) | | | 4.32 (*dd,* 5.3, 11.9) |

[a]For abbreviation see Table 1.

ment peaks at *m/z* 740 $[M - H]^-$, 579 [M – H – 162 (glucosyl)]$^-$, and 561 $[M - 1 - 179]^-$. The IR spectrum of **1** showed absorption bands ascribable to hydroxyl at 3393 cm$^{-1}$, glycosidic (C–O) at 1037 cm$^{-1}$, a secondary amide at 1540 and 1650 cm$^{-1}$, and long aliphatic chains at 2921, 1469, and 721 cm$^{-1}$. The $^1$H and $^{13}$C NMR spectral data of **1** indicated the presence of a sugar, an amide, and long-chain aliphatic moieties, strongly suggesting the glycolipid nature of the molecule (Table 1).

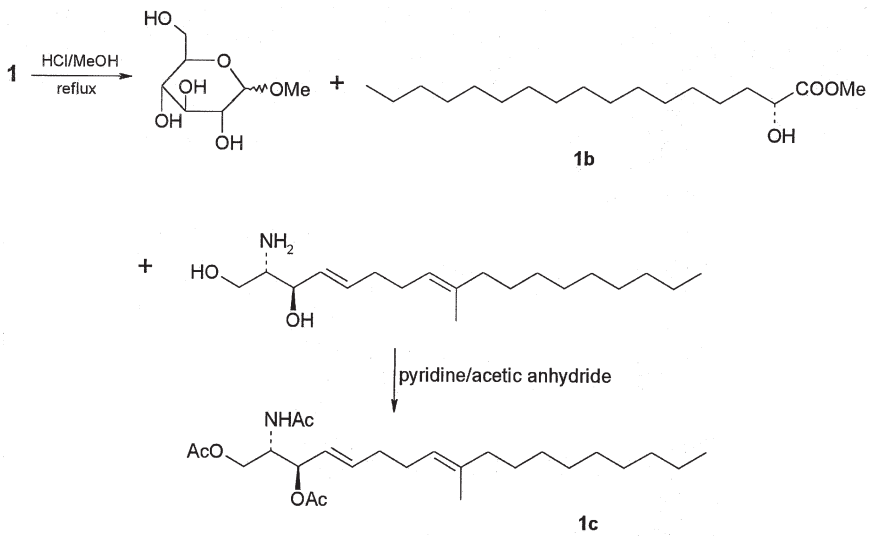
To determine the number of hydroxyl groups, compound **1** was acetylated with acetic anhydride/pyridine at room temperature to give its peracetate derivative **1a**, which showed a molecular ion peak at *m/z* 993 $[M]^+$ in its EI-MS, consistent with the composition $C_{54}H_{91}NO_{15}$ for **1a**. The existence of a fragment ion peak at *m/z* 663 [M – 331 (tetraacetyl hexose)]$^+$ confirmed hexose as the sugar residue. Meanwhile, the EI-MS data of **1a** also displayed the diagnostic fragments of the sugar moiety at *m/z* 331 (base peak), 271, 229, 211, 169, and 109, due to an acetylated glucopyranoside (13). Compound **1a** showed six acetyl signals at δ 2.15 (3H, *s*), 2.09 (3H, *s*), 2.03 (3H, *s*), 2.02 (6H, *s*), and 2.00 ppm (3H, *s*) in the $^1$H NMR spectrum and at δ 20.5 (four *C*$H_3$CO), 20.9 (two *C*$H_3$CO) and at δ ppm 169.3 (two $CH_3$*C*O), 169.6 (three $CH_3$*C*O) and 170.0 (one $CH_3$*C*O) in the $^{13}$C NMR spectrum, respectively. In addition to a fragment ion at *m/z* 663 [M – Glc(OAc)$_4$ + 1]$^+$ **1a** also provided typical fragment ions at *m/z* 933 $[M - HOAc]^+$ and 874 $[M - HOAc - OAc]^+$, thereby confirming the presence of six hydroxyl groups in the original structure of **1**.

On methanolysis (6,9), compound **1** yielded a fatty acid methyl ester, a mixture of α- and β-anomers of methyl glucoside, and an LCB (Scheme 1). The methyl ester **1b** was identified as methyl 2′**-**hydroxyheptanoate by the help of GC–MS analysis, with a molecular ion peak at *m/z* 300, corresponding to the composition $C_{18}H_{36}O_3$. Comparison of the $^1$H NMR and optical rotation data ($[\alpha]_D^{27}$ – 4.5°) with those reported in the literature (11) led us to propose that the relative stereochemistry at C-2′ of the fatty acid methyl ester was *R*. That the optical rotation of the methyl glucoside, $[\alpha]_D^{27}$ +74.2° (determined on the methanolysis product from **1**), was close

to that of the authentic sample, $[\alpha]_D^{25}$ + 77.3° (12), indicated that glucose was present as its D-isomer.

In the $^1$H NMR spectrum of **1** an anomeric signal indicative of the sugar moiety was observed at δ 4.91, and the coupling constant (*d, J* = 7.8 Hz) of this signal suggested the β-configuration of a glucoside linkage. The six oxygenated carbon signals at δ 105.4 (CH), 78.3 (CH), 78.3 (CH), 74.9 (CH), 71.5 (CH), and 62.6 ($CH_2$) in the $^{13}$C NMR spectrum also supported the presence of the β-glucopyranoside moiety in **1** by comparison of the observed and reported chemical shifts (14). In addition, from the heteronuclear multiple bond correlation spectrum, the correlation between H-1″ [δ 4.91 (1H, *d*)] and C-1 [δ 69.9 ($CH_2$)] suggested that the glucose was attached to the C-1 position of the LCB.

The $^1$H NMR data (Table 1) of **1** revealed the presence of two terminal methyls at δ 0.86 (6H, *t, J* = 7.0 Hz), an allylic methyl group at δ 1.61 (3H, *s*, H-19), methylene protons at δ 1.25 (*br s*), an amide proton signal at δ 8.36 (*d, J* = 8.6 Hz), an anomeric proton at δ 4.91 (*d, J* = 7.8 Hz), and carbinol protons appearing as multiplets between δ 3.90 and 4.75. A signal appearing at δ 4.81 (*m*, H-2) was assigned as a methine proton vicinal to the nitrogen atom, clearly suggesting a branched cerebroside containing a 2-hydroxy fatty acid (6,15). Furthermore, **1** was considered to possess a normal type of side chains since the carbon signals due to the terminal methyl groups were observed at δ 14.0 (normal form) in the $^{13}$C NMR spectrum (16). The $^{13}$C NMR spectrum of **1** exhibited carbon signals at δ 175.5 (carbonyl carbon), 54.5 (CHNH, C-2), 22.7–31.9 (methylene carbons), 14.0 (two terminal methyls, C-18 and C-17′), and 15.9 (an allylic methyl group, C-19), which further support the branching glycolipid nature of the molecule. Four olefinic carbon signals observed at δ 124.0 (CH), 131.7 (CH), 132.2 (CH), and 135.6 (quaternary carbon) suggested that **1** possessed two double bonds. In the $^1$H-$^1$H homonuclear correlation spectroscopy spectrum, the correlation between H-4 and H-3, H-4 and H-5, H-5 and H-6, H-6 and H-7, H-7 and H-8 was observed. The above correlation analysis has thus unambiguously assigned the position of the two double bonds at C-4 and C-8, respectively. The analysis was further supported by HMBC spectrum of **1**, which displayed the correlation between H-6 and C-4, H-3 and C-5, H-7 and C-9, H-10 and C-8. On the other hand, the presence of an allylic methyl group (C-19) in the branched LCB was also confirmed by the HMBC spectrum in which the correlation between H-19 and C-8 was observed. The geometry of the C-4/C-5 alkene bond was determined to be *E* by the large vicinal coupling constant (*J* = 15.3 Hz) displayed between H-4 and H-5, as also evidenced by the $^{13}$C NMR chemical shift of the methylene carbon C-6 (δ 32.9) next to the olefinic carbon (15) and the signals of olefinic protons (H-4 and H-5) that appear in the vicinity of δ 5.95 as a multiplet (17). When C-7 methylene protons were irradiated in an nuclear Overhauser effect (NOE) difference experiment, an NOE enhancement of the C-19 methyl protons was observed, arguing that the C-8/C-9 double bond was also assigned *E*. Furthermore, the $^{13}$C NMR chemical shift of the C-19 methyl group (δ 15.9) in turn supported the assignment of this *trans* isomer, as demonstrated by comparison with the chemical shifts of the C-3 methyl groups in *E* (δ 15.4) and *Z* (δ 22.7) isomers of 3-methyl-3-hexene (18). It is thus clear that **1** possesses a branched sphingoid moiety with (4*E*,8*E*) geometry, 2-amino-1,3-dihydroxy-9-methyl-4,8-octadecanediene. In addition, treatment of the methanolysis product of **1** with acetic anhydride/pyridine at 70°C afforded production of a triacetyl LCB **1c**, which we suggest is 2-acetoamino-1,3-diacetoxy-9-methyl-4,8-octadecanediene on the bases of the molecular ion at *m/z* 438 and the $^1$H NMR spectrum, which are consistent with those of the synthetic model compound (19). All of the above spectral evidence further supported that **1** is a cerebroside composed of a (4*E*,8*E*)-2-amino-1,3-dihydroxy-9-methyl-4,8-octadecanediene, (2*R*)-2-hydroxy fatty acid, and β-D-glucopyranose.




**SCHEME 1**

**TABLE 3**
**$^1$H NMR Data and Optical Rotations of Cerebroside 1, Natural 4, Synthetic 5, and Two Derivatives 1a and 6[a]**

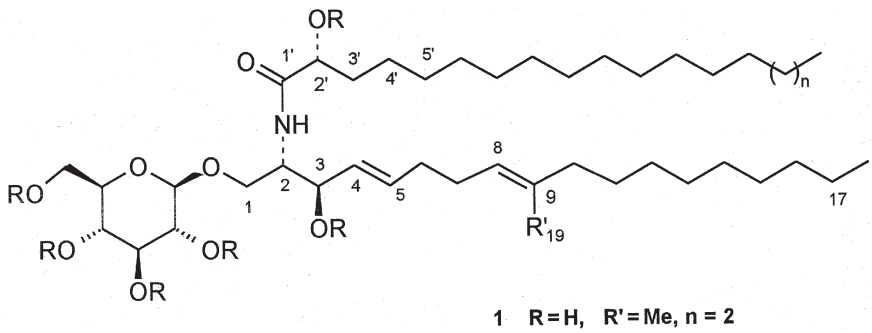
| δ $^1$H | **1** (pyridine-$d_5$) | **4** (pyridine-$d_5$) | **5** (pyridine-$d_5$) | **1a** ($CDCl_3$) | **6** ($CDCl_3$) |
|---|---|---|---|---|---|
| 1-Ha | 4.71 (*dd,* 6.0, 10.5) | 4.71 (*dd,* 5.9, 10.3) | 4.69 (*dd,* 5.4, 10.7) | 3.93 (*dd,* 4.0 10.3) | 4.04 (*dd,* 3.7, 10.2) |
| 1-Hb | 4.23 (*dd,* 6.8, 10.4) | 4.23 (*m*) | 4.20 (*m*) | 3.61 (*dd,* 4.5, 10.3) | 3.64 (*dd,* 4.4, 10.0) |
| 2-H | 4.81 (*m*) | 4.80 (*m*) | 4.76 (*m*) | 4.31 (*m*) | 4.31 (*m*) |
| 3-H | 4.75 (*m*) | 4.75 (*m*) | 4.76 (*m*) | 5.32 (*dd,* 5.3, 6.8) | 5.28 (*m*) |
| 2′-H | 4.57 (*dd,* 5.2, 7.8) | 4.57 (*m*) | 4.57 (*m*) | 5.15 (*dd,* 4.7, 7.1) | 4.95 (*dd,* 5.4, 6.3) |
| $[\alpha]_D$ | +4.9° (MeOH) | +7.0° (*n*-PrOH) | +5.4° (MeOH) | +5.6° ($CHCl_3$) | +8.8° ($CHCl_3$) |

[a]*J* is given in Hz, in parentheses. δ are in ppm. For abbreviation see Table 1

The relative stereochemistry at C-2 and C-3 in **1** was presumed as 2*S*,3*R* (*erythro*) which was shown to be the same as that of the natural cerebrosides **4**, which are phallusides isolated from the ascidian *Phallusia fumigata* (11) and of synthetic glucosyl-(2*S*,3*R*)-sphingadienine **5** (20). The chemical shifts and coupling constants of H-1, H-2, H-3, and H-2′ in **1** and **1a** are in agreement with those of natural **4** and synthetic **5**, synthetic precursor of **5**, (2*S*,3*R*,4*E*,8*E*, 2′*R*)-2-(2′-acetoxyhexadecanoyl)amino-3-*O*-acetyl-1-*O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl)-4,8-octadecadien-1,3-diol **6** (20) (Table 3). Moreover, the specific rotations of **1** ($[\alpha]_D^{26}$ +4.9°) and **1a** ($[\alpha]_D^{26}$ +5.6°) are also in accordance with those of natural **4** ($[\alpha]_D^{26}$ +7.0°) and synthetic **5** ($[\alpha]_D^{20}$ +5.4°) and **6** ($[\alpha]_D^{26}$ +8.8°). These data suggest that **1** has the same absolute configuration as that of natural **4** and synthetic **5** for the core structure at chiral centers 2, 3, and 2′. On the basis of the above evidence, the structure of **1** was therefore established as (2*S*,3*R*,4*E*,8*E*)-1-(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyheptadecanoyl]amino-9-methyl-4,8-octadecadiene (Scheme 2).

Compounds **2** and **3** have the same $^1$H and $^{13}$C NMR (Table 3) data in addition to IR absorptions as **1**, indicating that both **2** and **3** are 9-methyl-sphinga-4,8-dienine-type cerebrosides possessing 2-hydroxy fatty acid and β-D-glucopyranose moieties. The molecular formulas of **2** and **3** were determined as $C_{41}H_{77}NO_9$ and $C_{43}H_{81}NO_9$, respectively, by negative high-resolution FAB-MS and $^{13}$C NMR data. Further methanolysis of both yielded the corresponding fatty acid methyl esters, namely, methyl 2-hydroxy palmitate and methyl 2-hydroxy stearate, which were identified by GC–MS. From the above evidence and comparison of the physicochemical properties with the reported data, compounds **2** and **3** were characterized as (2*S*,3*R*,4*E*,8*E*)-1-(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxypalmitoyl]amino-9-methyl-4,8-octadecadiene (= cerebroside B), and (2*S*,3*R*,4*E*,8*E*)-1-(β-D-glucopyranosyl)-3-hydroxy-2-[(*R*)-2′-hydroxyoctadecanoyl] amino-9-methyl-4,8-octadecadiene (= cerebroside D) (Scheme 2), respectively, which were previously obtained from a basidiomycete, *R. ochroleuca* (8) and an imperfect fungus *Pachybasium* sp. (18); both had antifungal activity.

Sphingolipids are ubiquitous membrane constituents of animals, plants, and also lower forms of life, the principal component of which is the LCB or sphingoid base. In nature, the most widely occurring sphingoid base is D-*erythro*-4(*E*)-sphyingenine, whereas branched (4*E*,8*E*)-sphingadienines having two double bonds in the hydrocarbon chain are minor sphingoid bases. The present study has demonstrated the presence in *P. ellisii* of a previously unrecognized cerebroside and two known cerebrosides, consisting of 9-methyl-4,8-sphingadienine in amide linkage with a hydroxy fatty acid and in β-glycosidic bond with glucose, respectively. The new cerebroside belongs to the first class of GSL (which have their sugar portion linked directly to the ceramide by a glycoside), and contains a fairly unusual dienic LCB with a methyl branch at C-9. This cerebroside has been found in a unique marine microorganism *Thraustochytrium globosum* (15), an




**1** R = H, R' = Me, n = 2
**1a** R = Ac R' = Me, n = 2
**2** R = H, R' = Me, n = 1
**3** R = H, R' = Me, n = 3
**5** R = H, R' = H, n = 1

**SCHEME 2**

imperfect fungus *Pachybasium* sp. (18), a pathogenic fungus *Fusicoccum amygdali* (21), and a marine animal sea anemone *Metridium senile* (22). The natural occurrence of molecules of this species from higher fungi has also been reported (8). Thus, the branched nonadecasphingadienine is presumed to be a characteristic component in cerebrosides from lower organisms. From the viewpoint of comparative biochemistry, it will be of considerable interest to elucidate fully its distribution and also to investigate the physiological significance of the 9-methyl branch, as well as the biosynthetic pathway.

It should be noted that the occurrence of structurally closely related sphingolipid derivatives in taxonomically remote species is very intriguing and may indicate the connection with a common producer, probably symbiotic microorganisms.

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# Method to Produce 9(*S*)-Hydroperoxides of Linoleic and Linolenic Acids by Maize Lipoxygenase

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**ABSTRACT:** Seed from maize (corn) *Zea mays* provides a ready source of 9-lipoxygenase that oxidizes linoleic acid and linolenic acid into 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid and 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid, respectively. Corn seed has a very active hydroperoxide-decomposing enzyme, allene oxide synthase (AOS), which must be removed prior to oxidizing the fatty acid. A simple pH 4.5 treatment followed by centrifugation removes most of the AOS activity. Subsequent purification by ammonium sulfate fractional precipitation results in negligible improvement in 9-hydroperoxide formation. This facile alternative method of preparing 9-hydroperoxides has advantages over other commonly used plant lipoxygenases.

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Oxylipin research often requires the use of 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid [9*S*-HPODE; 9-HPODE is the abbreviation for stereochemically undefined 9-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid]. In our laboratory, 9*S*-HPODE was routinely produced by oxidation of linoleic acid by 9-lipoxygenase (9-LOX) from a tomato fruit extract (1). Although tomato fruit is readily available, this method has a few disadvantages. Extraction of the product by chloroform as outlined by the method often gives intractable emulsions, and a variety of carotenoids co-elute by chromatographic clean-up of 9*S*-HPODE. Further, the use of certain varieties of tomato has given us amounts of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid as high as 15%. In addition, the 1-mM solution of linoleic acid recommended (280 mg/L) gives only partial conversion to 9*S*-HPODE.

Probably because potato is a readily available commodity, potato 9-LOX has often been used to form 9*S*-HPODE (2). Our laboratory has limited experience with potato 9-LOX, but some purification (2) would be required in view of the biotransformations of 9*S*-HPODE known to occur in crude potato extracts (3,4). Although recombinant 9-LOX cloned into bacteria are known, these cultures must be maintained.

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Abbreviations: AOS, allene oxide synthase; CP–HPLC, chiral-phase high-performance liquid chromatography; EI, electron impact; FID–GC, flame-ionization detection–gas chromatography; GC–MS, gas chromatography–mass spectrometry; 9*S*-HPODE, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 9*S*-HPOTE, 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid; 9-LOX, 9-lipoxygenase; mes, 2-(*N*-morpholino)ethane sulfonic acid; OTMSi, trimethylsilyloxy; SP–HPLC, straight-phase high-performance liquid chromatography.

*Zea mays,* maize (corn), seed is another readily available commodity found worldwide. Although LOX from corn was the first 9-LOX identified (5), it has the disadvantage of possessing a highly efficient enzyme, allene oxide synthase (AOS, formerly known as hydroperoxide isomerase). AOS immediately transforms 9*S*-HPODE into 9,10- and 10,13-ketols. AOS can be mostly removed by selective ammonium sulfate precipitation to such an extent that 9*S*-HPODE can be isolated (6), but further improvement in the ease of preparation is desirable. The germs of other cereals, such as rice (7), barley (8) and wheat (9), contain 9-LOX; however, multistep partial purification of these 9-LOX is required.

In this report alternative methods are described to oxidize linoleic acid or linolenic acid into 9*S*-HPODE or 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9*S*-HPOTE), respectively, by facile procedures using either corn germ (embryonic axis plus scutellum) or whole corn seed.

## MATERIALS AND METHODS

*Materials.* The seed source was *Z. mays*, commonly known as maize (international terminology) or corn (U.S.A. terminology). Common field corn, otherwise known as "dent corn," was used. Corn seed preferably was from the most recent harvest. Ear corn, that is, "corn-on-the-cob," is recommended. The cob, upon which the kernels are attached, is a specialized receptacle with archaic origins from a stalk or tassel. In the past, corn was stored in bins as ear corn and permitted to air-dry. Recently, most corn grain is harvested by a combined picker/sheller, and occasionally this grain is dried with heated air. Although not proven, it is believed that ear corn may be a more reliable product, not unduly subjected to variable heat conditions. Ear corn was purchased from a local animal feed store, and then the seed was shelled from the cob by hand.

Almost all of the 9-LOX activity resides in the germ, whereas the endosperm has negligible activity (5). By weight, the germ makes up 10 to 14% of the total kernel and comprises several anatomically distinct tissues that are subcategorized by the general terminology of the embryonic axis plus the scutellum. The germ is identified as an oval depression on one side of the kernel that extends from the base of the kernel to about two-thirds upward. For a detailed explanation of germ tissue, consult the microscopic work of Wolf *et al.* (10). For those investigators who do not choose the tedium of hand-dissecting germ, an alternative method using whole corn kernels is outlined below (Procedure 3).

Linoleic and α-linolenic acids were obtained from Nu-Chek-Prep (Elysian, MN). 2-(*N*-Morpholino)ethane sulfonic acid (mes) buffer was purchased from Research Organics (Cleveland, OH) and was used as the K salt.

*Preparation of 9*S*-HPODE/9*S*-HPOTE: Procedure 1.* Acid treatment (pH 4.5) of a water extract of corn germ followed by centrifugation removed sufficient AOS activity to afford 9*S*-HPODE from 9-LOX activity. Hand-dissected corn germ (2 g of the embryonic axis plus scutellum) was homogenized using a Polytron homogenizer for 1 min at full speed with 20-mL deionized water. The homogenate was adjusted to pH 4.5 ± 0.2 with citric acid (~0.1 M). The acidified homogenate did not require an incubation time, and it was subjected to centrifugation at 10,000 × *g* for 30 min whenever convenient. The floating layer of lipid bodies was aspirated, and the supernatant was carefully decanted. To avoid minor amounts of suspended residue, the supernatant was filtered through a wad of cotton. The supernatant was adjusted to pH 6.5 with KOH (~0.1 M) giving 11–15 mL solution, which was brought up to 19 mL with 50 mM mes buffer at pH 6.5 (final reaction concentration of 10–20 mM mes). Linoleic/linolenic acid substrate (1 mL) (80 mM, 22.4/22.2 mg) and 2.24 wt% Tween 20 at pH 7 ± 0.2 were added to the buffered supernatant (final linoleic/linolenic acid concentration of 4 mM) in a 250-mL Erlenmeyer flask with vigorous magnetic stirring under a stream of pure oxygen for 30 min at room temperature. After reaction, the solution was adjusted to pH 4.5 with 1 M oxalic acid and immediately extracted with 60 mL chloroform/methanol (2:1, vol/vol). The chloroform layer was washed with 15 mL water and then was concentrated by rotary evaporator at about 37°C until nearly dry. The residue plus a small amount of solvent remaining was immediately taken up in about 1 mL of diethyl ether and was stored at –20°C for further processing. It is important to store hydroperoxides in the freezer dissolved in solvent. After chromatographic purification, methanol as a solvent is recommended for long-term storage.

A "control reaction" consisted of the identical treatment as Procedure 1, except the citric acid adjustment of the homogenate to pH 4.5 was omitted.

For preparation of substrate, either 112 mg linoleic acid or 111 mg linolenic acid plus 112 mg Tween 20 was stirred with 1 mL water. Enough 1 M KOH was added to achieve pH 12–13, at which point the solution should clarify. The solution was then acidified with 1 M citric acid to pH 7 to obtain a uniformly cloudy solution. This solution was then brought up to 5 mL with water (80 mM substrate).

*Preparation of 9*S*-HPODE: Procedure 2.* Corn germ was homogenized in water, adjusted to pH 4.5, centrifuged, and the pH of the supernatant was readjusted to 6.5 as in Procedure 1 described above. The supernatant was adjusted to 40% of $(NH_4)_2SO_4$ saturation and centrifuged at 10,000 × *g* for 30 min. This supernatant was adjusted to 60% $(NH_4)_2SO_4$ saturation and centrifuged as above. The pellet obtained (40 to 60% of saturation) was brought up to 19 mL with 50 mM mes buffer (pH 6.5). As described in Procedure 1, addition of 1 mL substrate (80 mM, 22.4 mg linoleic acid) containing 2.24 wt% Tween 20 commenced the reaction. The final reaction concentrations were 47.5 mM mes, 4 mM substrate, and 0.112 wt% Tween 20. Recovery of product was identical to Procedure 1.

*Preparation of 9*S*-HPODE: Procedure 3.* Whole corn kernels (15 g) were ground into a meal with an electric coffee grinder. The corn meal was homogenized with 25 mL water using a Polytron homogenizer (full speed for 1 min). The homogenate was adjusted to pH 4.5 with citric acid and centrifuged as in Procedure 1. As in Procedure 1, no particular incubation time at pH 4.5 was required. Usually 9–10 mL of supernatant was obtained which was diluted to 19 mL with mes buffer, pH 6.5. Substrate solution (1 mL) was added as before, and the reaction and recovery of product were the same as the other procedures. The final reaction concentrations were ~25 mM mes, 4 mM substrate, and 0.112 wt% Tween 20.

*Analytical methods.* Activity as a function of pH (4.5 to 8.25) was determined with 50 mM $KH_2PO_4/Na_2HPO_4$ buffer. Activity assays were accomplished by oxygen electrode (Gilson 5/6H oxygraph; Middleton, WI). Temperature was 25°C. Substrate was 1 mM linoleic acid emulsified with 0.028 wt% Tween 20.

In order to determine the yield of either linoleic or linolenic acid conversion, an aliquot of the extracted products was reduced by $NaBH_4$ in 1 mL methanol for 30 min. To extract products, 3 mL $CHCl_3/H_2O$ (2:1, vol/vol) was added, and the upper aqueous layer was adjusted to pH 4 with 1 M oxalic acid. The recovered $CHCl_3$ layer was washed with water, and the fatty acids extracted by $CHCl_3$ were esterified by diazomethane in diethyl ether/methanol (9:1, vol/vol) followed by formation of trimethylsilyloxy ethers (OTMSi) by pyridine/hexamethyldisilazane/trimethylchlorosilane (2:2:3, by vol) for 15 min. The reagent was evaporated, and the residue was taken up in hexane for determination of products by flame-ionization detection–gas chromatography (FID–GC) using a Hewlett-Packard model 5890 gas chromatograph (Palo Alto, CA). The capillary column used was an SPB-1 (dimethyl polysiloxane phase, 0.32 mm × 30 m, film thickness 0.25 μm) from Supelco (Bellefonte, PA). Temperature programming was from 160 to 260ºC at 5ºC/min with a hold at 260ºC for 5 min; He flow was 2 mL/min. The identity of the FID–GC peaks was confirmed by GC–mass spectrometry (MS) using a Hewlett-Packard Model 5890 gas chromatograph interfaced with a model 5971 mass-selective detector operating at 70 ev. The capillary column utilized was a Hewlett-Packard HP-5MS cross-linked 5% phenyl methyl silicone (0.25 mm × 30 m, film thickness 0.25 μm). Temperature programming was identical to the conditions used for FID–GC; He flow rate was 0.67 mL/min. The mixture containing 9*S*-HPOTE was also hydrogenated with $H_2$ and a catalyst (5% Pd on $CaCO_3$; Aldrich Chemical Co., Milwaukee, WI) for 1 h. The methyl ester/OTMSi derivative was produced as described above for analysis by GC–MS.

As outlined (11), regio- and stereoconfigurations were determined by straight-phase high-performance liquid chromatography (SP-HPLC) (regio-analysis) followed by stereo-

analysis by chiral-phase HPLC (CP-HPLC) of the methyl hydroxyoctadecadienoate derivative. The stereoconfiguration of 9*S*-HPOTE and 9*S*-HPODE was ascertained by an independent method of forming diastereomeric (–)-menthylcarbonyl derivatives (12) modified to use $KMnO_4$ in acetic acid in the place of oxidative ozonolysis (13). The (–)-menthylcarbonyl derivative of dimethyl 2-hydroxy-decanedioate from oxidative cleavage was separated into 2(*S*) and 2(*R*) isomers by GC (14).

## RESULTS

*Source of corn 9-LOX and pH optimum.* A full description of the source of maize (corn) used in the preparation of 9-LOX is outlined in the Materials and Methods section. In addition, a rationale is given for choosing between corn germ or whole corn kernels.

By use of the unpublished procedure of H.W. Gardner and D.D. Christianson, corn germ 9-LOX was purified 320-fold (reviewed in Ref. 15) in order to obtain a pH activity optimum at 6.5 (Fig. 1). Although LOX activity was shown to be very slight above pH 8.3, Veldink *et al.* (16) reported that a corn-germ preparation oxidized linoleic acid to mainly the 13(*S*)-hydroperoxide at pH 9. Whether this oxidation was promoted by higher pH or the result of another LOX isoenzyme is not known. In this method an optimum of pH 6.5 was maintained to favor the formation of 9*S*-HPODE.

*Procedure 1.* A simple water extraction of corn germ, adjustment to pH 4.5 with citric acid, and centrifugation removed most of the AOS activity. Thus, readjusting the pH with KOH and mes to the optimum pH 6.5 for corn 9-LOX and incubation with 4 mM linoleic acid afforded 9*S*-HPODE in good yield (70 ± 21%), while AOS-produced ketols amounted to only 4 ± 3% of the total (Table 1).

*Procedure 2.* After acidification and centrifugation by Procedure 1, further purification of corn 9-LOX with fractional precipitation between 40 and 60% of ammonium sulfate saturation resulted in an insignificant difference in yield of 9*S*-HPODE compared to Procedure 1 (Table 1).

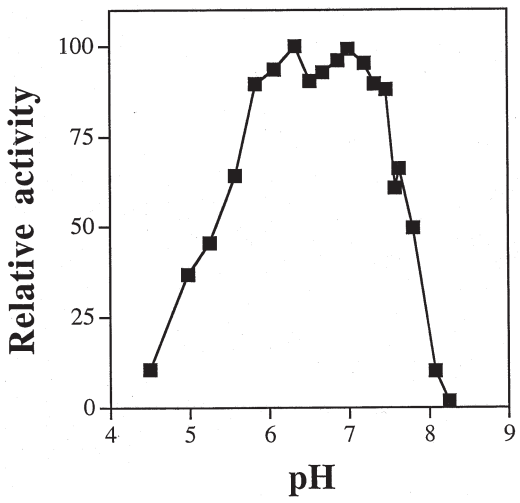


**FIG 1.** Corn-germ lipoxygenase activity (relative values) as a function of pH (50 mM $Na_2HPO_4/KH_2PO_4$ buffer).

**TABLE 1**
**Percentage Yield of 9-Hydroperoxide, Ketols, and Unreacted Linoleic Acid[a]**

| Procedure[b] | 9*S*-HPODE | Linoleic acid | α- Plus γ-ketols | Other[c] |
|---|---|---|---|---|
| 1 | 70 ± 21 | 22 ± 23 | 4.0 ± 3.4 | 3.7 ± 4.0 |
| 2 | 69 ± 25 | 15 ± 29 | 7.9 ± 8.8 | 7.1 ± 4.4 |
| 3 | 55 ± 17 | 31 ± 19 | 11 ± 20 | 1.9 ± 1.0 |
| Control[d] | 2.1 ± 0.2 | 18 ± 10 | 75 ± 7 | 4.7 ± 3.5 |

[a]Weight percentage determined by flame-ionization detection–gas chromatography of $NaBH_4$-reduced products after conversion to methyl esters/trimethylsilyloxy (OTMSi) ethers. Values are means and standard deviation (Procedure 1, $n = 6$; Procedure 2, $n = 4$; Procedure 3, $n = 5$; Control, $n = 2$). Each oxidation represents a 20 mL reaction containing 4 mM linoleic acid (22.4 mg linoleic acid). On a molar basis, the values would be modified by their molecular weight: 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9*S*-HPODE) as methyl 9-OTMSi-octadecadienoate, 382 mg/mmol; linoleic acid as methyl linoleate, 294 mg/mmol; α- plus γ-ketols as methyl 9,10-diOTMSi- and 10,13-diOTMSi-octadecenoate, 472 mg/mmol.
[b]Procedure 1: homogenized corn germ adjusted to pH 4.5, centrifuged, decanted, and readjusted to pH 6.5. Procedure 2: same as Procedure 1, except additionally fractionated by $(NH_4)_2SO_4$ (40 to 50% of saturation). Procedure 3: same as Procedure 1, except whole corn meal replaced corn germ.
[c]"Other" were isomeric HPODE other than 9*S*-HPODE, as well as oxidized fatty acids more polar than ketols.
[d]"Control reaction," that is, a water extract of corn germ adjusted to pH 6.5 with 2-(*N*-morpholino)ethane sulfonic acid; no pH 4.5 treatment.

*Procedure 3.* A comparatively larger quantity of whole corn was pretreated by grinding into a meal before using whole corn meal in the place of corn germ by Procedure 1. This method resulted in slightly less oxidation of linoleic acid as expected (Table 1). However, it eliminated the time required to dissect the germ. Further purification of the whole-corn preparation by $(NH_4)_2SO_4$ precipitation (40–60% of saturation; method not listed) did not significantly improve the method (53 ± 16% 9*S*-HODE, 42 ± 16% linoleic acid, 1.3 ± 1.1% ketols).

*Regio- and stereoconfiguration of 9-LOX product.* As shown in Table 2, the stereoconfiguration of the 9-LOX product was predominantly 9*S*-HPODE (97%) as determined by SP-HPLC followed by CP-HPLC. Chemical determination of stereoconfiguration was confirmatory (Table 2).

*Formation of 9S-HPOTE.* The oxidation of linolenic acid by corn germ has not been reported previously. Procedure 1 afforded a yield of 9*S*-HPOTE of 69%, with the remainder being unreacted linolenic acid (27%), AOS-generated ketols (3%), and other fatty acids (1%). The electron impact–mass spectrum (EI–MS) of the methyl esterified, $NaBH_4$-reduced, OTMSi derivative was as follows in *m/z* [ion structure] (% ion intensity): 380 $[M]^+$ (0.6), 365 $[M - CH_3]^+$ (0.2), 311 $[M - CH_3CH_2CH{=}CHCH_2]^+$ (30), 290 $[M - TMSiOH]^+$ (3), 223 $[M - (CH_2)_7COOCH_3]^+$ (44), 157 (12), 154 (10), 143 (15), 130 (17), 108 (23), 103 (20), 73 $[TMSi]^+$ (100). Since EI–MS of the unsaturated derivative did not precisely locate the position of the hydroxyl, the product mixture was hydrogenated and then examined by EI–MS as the methyl ester/OTMSi derivative, affording the precise location of the hydroxyl at C-9: 371

**TABLE 2**
**Regio- and Stereoconfiguration of Hydroperoxides from Corn Lipoxygenase**

| | 9-Hydroperoxides | | 13-Hydroperoxides | |
|---|---|---|---|---|
| Substrate | (10*E*,12*Z*) | (10*E*,12*E*)[a] | (9*Z*,11*E*)[a] | (9*E*,11*E*)[a] |
| Linoleic acid | | | | |
| HPLC[b] | 93.3 ± 1.7 | 1.52 ± 0.37 | 2.67 ± 1.17 | 2.68 ± 0.82 |
| | 9*S* | 9*R* | | |
| | 97 ± 3 | 3 ± 3 | | |
| Chemical[c] | 96.2 | 3.8 | | |

| | 9-Hydroperoxide (10*E*,12*Z*,15*Z*) | | Other isomers |
|---|---|---|---|
| Linolenic acid | | | |
| HPLC[b] | 96.5 | | 3.5 |
| | 9*S* | 9*R* | |
| | 99.3 | 0.7 | |
| Chemical[c] | 99 | <1 | |

[a]Stereoconfiguration of 13-hydroperoxides and (*E,E*)-isomers was not determined.
[b]Analysis by straight-phase–high-performance liquid chromatography (HPLC) and chiral-phase–HPLC of methyl hydroxyoctadecadienoates from triphenylphosphine reduction and methyl esterification of hydroperoxide products. For statistical analysis of linoleic acid products, $n = 8$.
[c]Analysis of stereoconfiguration of 9*S*-HPODE and 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid by a chemical/gas chromatography method (11). For abbreviation see Table 1.

$[M - CH_3]^+$ (2), 355 $[M - CH_3O]^+$ (3), 339 $[M - CH_3OH - CH_3]^+$ (6), 259 $[CHOTMSi(CH_2)_7COOCH_3]^+$ (100), 229 $[CH_3(CH_2)_8CHOTMSi]^+$ (76), 155 (15), 129 (9), 109 (10), 103 (10), 73 $[TMSi]^+$ (49).

The stereoconfiguration of 9*S*-HPOTE examined after triphenylphosphine reduction gave a chiral purity better than 99% 9(*S*) (Table 2).

## DISCUSSION

Although it was not specifically investigated, it is presumed that AOS precipitated after acid treatment. Because AOS is largely membrane-bound in corn seed, care should be taken to carefully aspirate the floating oil-body layer and decant and filter the supernatant after centrifugation. Accidental inclusion of precipitate may account for the variability in AOS-generated ketols in the products, which occasionally were higher than usual. An additional treatment with ammonium sulfate fractionation would give additional protection against AOS inclusion (6), but it can be seen from Table 1 that there was no significant improvement. The largest variability in yield was due to the relative percentages of unreacted substrate and hydroperoxide product because of inconsistent recovery of LOX activity.

*Chromatographic purification of 9S-HPODE.* SP–HPLC separation of fatty acid hydroperoxides has been reported using hexane/2-propanol/acetic acid eluting solvent (17). Although acetic acid in the solvent is satisfactory for analytical applications, it may be a problem in preparative applications. Strong acids are known to decompose linoleic acid hydroperoxides (18). Weak acids, such as acetic acid, are far less damaging, but should be a concern when they are concentrated by evaporation. For this reason, the use of a pH 4 silica is preferred in order to suppress ionization of the carboxylic acid without the use of organic acid. Such supports are available for ordinary column chromatography, such as SilicAR CC4 (Mallinckrodt, Phillipsburg, NJ) or silicic acid (100 mesh, Mallinckrodt). Normally, elution by a stepwise or gradient of 5 to 10% acetone in hexane is satisfactory (19), but we found that α-ketol has a propensity to migrate with 9*S*-HPODE in this solvent. However, a gradient of diethyl ether in hexane has been shown to be satisfactory in separating 9*S*-HPODE from α-ketol (5). This method excludes unreacted substrate and AOS-produced ketols but only partly separates hydroperoxide regio-isomers. Early-eluting 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid can be largely removed by discarding the very early portion of the eluting hydroperoxide peak. In our laboratory we usually apply 0.5–0.7 g crude oxidation product to a 2.5 cm i.d. column containing 50 g silicic acid. Very often losses, including those from selective collection of fractions, result in recovery of about 50% of the original hydroperoxide product. Appropriate scaleup of the reaction reported here would be required to obtain 0.5–0.7 g crude product; however, attention must be given to surface area and stirring in order to promote sufficient oxygen exchange.

SP–HPLC on silica columns is more easily accomplished with methyl esters. Because fatty acid hydroperoxides are decomposed by acid-catalyzed methylation, including alkali-catalyzed transmethylation, only diazomethane is suitable for methyl esterification. As originally reported by Chan and Prescott (20), purification of the methyl-esterified 9-HPODE was readily accomplished by SP–HPLC on silica columns using elution with hexane and 1% or less of either ethanol or 2-propanol.

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# Synthetic Routes and Lipase-Inhibiting Activity of Long-Chain α-Keto Amides

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**ABSTRACT:** Synthetic routes to primary and *N*-alkyl α-keto amides are presented in this paper. Primary α-keto amides may be prepared by using an aldehyde as starting material. Commercially available α-keto acids may be coupled in high yield with primary amines by the mixed carbonic anhydride method affording *N*-alkyl α-keto amides. Alternatively, *N*-alkyl α-keto amides may be prepared by coupling long-chain α-hydroxy acids with amino components, followed by oxidation with pyridinium dichromate or NaOCl in the presence of 4-acetamido-2,2,6,6-tetramethyl-1-piperidinyloxy free radical. The α-keto amide derivatives prepared according to these procedures were tested for their ability to form stable monomolecular films at the air/water interface. The inhibition of porcine pancreatic lipase by the α-keto amides, spread as mixed films with 1,2-dicaprin, was studied with the monolayer technique. Among the compounds tested in this study, methyl 2-[(2-ketododecanoyl)amino]hexadecanoate was shown to be the most potent inhibitor, causing a 50% decrease in lipase activity at a 0.09 molar fraction.

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Inhibitors of pancreatic lipase hold great promise as antiobesity agents, because nonhydrolyzed triacylglycerols cannot be absorbed through the intestine and thus cannot contribute to the energy intake of the body. The β-lactone-containing inhibitor tetrahydrolipstatin is already a registered drug for the treatment of obesity (1,2). On the other hand, synthetic inhibitors of lipases are of interest because they may contribute to a better understanding of the mechanism of lipase action (3–5). Alkyl phosphonates of simple or more complicated structure are the main representative class of such synthetic inhibitors (6–10).

The enzymatic mechanism as well as the catalytic site residues of pancreatic lipase are similar to those of serine proteases, consisting of a Ser-His-Asp triad. Many inhibitors of serine proteases consist of a substrate-like structure incorporating an activated carbonyl group at the site of the scissile amide bond. A number of reactive carbonyl groups, such as fluorinated ketones (11), α-keto esters (12), α-keto amides (13) and α-keto heterocycles (14), have been successfully used in the design of protease inhibitors.

Synthetic compounds containing electrophilic carbonyl groups, such as fatty alkyl trifluoromethyl ketones (15,16) and tricarbonyl derivatives of arachidonic and palmitic acids (17), have been reported to inhibit cytosolic phospholipase $A_2$ and calcium-independent phospholipase $A_2$, enzymes containing a Ser residue in their active site. Recently, we reported that a lipophilic α-keto amide inhibits pancreatic lipase (18). In this paper we describe synthetic routes to primary and *N*-alkyl α-keto amides. Furthermore, we present that long-chain α-keto amides of simple structure may inhibit pancreatic lipase.

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Abbreviations: AcNH-TEMPO, 4-acetamido-2,2,6,6-tetramethyl-1-piperidinyloxy; COSY, correlation spectrometry; $Et_3N$, triethylamine; FAB, fast atom bombardment; HOBt, 1-hydroxybenzotriazole; IR, infrared; MS, mass spectrometry; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; PDC, pyridinium dichromate; PPL, porcine pancreatic lipase; THF, tetrahydrofuran; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; WSCI, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide.

## EXPERIMENTAL PROCEDURES

*Materials and methods.* 1,2-Dicaprin was purchased from Serdary Research Laboratory (London, Canada). 4-Acetamido-2,2,6,6-tetramethyl-1-piperidinyloxy free radical (AcNH-TEMPO) was purchased from Aldrich (Milwaukee, WI). Analytical thin-layer chromatography plates (silica gel 60 $F_{254}$) and silica gel 60 (70–230 mesh) were purchased from Merck (Darmstadt, Germany). Porcine pancreatic lipase (PPL) was purified at the laboratory using previously described procedures (19). Tetrahydrofuran (THF) was passed through a column of aluminum oxide and distilled from $CaH_2$. Isobutyl chloroformate was distilled and stored over $CaCO_3$. *N*-Methylmorpholine (NMM) and triethylamine ($Et_3N$) were distilled from ninhydrin. All other solvents and chemicals were of reagent grade and used without further purification. Melting points were determined on a Büchi 530 apparatus and are uncorrected. Infrared (IR) spectra were recorded on a PerkinElmer 841 spectrometer (Palo Alto, CA). $^1H$ nuclear magnetic resonance (NMR), $^{13}C$ NMR, distortionless enhancement by polarization transfer, correlation spectrometry (COSY) and $^{13}C$-$^1H$ COSY spectra were obtained in $CDCl_3$ using a Varian Mercury (200 MHz) or Varian Unity (300 MHz) spectrometer (Palo Alto, CA). Fast atom bombardment (FAB) mass spectra were obtained on a VG Analytical ZAB-SE instrument (Manchester, United Kingdom). Elemental analyses were performed on a PerkinElmer 2400 instrument. Monomolecular film experiments as well as enzyme kinetics experiments were performed with a KSV 2200 Barostat equipment (KSV-Helsinki, Finland).

*Synthetic procedures. (i) 2-Hydroxypentadecanenitrile (**2**).* To a stirred solution of tetradecanal (0.64 g, 3.0 mmol) in $CH_2Cl_2$ (4 mL), $NaHSO_3$ (0.47 g, 4.5 mmol) in water (1 mL) was added at room temperature. A white solid started to precipitate upon the addition. After stirring for 30 min at room temperature, $CH_2Cl_2$ was evaporated under reduced pressure, and water (3 mL) was added. To the resulting suspension, cooled at 0°C, a solution of KCN (0.29 g, 4.5 mmol) in $H_2O$ (1 mL) was added dropwise over a period of 3 h at 0°C. After

stirring at room temperature overnight, $CH_2Cl_2$ was added followed by extraction. The organic phase was washed with brine solution and dried ($Na_2SO_4$). The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography using petroleum ether 40–60°C/EtOAc (9:1, vol/vol) as eluent. Yield 0.545 g (76%); m.p. 53.5–55°C; $^1$H NMR (300 MHz) δ 4.5 (1H, *m*, CHCN), 2.4 (1H, *br*, OH), 1.8 (2H, *m*, C$H_2$CH), 1.6–1.3 (22H, *m*, 11 × $CH_2$), 0.9 (3H, *t, J* = 7 Hz, $CH_3$); $^{13}$C NMR (75 MHz) δ 120.03 (CN), 61.34 (*C*HCN), 35.18 [*C*$H_2$CH(OH)], 32.00, 29.72, 29.56, 29.43, 29.01, 24.62, 22.77 (*C*$H_2$CH$_3$), 14.20 ($CH_3$); IR (ν cm$^{-1}$, KBr): 3397 (O–H), 2254 (C≡N), 1074 (C–O); FAB-mass spectrometry (MS) *m/z* 262 (M + $Na^+$, 100%), 240 (M + $H^+$, 21%), 222 (21), 213 (97). Calc. for $C_{15}H_{29}NO$: C 75.26; H 12.21; N 5.82; MW 239.40. Found: C 75.12; H 12.51; N 5.93.

*(ii) 2-Hydroxypentadecanamide (**3**).* A suspension of compound **2** (0.48 g, 2.0 mmol) in concentrated hydrochloric acid (5 mL) was stirred at room temperature overnight. $CHCl_3$ (3 × 30 mL) was added, and the organic phase was washed with brine solution and dried ($Na_2SO_4$). The solvent was evaporated to a small volume and the white solid that precipitated after cooling was filtered and dried. Yield 0.345 g (67%); m.p. 151–152°C; $^1$H NMR (300 MHz) δ 4.5 (1H, *m*, C*H*CONH$_2$), 2.8 (1H, *m*, OH), 1.9–1.6 (2H, *m*, C$H_2$CH), 1.6–1.3 (22H, *m*, 11 × $CH_2$), 0.9 (3H, *t, J* = 7 Hz, $CH_3$); IR (ν cm$^{-1}$, KBr): 3384 and 3278 (NH, OH), 1639 (C=O), 1098 (C–O); FAB-MS *m/z* 280 (M + $Na^+$, 13%), 258 (M + $H^+$, 100%). Calc. for $C_{15}H_{31}NO_2$: C 69.99; H 12.14; N 5.44; MW 257.41. Found: C 70.22; H 12.43; N 5.63.

*General procedure for the amide bond formation* via *the mixed anhydride method.* To a stirred solution of the appropriate α-keto acid (1.0 mmol) in THF (5 mL) cooled at −10°C, NMM (0.11 mL, 1 mmol) and subsequently isobutyl chloroformate (0.13 mL, 1.0 mmol) were added dropwise. After stirring for 5 min at −10°C, an ice-cooled solution of the amino component (1.0 mmol) in THF (5 mL) was added dropwise. The reaction mixture was stirred for 1 h at −10°C and then at room temperature overnight. The solvent was evaporated and the residue was dissolved in EtOAc. The organic phase was washed with water, 0.5 N HCl, water, 5% $NaHCO_3$, brine solution, and dried ($Na_2SO_4$). The solvent was removed under reduced pressure and the residue was purified by column chromatography using petroleum ether 40–60°C/EtOAc (8:2, vol/vol) as eluent.

N-*hexadecyl-2-ketobutanamide (**7a**)*. Yield 0.296 g (91%); m.p. 49–50°C; $^1$H NMR (300 MHz) δ 6.9 (1H, *br*, CONH), 3.3 (2H, *m*, C$H_2$NH), 2.9 (2H, *q, J* = 7 Hz, C$H_2$CO), 1.6 (2H, *m*, C$H_2$CH$_2$NH), 1.3 (26H, *m*, 13 × $CH_2$), 1.1 (3H, *t, J* = 7 Hz, C$H_3$CH$_2$CO), 0.9 (3H, *t, J* = 7 Hz, $CH_3$). Calc. for $C_{20}H_{39}NO_2$: C 73.79; H 12.08; N 4.30; MW 325.54. Found: C 73.95; H 12.36; N 4.22.

N-*Hexyl-2-ketooctanamide (**7b**)*. Yield 0.210 g (87%); oil; $^1$H NMR (300 MHz) δ 6.9 (1H, *br*, CONH), 3.3 (2H, *m*, C$H_2$NH), 2.9 (2H, *t, J* = 7 Hz, C$H_2$CO), 1.5 (4H, *m*, C$H_2$CH$_2$CO, C$H_2$CH$_2$NH), 1.3 (12H, *m*, 6 × $CH_2$), 0.9 (6H, *m*, 2 × $CH_3$).

N-*hexadecyl-2-ketooctanamide (**7c**)*. Yield 0.328 g (86%); m.p. 60–61°C; $^1$H NMR (300 MHz) δ 6.9 (1H, *br,* CONH), 3.3 (2H, *m*, C$H_2$NH), 2.9 (2H, *t, J* = 7 Hz, $CH_2$CO), 1.5 (4H, *m*, C$H_2$CH$_2$NH, C$H_2$CH$_2$CO), 1.3 (32H, *m*, 16 × $CH_2$), 0.9 (6H, *m*, 2 × $CH_3$). Calc. for $C_{24}H_{47}NO_2$: C 75.53; H 12.41; N 3.67; MW 381.64. Found: C 75.70; H 12.78; N 3.42.

N-cis-*9-Octadecenyl-2-ketooctanamide (**7d**)*. Yield 0.367 g (90%); oil; $^1$H NMR (200 MHz) δ 6.9 (1H, *br*, CONH), 5.4 (2H, *m*, CH=CH), 3.3 (2H, *m*, C$H_2$NH), 2.9 (2H, *t, J* = 7 Hz, $CH_2$CO), 2.0 (4H, *m*, C$H_2$CH=CHC$H_2$), 1.6 (4H, *m*, C$H_2$CH$_2$NH, C$H_2$CH$_2$CO), 1.3 (28H, *m*, 14 × $CH_2$), 0.9 (6H, *m*, 2 × $CH_3$); $^{13}$C NMR (50 MHz) δ 199.47 (*C*OCONH), 160.09 (CONH), 129.92 and 129.68 (CH=CH), 39.28 ($CH_2$NH), 36.67 (*C*$H_2$CO), 32.55, 31.86, 31.47, 29.72, 29.66, 29.48, 29.35, 29.28, 29.22, 29.16, 28.89, 27.17, 27.12, 26.81, 23.13, 22.64 and 22.41 (*C*$H_2$CH$_3$), 14.06 and 13.95 ($CH_3$); FAB-MS *m/z* 409 (M + 2$H^+$, 100%), 269 (96).

*2-Keto*-N-*{2-[(2-ketobutanoyl)amino]ethyl}butanamide (**8a**)*. Yield 0.162 g (71%); m.p. 153–155°C; $^1$H NMR (300 MHz) δ 3.5 (4H, m, C$H_2$NH), 2.9 (4H, *q, J* = 7 Hz, $CH_2$CO), 0.9 (6H, *t, J* = 7 Hz, 2 × $CH_3$). Calc. for $C_{10}H_{16}N_2O_4$: C 52.62; H 7.07; N 12.27; MW 228.25. Found: C 52.94; H 7.12; N 11.95.

*2-Keto*-N-*{2-[(2-ketooctanoyl)amino]ethyl}octanamide (**8b**)*. Yield 0.269 g (79%); m.p. 121–122°C; $^1$H NMR (300 MHz) δ 3.5 (4H, *m*, C$H_2$NH), 2.9 (4H, *t, J* = 7 Hz, $CH_2$CO), 1.6 (4H, *m*, C$H_2$CH$_2$CO), 1.3 (12H, *m*, 6 × $CH_2$) 0.9 (6H, *t, J* = 7 Hz, 2 × $CH_3$). Calc. for $C_{18}H_{32}N_2O_4 \cdot 0.5H_2O$: C 61.86; H 9.52; N 8.02; MW 349.47. Found: C 62.22; H 9.62; N 7.73.

*General procedure for the deamination of lipidic α-amino acids.* To a stirred suspension of the lipidic α-amino acid (10 mmol) in 1 M $H_2SO_4$ (120 mL), a solution of $NaNO_2$ (1.45 g, 21 mmol) in water (38 mL) was added dropwise over a period of 2 h with heating at 80–90°C. The mixture was stirred for 3 h at 80–90°C and overnight at room temperature. It was then extracted with $Et_2O$ (4 × 200 mL), and the combined organic layers were washed with brine solution and dried ($Na_2SO_4$). The solvent was evaporated under reduced pressure, and the residue was purified by recrystallization from $CHCl_3$.

*2-Hydroxydodecanoic acid (**10a**)*. Yield 1.12 g (52%); m.p. 71–73°C; $^1$H NMR (200 MHz) δ 4.3 (1H, *m*, C*H*COOH), 1.8 (2H, *m*, C$H_2$CH), 1.3 (16H, *m*, 8 × $CH_2$), 0.9 (3H, *t, J* = 7 Hz, $CH_3$); IR (ν cm$^{-1}$, KBr): 3518 (OH), 3600–2500 (COOH), 1701 (C=O).

*2-Hydroxyhexadecanoic acid (**10b**)*. Yield 1.28 g (47%); m.p. 82–84°C. The NMR spectrum of **10b** was not obtained because of its low solubility in any of the deuterated solvents.

*General procedure for amide bond formation* via *the water-soluble carbodiimide method.* To a stirred suspension of the appropriate α-hydroxy acid (2.0 mmol) and amine (2.0 mmol) in $CH_2Cl_2$ (8 mL), $Et_3N$ (0.31 mL, 2.2 mmol) and subsequently 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (WSCI: 0.42 g, 2.2 mmol) and 1-hydroxybenzotriazole (HOBt: 0.27 g, 2.0 mmol) were added at 0°C. The reaction mixture was stirred for 1 h at 0°C and overnight at room temperature. The organic layer was washed with brine solution, dried ($Na_2SO_4$), and

evaporated under reduced pressure. The residue was purified by column chromatography using petroleum ether 40–60°C/EtOAc (3:2, vol/vol) as eluent.

N-*Hexyl-2-hydroxyhexadecanamide (**11a**)*. Yield 0.511 g (72%); m.p. 96–97°C; FAB-MS *m/z* 378 (M + $Na^+$, 5%), 356 (M + $H^+$, 100%). Calc. for $C_{22}H_{45}NO_2 \cdot 0.5H_2O$: C 72.47; H 12.72; N 3.84; MW 364.62. Found: C 72.49; H 12.90; N 3.55.

N-*Hexadecyl-2-hydroxyhexadecanamide (**11b**)*. Yield 0.525 g (53%); m.p. 94–96°C; $^1$H NMR (200 MHz) δ 6.4 (1H, *br*, CONH), 4.1 (1H, *m*, C*H*OH) 3.3 (2H, *m*, C$H_2$NH), 2.4 (1H, *br*, OH), 1.9–1.5 (4H, *m*, C$H_2$CHOH, C$H_2$C$H_2$NH), 1.3 (50H, *m*, 25 × $CH_2$) 0.9 (6H, *m*, 2 × $CH_3$); FAB-MS *m/z* 496 (M + $H^+$, 100%). Calc. for $C_{32}H_{65}NO_2 \cdot H_2O$: C 75.45; H 13.16; N 2.75; MW 513.90. Found: C 75.22; H 13.00; N 2.83.

N-cis-*9-Octadecenyl-2-hydroxyhexadecanamide (**11c**)*. Yield 0.699 g (67%); m.p. 80–81°C; $^1$H NMR (200 MHz) δ 6.5 (1H, *br*, CONH), 5.3 (2H, *m*, CH=CH), 4.1 (1H, *m*, C*H*OH) 3.3 (2H, *m*, C$H_2$NH), 2.6 (1H, *br*, OH), 2.0 (4H, *m*, C$H_2$CH=CHC$H_2$), 1.9–1.5 (4H, *m*, C$H_2$C$H_2$NH, C$H_2$CHOH), 1.3 (46H, *m*, 23 × $CH_2$), 0.9 (6H, *m*, 2 × $CH_3$); $^{13}$C NMR (50 MHz) δ 173.72 (CONH), 129.95 and 129.74 (CH=CH), 72.14 (CHOH), 39.12 ($CH_2$NH), 34.98 (*C*$H_2$CHOH), 31.91, 29.68, 29.65, 29.58, 29.54, 29.42, 29.35, 29.31, 29.25, 27.19, 26.88, 24.95, 22.67 (*C*$H_2$$CH_3$), 14.10 ($CH_3$). Calc. for $C_{34}H_{67}NO_2 \cdot 0.5H_2O$: C 76.92; H 12.91; N 2.64; MW 530.92. Found: C 76.92; H 13.08; N 2.68.

*Methyl 2-[(2-hydroxydodecanoyl)amino]hexadecanoate (**11d**)*. Yield 0.639 g (66%); m.p. 61–63°C; $^1$H NMR (200 MHz) δ 6.9 (1H, *d, J* = 8 Hz, CONH), 4.6 (1H, *m*, C*H*NH), 4.2 (1H, *m*, C*H*OH), 3.8 (3H, *s*, $COOCH_3$), 2.7 (1H, *br*, OH), 2–1.6 (4H, *m*, C$H_2$CHOH, C$H_2$CHNH), 1.3 (40H, *m*, 20 × $CH_2$), 0.9 (6H, *m*, 2 × $CH_3$); $^{13}$C NMR (50 MHz) δ 173.60 and 172.87 (CONH, *C*OOC$H_3$), 72.00 (CHOH), 52.34 (COO*C*$H_3$), 51.81 (CHNH), 34.87 and 32.36 (*C*$H_2$CHNH, *C*$H_2$CHOH), 31.89, 29.64, 29.58, 29.52, 29.38, 29.35, 29.16, 25.25, 24.81, 22.67 (*C*$H_2$$CH_3$), 14.11 ($CH_3$). Calc. for $C_{29}H_{57}NO_4 \cdot H_2O$: C 69.41; H 11.85; N 2.79; MW 501.78. Found: C 69.68; H 11.97; N 2.82.

*General procedures for the oxidation. (i) Procedure A: Oxidation using pyridinium dichromate (PDC)*. To a solution of α-hydroxy amide (1.0 mmol) in glacial acetic acid (5 mL), PDC (1.13 g, 3.0 mmol) was added. After stirring for 2 h at room temperature, the mixture was neutralized with 5% aqueous $NaHCO_3$ and extracted with EtOAc (3 × 15 mL). The combined organic layers were washed with brine solution and dried ($Na_2SO_4$). The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography using petroleum ether 40–60°C/EtOAc (9:1, vol/vol) as eluent.

*(ii) Procedure B: Oxidation using NaOCl/AcNH-TEMPO*. To a stirred solution of α-hydroxy amide (1.0 mmol) in a mixture of EtOAc/toluene/$CH_2Cl_2$ (1:1:0.1, by vol) (6 mL) was added a solution of NaBr (113 mg, 1.1 mmol) in water (0.5 mL), followed by AcNH-TEMPO (2 mg, 0.01 mmol), at 0°C. To the resulting biphasic system was added with vigorous stirring a solution of NaOCl (82 mg, 1.1 mmol) and $NaHCO_3$ (252 mg, 3.0 mmol) in $H_2O$ (3 mL) dropwise at 0°C over a period of 1 h. After stirring for 15 min at 0°C, EtOAc (15 mL) and water (5 mL) were added. The organic layer was washed first with 10% aqueous citric acid (10 mL) that contained KI (60 mg), then 10% aqueous $Na_2S_2O_3$ (10 mL), then brine solution (10 mL), and then it was dried ($Na_2SO_4$). The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography using petroleum ether 40–60°C/EtOAc (9:1, vol/vol) as eluent.

*2-Ketopentadecanamide (**4**)*. Procedure A: Yield 0.179 g (70%); m.p. 114–116°C; $^1$H NMR (200 MHz) δ 2.9 (2H, *t, J* = 8 Hz, $CH_2CO$), 1.6 (2H, *m*, C$H_2$$CH_2$CO), 1.3 (20H, *m*, 10 × $CH_2$), 0.9 (3H, *t, J* = 7 Hz, $CH_3$); IR (ν $cm^{-1}$, KBr): 3401 (NH), 1717 (C=O), 1670 (CONH); FAB-MS *m/z* 278 (M + $Na^+$, 10%), 256 (M + $H^+$, 28%), 211 (19). Calc. for $C_{15}H_{29}NO_2$: C 70.54; H 11.45; N 5.48; MW 255.40. Found: C 70.81; H 11.34; N 5.33.

N-*Hexyl-2-ketohexadecanamide (**12a**)*. Procedure B: Yield 0.335 g (95%); m.p. 55–56°C; $^1$H NMR (200 MHz) δ 6.9 (1H, *br*, CONH), 3.3 (2H, *m*, C$H_2$NH), 2.9 (2H, *t, J* = 7 Hz, $CH_2CO$), 1.6 (4H, *m*, C$H_2$$CH_2$CO, C$H_2$C$H_2$NH), 1.3 (28H, *m*, 14 × $CH_2$), 0.9 (6H, *m*, 2 × $CH_3$); $^{13}$C NMR (50 MHz) δ 190.47 (*C*OCONH), 161.61 (CONH), 39.30 ($CH_2$NH), 34.05 (*C*$H_2$CO), 31.42, 29.11, 28.57, 25.21, 22.88, 22.40 (*C*$H_2$$CH_3$), 13.96 ($CH_3$); IR (ν $cm^{-1}$, KBr): 3330 (NH), 1722 (C=O), 1658 (CONH); FAB-MS *m/z* 376 (M + $Na^+$, 7%), 354 (M + $H^+$, 100%). Calc. for $C_{22}H_{43}NO_2$: C 74.73; H 12.26; N 3.96; MW 353.58. Found: C 74.78; H 12.06; N 3.87.

N-*Hexadecyl-2-ketohexadecanamide (**12b**)*. Procedure A: Yield 0.257 g (52%); $^1$H NMR (200 MHz) δ 6.9 (1H, *br*, CONH), 3.3 (2H, *m*, C$H_2$NH), 2.9 (2H, *t, J* = 7 Hz, $CH_2CO$), 1.6 (4H, *m*, C$H_2$$CH_2$CO, C$H_2$C$H_2$NH), 1.3 (48H, *m*, 24 × $CH_2$), 0.9 (6H, *m*, 2 × $CH_3$); $^{13}$C NMR (50 MHz) δ 190.54 (*C*OCONH), 160.12 (CONH), 39.32 ($CH_2$NH), 36.72 (*C*$H_2$CO), 31.91, 29.67, 29.64, 29.47, 29.43, 29.35, 29.22, 29.20, 29.06, 26.83, 23.21, 22.68 (*C*$H_2$$CH_3$), 14.10 ($CH_3$). Calc. for $C_{32}H_{63}NO_2 \cdot 0.5H_2O$: C 76.43; H 12.83; N 2.78; MW 502.88. Found: C 76.62; H 12.45; N 2.49.

N-cis-*9-Octadecenyl-2-ketohexadecanamide (**12c**)*. Procedure A: Yield 0.338 g (65%), Procedure B: Yield 0.493 g (93%); m.p. 55.5–57°C; $^1$H NMR (200 MHz) δ 6.9 (1H, *br*, CONH), 5.4 (2H, *m*, CH=CH), 3.3 (2H, *m*, C$H_2$NH), 2.9 (2H, *t, J* = 7 Hz, $CH_2CO$), 2.0 (4H, *m*, C$H_2$CH=CHC$H_2$), 1.6 (4H, *m*, C$H_2$$CH_2$CO, C$H_2$C$H_2$NH), 1.3 (44H, *m*, 22 × $CH_2$), 0.9 (6H, *m*, 2 × $CH_3$); $^{13}$C NMR (50 MHz) δ 199.49 (*C*OCONH), 160.10 (CONH), 129.94 and 129.71 (CH=CH), 39.29 ($CH_2$NH), 36.70 (*C*$H_2$CO), 31.89, 29.62, 29.57, 29.50, 29.41, 29.32, 29.22, 29.16, 29.05, 27.18, 27.14, 26.82, 23.19, 22.66 (*C*$H_2$$CH_3$), 14.08 ($CH_3$). Calc. for $C_{34}H_{65}NO_2$: C 78.55; H 12.60; N 2.69; MW 519.83. Found: C 78.70; H 12.56; N 2.71.

*Methyl 2-[(2-ketododecanoyl)amino]hexadecanoate (**12d**)*. Procedure B: Yield 0.477 g (99%); m.p. 46.5–47.5°C; $^1$H NMR (200 MHz) δ 7.4 (1H, *d, J* = 9 Hz, CONH), 4.5 (1H, *m*, C*H*NH), 3.8 (3H, *s*, $COOCH_3$), 2.9 (2H, *t, J* = 7 Hz, $CH_2CO$), 1.9–1.6 (4H, *m*, C$H_2$$CH_2$CO, C$H_2$CHNH), 1.3 (38H, *m*, 19 × $CH_2$), 0.9 (6H, *m*, 2 × $CH_3$); $^{13}$C NMR (50 MHz) δ 198.52

(*C*OCONH), 171.95 (*C*OOCH$_3$), 159.70 (CONH), 52.48 (COO*C*H$_3$), 52.14 (CHNH), 36.73 (*C*H$_2$CO), 32.24, 31.86, 29.62, 29.56, 29.52, 29.47, 29.41, 29.30, 29.07, 29.02, 25.20, 23.12, 22.66 (*C*H$_2$CH$_3$), 14.10 (CH$_3$). Calc. for $C_{29}H_{55}NO_4$: C 72.30; H 11.51; N 2.91; MW 481.75. Found: C 71.97; H 11.30; N 2.56.

*Monomolecular film experiments.* Surface pressure/area curves as well as inhibition experiments were performed with a Teflon trough. Before each experiment the trough was first washed with tap water, then gently brushed in the presence of distilled ethanol, washed again with plenty of tap water, and finally rinsed with double-distilled water. The lipidic film, from a solution in $CHCl_3$ (approximately 1 mg·mL$^{-1}$), was spread with a Hamilton syringe over an aqueous subphase composed of 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (Tris/HCl) 10 mM, pH 8.0, NaCl 100 mM, $CaCl_2$ 21 mM, and EDTA 1 mM. The above buffer solution was prepared with double-distilled water and filtered through a 0.22 µm Millipore (Milford, MA) membrane. Before each utilization, residual surface-active impurities were removed by sweeping and suction of the surface (20). The force/area curves were automatically recorded upon a continuous compression rate at 4.8 cm·min$^{-1}$.

*Enzyme kinetics experiments.* The inhibition experiments were performed using the monolayer technique. The principle of this method has been previously described (20).

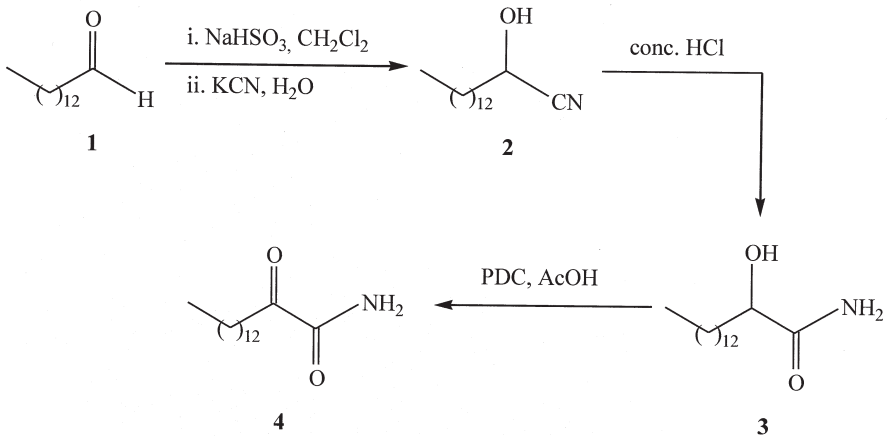
For the inhibition studies the method of mixed monomolecular films was used. This method involves the use of a zero-order trough, consisting of two compartments: a reaction compartment, where mixed films of substrate and inhibitor are spread, and a reservoir compartment, where only a pure film of substrate is spread. The two compartments are connected to each other by narrow surface channels. The aqueous subphase was composed of Tris/HCl 10 mM, pH 8.0, NaCl 100 mM, $CaCl_2$ 21 mM, and EDTA 1 mM. PPL (final concentration 4.0 ng·mL$^{-1}$) was injected into the subphase of the reaction compartment, where efficient stirring was applied. When, owing to the lipolytic action of the enzyme, the surface pressure decreased, a mobile barrier moving over the reservoir compartment compressed the film, thus keeping the surface pressure constant. The surface pressure was measured on the reservoir compartment with a Wilhelmy plate. The surface of the reaction compartment was 100 cm$^2$ and its volume 120 mL. The reservoir compartment was 14.8 cm wide and 24.9 cm long. The lipidic films were spread from a chloroform solution (approximately 1 mg·mL$^{-1}$). The kinetics were recorded for 20 min. In all cases linear kinetics were obtained. Each experiment was duplicated.

## RESULTS AND DISCUSSION

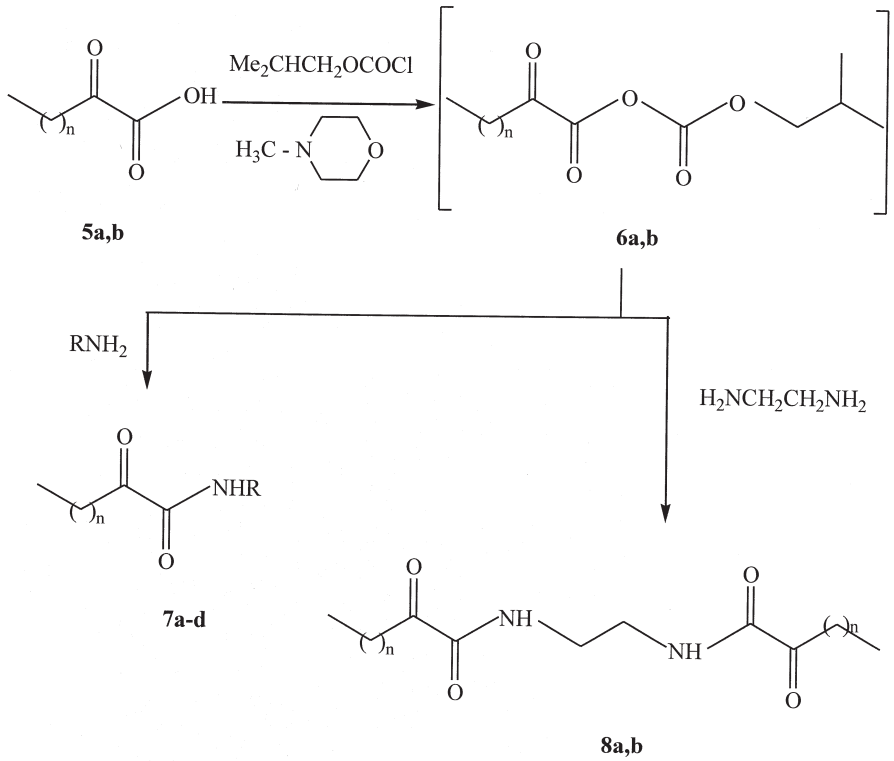
*Synthesis of various α-keto amides.* A number of primary and *N*-alkyl α-keto amides with small, medium, or long chains were synthesized following various routes. 2-Ketopentadecanamide (**4**) was prepared from tetradecanaldehyde (**1**) as described in Scheme 1. Addition of KCN to aldehyde **1** afforded cyanohydrin **2**, which was mildly hydrolyzed to 2-hydroxypentadecanamide (**3**). Oxidation of **3** using PDC afforded primary α-keto amide **4** in high yield.

Commercially available α-keto acids can be directly coupled with primary amines by the mixed carbonic anhydride method (21) in high yield. The mixed anhydrides of 2-ketobutanoic acid (**5a**) and 2-ketooctanoic acid (**5b**) with isobutyl chloroformate (**6a**,**b**) were prepared *in situ* and were used for coupling with hexylamine, hexadecylamine, *cis*-9-octadecenylamine, and 1,2-ethylenediamine to afford *N*-alkyl α-keto amides **7a–d** and **8a**,**b** (Scheme 2). The use of dicyclohexylcarbodiimide as a condensing agent in the presence of HOBt proved to be unsuccessful for the coupling of α-keto acids, leading to moderate yields.

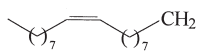
However, long-chain α-keto acids are not commercially available. Thus, we developed a general method for the synthesis of *N*-alkyl α-keto amides using lipidic α-amino acids as starting material. The lipidic α-amino acids (22) are a class of compounds combining structural features of amino acids with those of fatty acids. They are nonnatural α-amino acids with saturated or unsaturated long side chains and can be prepared by classical methods in racemic form. New methods for the




**SCHEME 1**




| 5, 6, 8 | n |
|---|---|
| a | 1 |
| b | 5 |

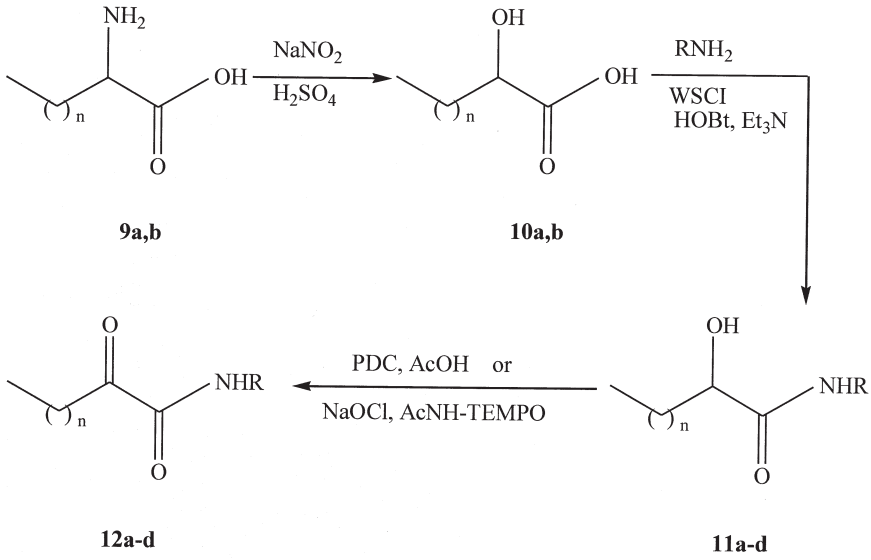
| 7 | n | R |
|---|---|---|
| a | 1 | $CH_3(CH_2)_{15}$ |
| b | 5 | $CH_3(CH_2)_5$ |
| c | 5 | $CH_3(CH_2)_{15}$ |
| d | 5 |  |

**SCHEME 2**

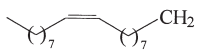
enantiomeric synthesis of saturated (23) or unsaturated derivatives, like 2-aminoarachidonic acid (24), have been recently presented. 2-Aminododecanoic acid (**9a**) and 2-aminohexadecanoic acid (**9b**) were prepared as described in the literature (22) and deaminated by treatment with $NaNO_2$ under acidic conditions (Scheme 3). 2-Hydroxy acids **10a**,**b** were coupled with various amino components using WSCI (25) as a condensing agent in the presence of HOBt. Apart from linear saturated or unsaturated amines, methyl 2-amino hexadecanoate was used as amino component. The lipophilic *N*-alkyl α-keto amides **12a–d** were obtained by oxidation of **11a–d** with either PDC or NaOCl in the presence of catalytic amounts of AcNH-TEMPO free radical (26,27). In most cases PDC was an effective reagent for the oxidation, affording the products in good yields. However, the conversion of products with long aliphatic chains proceeded only in moderate yields. In these cases, the use of NaOCl in the presence of AcNH-TEMPO in a biphasic system of toluene, ethyl acetate, dichloromethane, and aqueous NaBr proved to be advantageous, affording the oxidized products in almost quantitative yields.

All intermediates and final products gave satisfactory analytical and spectroscopic data, which are described in detail in the experimental part. In the $^{13}C$ NMR spectra of the α-keto amide derivatives, two signals corresponding to carbon atoms of COCONH were clearly assigned. The carbon atom of the α-keto group was shifted at 199.5–190.5 ppm owing to the presence of the adjacent amide function. The signal of the amide group carbon atom appeared at 160 ppm, whereas in the case of α-hydroxy amides it was at 173 ppm. Furthermore, $^{13}C$ NMR spectra of α-hydroxy amides showed a signal at 72 ppm that was assigned to the carbon atom of the methine group.

*Force/area curves of the α-keto amides prepared.* The use of the monolayer technique, which is based upon surface pressure decrease due to film hydrolysis, is advantageous for the study of lipase inhibition, because with conventional emulsified systems it is not possible to control their "interfacial quality" (28). The monolayer technique is applicable to those cases of enzymatic studies where the lipid film forms a stable monomolecular film at the air/water interface and where the reaction products are freely water-soluble and dif-




| 9, 10 | n |
|---|---|
| **a** | 9 |
| **b** | 13 |

| 11, 12 | n | R |
|---|---|---|
| **a** | 13 | $CH_3(CH_2)_5$ |
| **b** | 13 | $CH_3(CH_2)_{15}$ |
| **c** | 13 |  |
| **d** | 9 | $CH_3(CH_2)_{13}CH(COOMe)$ |

**SCHEME 3**

fuse away rapidly into the aqueous phase (29,30). To determine the film stability and the interfacial properties of the compounds synthesized, we recorded their force/area curves at the air/water interface by a continuous compression.

Compounds **7a**,**c**, bearing a two- and a six-carbon atom chain, respectively, in the α-keto acid moiety and a 16-carbon-atom aliphatic chain in the amine moiety, were not able to form stable films. A chain of six carbon atoms in the amine moiety was not able to stabilize the film, even when a chain of 14 carbon atoms was present in the α-keto acid moiety (compound **12a**). Compounds **4**, **7b,** and **8a**,**b** were not proven able to form stable thin films, in agreement with the above findings.

*N*-Alkyl α-keto amides **7d**, and **12b–d** were proven able to form stable thin films. Figure 1 illustrates representative recordings of surface pressure dependency as a function of the molecular area of four different compounds spread over a buffered subphase at pH 8.0. The collapse pressures of compounds **12d** and **7d** are observed at surface pressure values of 20.9 and 13.2 $mN \cdot m^{-1}$, respectively. By comparing the force/area curves obtained for compounds **7d** and **12c**, one can notice a significant decrease in the molecular area, as the alkyl chain length in the α-keto acid moiety increases. An expanded film was obtained for compound **7d**, having a *cis* double bond in the amine moiety and an aliphatic chain of six carbon atoms in the α-keto acid moiety, which may be attributed to the presence of the double bond. However, in the case of compound **12c**, bearing the same unsaturated chain in the amine moiety and a chain of 14 carbon atoms in the α-keto acid moiety, the film was condensed. This indicates that in the latter case the predominant factor is the long saturated alkyl chain in the α-keto acid moiety, resulting in a different mode of molecular packing.

*Pancreatic lipase activity on mixed films containing α-keto amide inhibitors.* The α-keto amides, which formed stable monomolecular films at the air/water interface, were studied as potential inhibitors for PPL. There are at least five major reasons

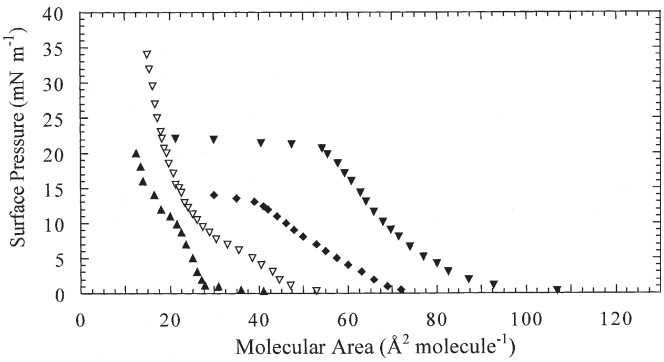



**FIG. 1.** Force/area curves of *N-cis*-9-octadecenyl-2-ketooctanamide (**7d**) (◆); *N*-hexadecyl-2-ketohexadecanamide (**12b**) (▽); *N-cis*-9-octadecenyl-2-ketohexadecanamide (**12c**) (▲); methyl 2-[(2-ketododecanoyl)amino]hexadecanoate (**12d**) (▼). The aqueous subphase was composed of Tris/HCl 10 mM, pH 8.0, NaCl 100 mM, $CaCl_2$ 21 mM, EDTA 1 mM. The continuous compression experiments were performed in the rectangular reservoir of the zero-order trough (20).

for using lipid monolayers as substrates for lipolytic enzymes (31): (i) It is easy to follow the course of the reaction by monitoring one of several physicochemical parameters characteristic of the monolayer film: surface pressure, potential, density, etc. (ii) Probably the most important reason is that it is possible with lipid monolayers to vary and control the "interfacial quality," which depends on the nature of the lipids forming the monolayer, the orientation and conformation of the molecules, the molecular and charge densities, the water structure, the viscosity, etc. (iii) Using the surface barostat balance, the lipid packing of a monomolecular film of substrate can be maintained constant during the course of hydrolysis, and it is therefore possible to obtain accurate pre-steady-state kinetic measurements with minimal perturbation caused by increasing amounts of reaction products. (iv) The monolayer technique is highly sensitive and very little lipid is needed to obtain kinetic measurements. This advantage can often be decisive in the case of synthetic or rare lipids. (v) Inhibition of lipase activity by water-insoluble substrate can be precisely estimated using the zero-order trough and mixed monomolecular films in the absence of any synthetic, nonphysiological detergent. The monolayer technique is therefore suitable for modeling *in vivo* situations.

The inhibition of PPL was studied using the monolayer technique (20) and mixed monomolecular films of lipase substrate (1,2-dicaprin) containing variable proportions of each inhibitor.

Depending upon the film stability and the collapse pressure of each compound, we chose the appropriate surface pressure at which the inhibition kinetics were performed. Compound **7d** was studied at 10 $mN \cdot m^{-1}$ and compound **12b** at 20 $mN \cdot m^{-1}$. Compound **12d** was studied at both 15 and 20 $mN \cdot m^{-1}$. PPL was active, and linear kinetics were recorded at 10, 15, and 20 $mN \cdot m^{-1}$.

The decrease in the enzymatic activity caused by the presence of each of the α-keto amide inhibitors mixed with 1,2-dicaprin monolayers at a molar fraction of 10% was measured. The remaining enzymatic activity of PPL was 65.1 ± 3.8% for compound **7d** and 70.2 ± 4.8% for compound **12b**. At this molar fraction, the remaining enzymatic activity found in the presence of compound **12d** was 47.6 ± 3.2% at a surface pressure of 15 $mN \cdot m^{-1}$ and 50.1 ± 3.7% at 20 $mN \cdot m^{-1}$. Since among the compounds tested, compound **12d** was the best inhibitor of PPL, it was further studied at various molar fractions. Remaining lipase activity was plotted as a function of the inhibitor **12d** molar fraction, at both 15 and 20 $mN \cdot m^{-1}$, and the results obtained are presented in Figure 2. The dotted line corresponds to surface dilution phenomena, which reflects the decrease in lipase activity that would be observed if a nonsubstrate, noninhibitor compound, that is, a so-called surface dilutor, were present in the monomolecular film. From these curves the $\alpha_{50}$ value, which is the inhibitor molar fraction present in 1,2-dicaprin monolayers that causes a 50% decrease in the enzymatic activity, was calculated. The $\alpha_{50}$ values were found to be 0.094 ± 0.022 at 15 $mN \cdot m^{-1}$ and 0.100 ± 0.018 at 20 $mN \cdot m^{-1}$, indicating that within the range of surface pressure investigated, there was a slight effect of surface pressure on inhibition.

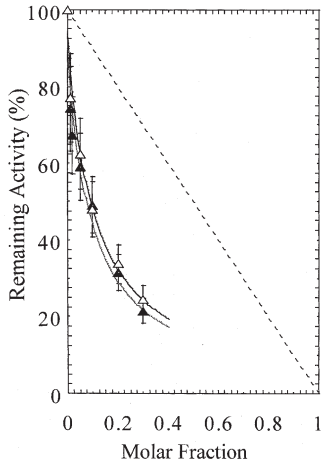



**FIG. 2**. Effect of increasing the concentration of **12d** on the hydrolysis rate by porcine pancreatic lipase of 1,2-dicaprin monolayer maintained at a constant surface pressure of 15 $mN \cdot m^{-1}$ (▲) or 20 $mN \cdot m^{-1}$ (▽). The aqueous subphase was composed of Tris/HCl 10 mM, pH 8.0, NaCl 100 mM, $CaCl_2$ 21 mM, EDTA 1 mM. The kinetics of hydrolysis were recorded for 20 min. For **12d** see Figure 1.

Although these values indicate a rather weak inhibition of pancreatic lipase, the α-keto amide **12d** exhibits a stronger inhibitory effect as compared to recently reported chiral acylglycerol analogs belonging to phosphonate-type inhibitors ($\alpha_{50}$ values 0.13–0.20) (32). Up to now, the best synthetic inhibitor of HPL reported in the literature is *O*-hexadecyl-*O*-(*p*-nitrophenyl) *n*-undecyl phosphonate, with an $\alpha_{50}$ value of 0.003 (10).

In the case of serine proteases, the mechanism of action of inhibitors containing electrophilic carbonyl groups most likely involves a nucleophilic addition of the active site serine hydroxyl group of the enzyme to the carbonyl group of the inhibitor, with formation of a metastable hemiacetal adduct that mimics the tetrahedral species involved in the enzymatic cleavage of peptide bonds. In the case of some proteases, X-ray (33,34) and $^{13}C$ NMR (35) studies have demonstrated the formation of such enzyme–inhibitor hemiacetal adducts. Based on the fact that the catalytic triad of lipases is homologous to that described in serine proteases as well as on their similar mechanism of action, the above findings may be extrapolated to lipases. Thus, the inhibitory effect of the α-keto amides on pancreatic lipase may be attributed to the interaction of the active-site serine hydroxyl group with the carbonyl group of the synthetic compound.

In conclusion, three synthetic routes to primary and *N*-alkyl α-keto amides were developed. α-Keto amides of simple structure, like compound **12d**, are able to inhibit pancreatic lipase, indicating that the α-keto amide group may be used as a reactive functionality in the design and synthesis of potent inhibitors of lipolytic enzymes.

## ACKNOWLEDGMENTS

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COMMUNICATION

# Inhibition of Purified Pig and Human Liver Retinyl Ester Hydrolase by Pharmacologic Agents

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**ABSTRACT:** Identification of inhibitors of retinyl ester hydrolase (REH) would help to elucidate its role in vitamin A metabolism *in vivo*. By using standard incubation conditions, the effects of 215 drugs as potential inhibitors of purified pig and human liver REH when acting on micellar substrate retinyl palmitate were evaluated at 16.7, 167, and 1670 μM. Out of the compounds tested, 103 were inhibitors of the pig liver enzyme. The most potent compounds, in order of decreasing activity, were chloral hydrate, lovastatin, phytomenadione, alimemazine, physostigmine, thioridazine, phenoxybenzamine, probucol, cinnarizine, cyclandelate, amiodarone, flupenthixol, and naftidrofuryl; this order is roughly similar to that of their inhibition of human liver REH. Of the 10 tricyclic ring-containing drugs tested, alimemazine was the most potent enzyme inhibitor. The concentrations necessary for 50% enzyme inhibition ranged from <2.6 up to >540 μM. Moreover, inhibitory kinetic studies showed that at least two pharmaceuticals, chloral hydrate and amiodarone, are potent REH inhibitors at therapeutically achievable serum concentrations. First-pass metabolites were inactive as REH inhibitors compared to that of the parent compounds, in the cases of chloral hydrate, lovastatin, and cyclandelate.

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The liver plays a central role both in the regulation of plasma retinol (vitamin A alcohol) concentration and in the maintenance of whole-body vitamin A homeostasis *via* delivery of retinyl esters in chylomicron remnants and the retinol–retinol-binding-protein complex (1,2). Once inside the hepatocytes, retinol molecules released by cellular processes, i.e., hydrolysis of gut-derived retinyl esters (1) or desorption from its carrier protein (2), enter the pool of free retinol. The major route of egress of vitamin A from this pool is into the retinyl ester storage compartment. The formation of esterified retinol takes place mainly in stellate cells (3,4) through the action of acyl-CoA:retinol acyltransferase (5) or lecithin:retinol acyltransferase (6), the resulting retinyl esters being found in the cytoplasm as large lipid droplets (3). In response to changes in hepatic retinol concentration, vitamin A balance across the liver is maintained by regulating the rates of hydrolysis and formation of retinyl ester.

In well-nourished populations, the amount of retinoids in plasma and bile reflects the intrahepatic formation of retinol, which is likely controlled by a number of factors including the activities and selectivities of the key enzymes in the pathway from retinyl ester to retinol. Several enzymes are able to catalyze such a reaction *in vitro* (see Ref. 7 for recent review). Among these ubiquitous retinyl ester-utilizing enzymes, we have recently (8) demonstrated the presence of a retinyl ester hydrolase (REH) (EC 3.1.1.-) in liver that is, in some aspects [e.g., primary structure, immunological behavior, specific activity, pH dependence, and sensitivity to bis-(4-nitrophenyl) phosphate] similar to liver microsomal esterase ES-4 (EC 3.1.1.1) but exhibits some distinct characteristics, including bimodal distribution of activity between cytosolic and membrane fraction, detergent {e.g., 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS)}-dependent esterase activity against retinyl palmitate (a hydrophobic substrate molecule), and higher molecular mass. ES-4 (also called the pI 6.2/6.4 esterase or microsomal hydrolase) belongs to the nonspecific carboxylesterases supergene family. This is an extremely large family of enzymes that are involved in detoxification of many xenobiotics including drugs containing ester, thioester, and amide functional groups.

Although the function of REH in vitamin A metabolism remains incompletely understood, its existence in liver cytosol (9) suggests that this enzyme may act at the lipid-water interface of intracellular fat bodies to hydrolyze retinyl esters, a step in retinol mobilization (3,7). One method for studying the physiological role of an enzyme is through the use of inhibitors. However, no biocompatible inhibitors of soluble REH have yet been reported. Therefore, the purpose of this study was to evaluate a series of widely used drugs ($n$ = 215) as inhibitors for further characterizing this enzyme.

## EXPERIMENTAL PROCEDURES

Pig and human liver REH enzymes were prepared by methods described previously (8). Two hundred fifteen drugs from different pharmacological classes were studied in the pig REH assay system; only 18 of these substances were examined in both human and pig assay systems. The test compounds were purchased or generously obtained in their chemically pure form from the various manufacturers. Retinyl

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; ES, nonspecific carboxylesterase; $IC_{50}$, inhibitor concentration giving 50% inhibition of enzyme activity; REH, retinyl ester hydrolase.

palmitate was a gift from Hoffman-La Roche (Basel, Switzerland), Triton X-100 was from Serva (Heidelberg, Germany), CHAPS was from Boehringer (Mannheim, Germany), and bovine serum albumin was from Sigma (Deisenhofen, Germany). Inorganic substances, organic solvents (e.g., dimethyl sulfoxide), and antioxidants (e.g., ascorbic acid and butylated hydroxytoluene) were purchased from Merck (Darmstadt, Germany). All chemicals and solvents were of analytical grade.

REH activity was determined by the method of Cooper and Olson (9). The standard mixture solution consisted of 2 mg/mL Triton X-100 and 100 mM CHAPS in 0.05 M Tris-maleate buffer, pH 8.0, in a total volume of 0.5 mL. The final concentration of the substrate retinyl palmitate in the assay was 0.05 mM. The reaction was started by adding the enzyme (170–220 μg protein/mL), and the mixture was incubated with gentle shaking for 60 min at 37°C. The incubations were terminated by addition of 0.5 mL ethanol. Liberated retinol was extracted with 2 mL *n*-hexane containing 0.1% (wt/vol) butylated hydroxytoluene. The organic phase was evaporated to dryness and dissolved in chloroform/methanol (1.5:4, vol/vol). The amount of retinol formed by REH was quantified fluorimetically (excitation at 325 nm, emission at 480 nm) by reversed-phase high-performance liquid chromatography, as described previously (8), by using a Bio-Tek Kontron (Neufahrn, Germany) Spherisorb ODS (5 mm × 25 cm) column eluted with methanol at a flow rate of 1.8 mL/min (8).

To test the effect of several drugs as potential inhibitors on purified human REH, the enzyme reaction was carried out according to the standard REH assay, except that 0.05 M Tris-maleate buffer, pH 8.0, was replaced with 0.05 M Tris-maleate buffer, pH 6.5 (10). In assays involving the drugs under investigation, the agents were incubated with the enzyme for 30 min before addition of the substrate. These experiments were performed in the presence of three different drug concentrations, 16.7, 167, and 1670 μM, and each inhibitor was evaluated in triplicate. All assay controls were adjusted for effects of solvents on enzyme activity. Solutions of the drugs were made fresh for each experiment. The property of each solvent that allowed solubilization of the drugs was apparently related to both its hydrogen ion concentration and polarity. Several drugs were readily solubilized by either 0.05 M Tris-maleate buffer, pH 6.0, or 0.05 M glycine-NaOH buffer, pH 9.5, but not by 0.05 M Tris-maleate buffer, pH 8.0. To examine the effects of these drugs on the activity of REH, the enzyme reaction was carried out according to the standard assay used, except that 0.05 M Tris-maleate buffer, pH 8.0, was replaced with one of the two buffers mentioned before. Water-insoluble drugs were dissolved in dimethyl sulfoxide, methanol, or ethanol and then diluted with the enzyme diluent. Final concentration of these organic solvents in assay mixture never exceeded 2% (by vol).

Drug concentrations for half-maximal inhibition of the REH activity ($IC_{50}$) were determined for the specified conditions. The inhibitor $IC_{50}$ values were calculated from individual concentration-effect curves as suggested by Hafner *et al.* (10).

Protein concentration was determined by measuring the absorbance at 595 nm by the Bradford dye-binding assay (11). The reference protein for all assays was bovine serum albumin. Retinol was prepared from retinyl palmitate by standard procedures (8). The concentration of pure retinol dissolved in petroleum ether was determined spectrophotometrically using an extinction coefficient of 1830 at 325 nm ($E_{325nm}^{1\%,1\ cm}$; Ref. 12).

## RESULTS

In a preliminary approach, 215 pharmacologic drugs from different chemical classes were tested, each at 16.7, 167, and 1670 μM, for their effects on REH activity. Numerous drugs ($n$ = 103) were able to inhibit the pig form of liver REH to various degrees. Only the 33 most active compounds are shown in Table 1. Under these conditions, the strongest inhibition observed in the micromolar range (at 167 μM) was by chloral hydrate and lovastatin, two distinctly different compounds.

To establish whether these findings would hold true for human liver REH, a number of compounds ($n$ = 18) listed in Table 1 were evaluated for their effects on this REH form, under the same conditions. All 18 drugs inhibited purified human REH activity, and chloral hydrate was again found to be most potent. For these drugs the rank order of potency for inhibition (number in parentheses following residual REH activity; Table 1) was similar to that seen with purified pig REH, with some notable exceptions; miconazole, diclofenac, and amiodarone exhibited human REH-selectivity, whereas flupenthixol and physostigmine exhibited pig REH-selectivity. However, the REH inhibitory effects of the drugs (Table 1) should be viewed as relative ranges of potency or selectivity rather than absolute rankings.

The results of inhibitory kinetic studies of pig liver REH are shown in Table 2. To evaluate whether the drug concentration used in these experiments and the calculated $IC_{50}$ values were comparable with the serum and tissue concentrations seen after therapeutic dosage, we compiled data for the test compounds from a review of the available literature (Table 2; Refs. 13–22). For all drugs, except two, for which data could be compared, their expected clinical serum concentration was below their $IC_{50}$ values for hepatic REH inhibition. Exceptions are chloral hydrate and amiodarone, whose $IC_{50}$ values are within the range found in human serum. However, some drugs will concentrate in the liver during long-term therapy, reaching tissue concentrations 4- to 11-fold higher than in plasma, as has been shown for drugs such as phytomenadione, probucol, and amiodarone (Table 2). Thus, mainly within the liver, regional concentrations of these drugs comparable to those producing REH inhibition in our *in vitro* experiments could occur in *in vivo* situations.

Among the pharmacologic agents tested, chloral hydrate was the most potent inhibitor, with an $IC_{50}$ value of 2.5 μM. This value is in the concentration range achieved clinically (Table 2). The aim of the next set of experiments was therefore to investigate whether chloral hydrate (a compound with

**TABLE 1**
**Therapeutic Agents with Inhibitory Effects on Pig and Human Liver Retinyl Ester Hydrolase (REH)[a]**

| | | Remaining REH activity (% of control)[b] in the presence of the drug (μM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Source: From pig liver | | | Source: From human liver | | |
| International nonproprietary name of drugs | Drug type | 16.7 | 167.0 (rank) | 1670.0 | 16.7 | 167.0 (rank) | 1670.0 |
| Chloral hydrate[c] | NEA | 16 | 2 (1) | 2 | 3 | 0 (1) | 0 |
| Lovastatin | E | 43 | 16 (2) | 6 | 89 | 60 (10) | ND |
| Phytomenadione | NEA | 77 | 40 | 16 | ND | ND | ND |
| Alimemazine | NEA | 79 | 45 (3) | 21 | 76 | 48 (4) | 17 |
| Physostigmine | E | 87 | 54 (4) | 23 | 100 | 87 (16) | 44 |
| Thioridazine | NEA | 92 | 54 (5) | 23 | 82 | 49 (8) | 30 |
| Phenoxybenzamine | NEA | 85 | 57 (6) | 23 | 84 | 58 (9) | 32 |
| Probucol | NEA | 85 | 61 | 28 | ND | ND | ND |
| Cinnarizine | NEA | 87 | 68 (7) | ND | 74 | 47 (3) | ND |
| Cyclandelate | E | 90 | 70 (8) | 27 | 70 | 44 (2) | 7 |
| Amiodarone | NEA | 90 | 80 (9) | 33 | 76 | 48 (5) | 38 |
| Flupenthixol | NEA | 92 | 80 (10) | 36 | 100 | 91 (18) | 51 |
| Naftidrofuryl | E | 95 | 80 (11) | 36 | 88 | 71 (11) | 37 |
| Nifedipine | E | 91 | 81 (12) | 37 | 91 | 82 (14) | 43 |
| Hydroxyzine | NEA | 92 | 81 | 40 | ND | ND | ND |
| Quinidine | NEA | 90 | 82 | 38 | ND | ND | ND |
| Bencyclane | NEA | 91 | 83 | 40 | ND | ND | ND |
| Clemastine | NEA | 93 | 83 | 41 | ND | ND | ND |
| Cyclophosphamide | NEA | 98 | 83 | 43 | ND | ND | ND |
| Amitriptyline | NEA | 95 | 84 | 43 | ND | ND | ND |
| Erythromycin | NEA | 91 | 85 (13) | 41 | 100 | 91 (17) | 46 |
| Bromocryptine | A | 100 | 85 (14) | 50 | 94 | 87 (15) | 45 |
| Diclofenac | NEA | 93 | 86 (15) | 48 | 79 | 48 (6) | 20 |
| Chlorprothixene | NEA | 91 | 88 (16) | 49 | 91 | 82 (13) | 40 |
| Cefotiame | A | 93 | 88 (17) | 41 | 90 | 82 (12) | 37 |
| Sulfinpyrazone | A | 90 | 89 | 51 | ND | ND | ND |
| Miconazole | NEA | 100 | 89 (18) | 32 | 79 | 49 (7) | 21 |
| Glibornuride | A | 100 | 91 | 59 | ND | ND | ND |
| Clopenthixol | NEA | 97 | 93 | 51 | ND | ND | ND |
| Fluphenazine | NEA | 98 | 94 | 65 | ND | ND | ND |
| Etidocaine | A | 100 | 100 | 59 | ND | ND | ND |
| Chlorambucil | NEA | 100 | 100 | 63 | ND | ND | ND |
| Triflupromazine | NEA | 100 | 100 | 65 | ND | ND | ND |

[a]Purified enzyme was incubated with the inhibitor at the indicated concentration for 30 min at 37°C prior to initiating the enzyme reaction by addition of 0.05 mM retinyl palmitate. The activities were assayed as described in the Experimental Procedures section.
[b]Results are presented as the percentage relative REH activity when compared with that in a control reaction in which the inhibitor was omitted. The data are means for triplicate experiments.
[c]Position of the drug in the rank list is given in parentheses; higher-ranking numbers are associated with decreased potency for inhibition. Abbreviations: A, amide-type drug; E, ester-type drug; NEA, nonester/nonamide drug; ND, not determined.

a short half-life) or its metabolites could interfere in hepatic vitamin A metabolism. In these experiments we tested directly for inhibition of REH activity by chloral hydrate, trichloroethanol, or trichloroacetic acid (13). Chloral hydrate and trichloroethanol were tested above or at the clinical peak plasma concentration found in the *in vitro* REH assay; REH activity was inhibited by 85% and >95% at chloral hydrate concentrations of 16.7 and 167 μM, respectively. In contrast, weak (<30%) inhibition of retinyl palmitate hydrolysis was observed by the degradation products, even at a concentration of 1670 μM. Thus, although chloral hydrate is a potent REH inhibitor *in vitro*, this is less likely to occur *in vivo* because chloral hydrate undergoes extensive first-pass metabolism to products without inhibitory potency on REH at clinically relevant concentrations. Similar observations were made comparing REH inhibition by lovastatin and cyclandelate with their metabolites (data not shown).

## DISCUSSION

Screening common pharmacologic agents for inhibition of hepatic REH has identified 103 as inhibiting REH activity to varying extents. In general, compounds containing amide bonds ($n$ = 25) were less potent as REH inhibitors than were ester-type drugs ($n$ = 16) (Table 1). Because the structures of these agents are very diverse, they may exert their effect through many different mechanisms. One mode of inhibition is *via* direct interaction with the catalytic site of the enzyme. An example of an active site-directed enzyme blocker is chloral hydrate, known to inhibit chicken liver carboxylesterase

**TABLE 2**
**Inhibitory Potency and Tissue Concentrations of REH Blockers**

| Compound | Inhibitory potency (μM) $IC_{50}$[a] | Tissue concentration (μM) Serum | Liver | References[b] |
|---|---|---|---|---|
| Chloral hydrate | 2.5 | 6.4 | DNA | 13 |
| Lovastatin | 10.0 | 1.1 | DNA | 14 |
| Phytomenadione | 90.0 | 0.0086 | 0.051 | 15, 16 |
| Physostigmine | 190.0 | 0.012 | DNA | 17 |
| Probucol | 260.0 | 14 | 56 | 18 |
| Amiodarone | 380.0 | 220 | 2500 | 19, 20 |
| Flupenthixol | 430.0 | 0.073 | DNA | 21 |
| Naftidrifuryl | 510.0 | 1.2 | DNA | 22 |

[a]Inhibitor concentration giving 50% inhibition of pig liver REH ($IC_{50}$) values were measured using 0.5 mM retinyl palmitate as substrate.
[b]Comparison of the concentration of tested drugs in serum and liver (assuming that 1 g of tissue approximates 1 mL in volume) as adapted from the literature (13–22). Abbreviation: DNA, data not available; for other abbreviation see Table 1.

(EC 3.1.1.1) (23). A mechanism has been proposed (25) whereby chloral hydrate is dehydrated in a rate-limiting step by the enzyme to form trichloroacetaldehyde, which rapidly adducts the protein *via* its active-site serine, yielding an inactive enzyme. It is to be expected that the same mode of inhibition could also take place with REH, another serine esterase. Physostigmine is a reversible inhibitor of acetylcholinesterase (EC 3.1.1.7) and other serine esterases (24). The REH inhibitory activity of physostigmine therefore is likely *via* carbamoylating the catalytic serine. Physostigmine also discriminates between carboxylesterase subtypes (25). For example, ES-4 (pI 6.2/6.4 esterase, closely related to REH) and ES-10 (pI 6.0/6.1 esterase) were significantly inhibited, but ES-3 (pI 5.6 esterase) was not inhibited at the concentration used. ES-4 also shares long-chain fatty acyl coenzyme A-splitting activity with acyl coenzyme A:cholesterol acyltransferase (EC 2.3.1.26; Refs. 25,26). However, isoforms of this enzyme are sensitive to inhibition by ester drugs, namely, cyclandelate and lovastatin (27,28), as is the case for REH. Lovastatin appears to function as an inhibitor of retinyl ester hydrolysis by acting as an alternative substrate; as an ester-type drug it requires carboxylesterase-mediated bioactivation in order to elicit pharmacological activity (29). It can be anticipated that acting as an alternative substrate also applies to the mode of inhibition of REH by naftidrofuryl, a known substrate for butyl cholinesterase (EC 3.1.1.8) (30). The same may be true for dilazep (not shown), which is a substrate for the human liver carboxylesterase subtypes pI 4.5 and pI 5.3 (31). On the other hand, metoclopramide, an amide-type drug, differentiates among carboxylesterases by inhibiting ES-2 (32) but not REH.

The REH inhibitory activities of erythromycin and alimemazine appear to proceed by a mechanism that was different from the anti-REH action of the active site-directed enzyme blockers. Erythromycin is an inhibitor and a substrate of CYP 3A4 (33). It contacts the enzyme at the catalytic site through hydrophobic interactions (34). By analogy, erythromycin may occupy the substrate-binding pocket of REH such that it interferes with the hydrolysis of retinyl palmitate. A similar mechanism of action may be operative in REH inactivation by alimemazine and related phenothiazine and thioxanthene neuroleptic drugs. In this context, it is interesting that insertion of a methylene group and rearrangement of another one in the triacylamine-containing side chain of alimemazine leads to ethopropazine. The latter compound is a reversible tricyclic inhibitor of acetylcholinesterase, and its main known mode of action is *via* blocking the access of the substrate to the esterolytic site (24).

The REH reaction occurs at a lipid-water interface, and it depends upon adsorption of the enzyme to the surface of the substrate-carrier. The interfacial binding site involved in the formation of enzyme-substrate-carrier complex may be part of a hydrophobic domain at or close to the active center of the enzyme. Lipophilic drugs thus may function as inhibitors of REH by binding to a nonpolar area of this enzyme. Likely candidates with submillimolar potency in REH assays that meet this description are phytomenadione (a carboxylase cofactor) and the nonsteroidal anti-inflammatory drug diclofenac (Table 1). The concept of diclofenac as a noncompetitive inhibitor of REH is supported by its high binding affinity to proteins (35). At the same time, we demonstrated that alkylating agents such as phenoxybenzamine, cyclophosphamide and chlorambucil are also powerful inhibitors of REH. At least one of these lipophilic compounds, chlorambucil, may also possess inhibitory activity by virtue of its ability to bind to proteins, but, unlike phytomenadione and diclofenac, it might act by irreversible formation of an enzyme-drug complex (36).

Inhibition of REH by some amphiphilic drugs may be due to rendering the substrate at the lipid-water interface less susceptible to the enzyme. Amiodarone, for example, is a cationic as well as an amphiphilic iodinated benzofuran derivative. These properties make the drug capable of entering into both lipophilic interactions with the substrate and electrostatic interactions with anionic interfaces, such as polar headgroup regions of substrate monolayer. Amiodarone could also inhibit enzymic activity by a direct interaction with the polypeptide chain at the interface or by modifications of surface charge, as amiodarone is an ionized amine at pH 7.4 (37). Moreover, amiodarone has a very long half-life (38) and extensive distribution to the liver and other organs, reaching tissue concentrations up to 11-fold higher than in plasma (Table

2). However, intracellular accumulation of large amounts of amiodarone is associated with the appearance of phospholipidosis and consequently fibrosis within the liver, both of which are likely secondary to drug-induced phospholipase inhibition (39,40). Thus, it is probable that amiodarone reaches intrahepatic concentrations adequate to inhibit not only phospholipase but also REH, as indicated by a comparison of its REH inhibitory potency ($IC_{50}$ of 380 μM) and its liver concentration (≤2500 μM) during long-term therapy (Table 2).

In summary, a screening of a range of common pharmacologic agents for inhibition of hepatic REH has led to the identification of numerous drugs as active compounds. Some of these agents may be useful tools for distinguishing among esterase enzymes and for probing their physiological relevance *in vivo*.

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# Plasma Cholesteryl Ester Transfer Protein and Lipoprotein Levels During Treatment of Growth Hormone-Deficient Adult Humans

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**ABSTRACT:** The incidence of atherosclerosis is increased in growth hormone (GH) deficient-individuals. Nonetheless, the antiatherogenic benefits of GH replacement therapy remain uncertain. In this study the effect of human recombinant growth hormone (hrGH) replacement therapy administered to GH-deficient adults on the plasma cholesteryl ester transfer protein (CETP) concentration and activity was analyzed. These findings were related to changes in the concentrations of the plasma lipoproteins. The hrGH was administered for 12 mon to human GH-deficient patients ($n$ = 13; 8 men, 5 women). During the study plasma lipoproteins were separated by ultracentrifugation, and plasma cholesterol esterification rate (CER), endogenous CETP activity, and CETP concentration were measured. GH replacement therapy transiently (at 3 mon) lowered plasma concentration of CETP and low density lipoprotein-cholesterol (LDL-C) and raised total triglycerides. Furthermore, hrGH permanently increased both the plasma lipoprotein(a) [Lp(a)] concentration, which is known as atherogenic, and the proportion of cholesteryl ester in the high density lipoprotein$_2$ ($HDL_2$) particles, which is potentially atheroprotective. The simultaneous decrease of the plasma CETP and LDL-C concentrations elicited by hrGH indicated a close relationship between LDL metabolism and the regulation of the CETP gene expression. Endogenous CETP activity and the CER were not modified because these parameters are regulated in opposite ways by plasma levels of triglycerides; that is, CER increased and CETP decreased.

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Patients with hypopituitarism are at a greater risk than normal subjects of developing premature cardiovascular disease, which has been related to growth hormone (GH) deficiency (1,2). Cardiovascular risk factors such as decreased plasma fibrinolytic activity, glucose intolerance and insulin resistance as well as increased abdominal adiposity and plasma levels of low density lipoprotein cholesterol (LDL-C) (3,4), together with carotid intima-media thickness (5) and abnormal carotid arterial wall dynamics (6), have been identified in these subjects. Nonetheless, the antiatherogenic benefits of human recombinant growth hormone (hrGH) replacement therapy remain uncertain. In hypopituitarism hrGH treatment has been shown to lower the plasma LDL-C level in several (4,7–10), but not all studies (4,11,12). This treatment often increases the serum lipoprotein(a) [Lp(a)] concentration (4,8–10,13, 14), a condition related to premature atherosclerosis (15), although it reduces visceral adiposity (4) and seemingly reverses early morphological and functional atherogenic changes in major arteries (11).

Reverse cholesterol transport (RCT) is a mechanism that protects against premature atherosclerosis. Accordingly, the liver excretes cholesterol removed from peripheral cells, including the arterial wall intima. Unesterified cell membrane cholesterol taken up by nascent high-density lipoprotein (HDL) particles is esterified by lecithin cholesterol acyltransferase (LCAT) and reaches the liver either directly or indirectly upon transfer of HDL-cholesteryl ester to apolipoprotein B (apoB)-containing lipoprotein. The latter pathway is mediated by cholesteryl ester transfer protein (CETP), which exchanges HDL-cholesteryl ester with triglycerides from apoB-containing lipoproteins, bringing about a net transfer of cholesteryl ester from HDL to LDL and very low density lipoprotein (VLDL) (16–18). Although plasma CETP activity depends more on the availability of donor- and acceptor-lipoproteins than on the CETP mass itself (19,20), cholesteryl ester transfer is negligible in the absence of CETP, a condition that raises plasma HDL-C concentration (21).

Evidence for an interrelationship between cholesterol metabolism and the regulation of plasma CETP levels is supported by the following observations: (i) in a variety of species, plasma CETP concentration and activity, as well as hepatic and peripheral CETP mRNA levels, increase in response to a high-cholesterol diet (22–24) whereas the number of hepatic LDL receptors decreases (25); (ii) subjects with family-related hypercholesterolemia have high plasma CETP concentrations (26,27); (iii) simvastatin (28) and cholestyramine (29), well-known hypocholesterolemic drugs, increase the expression of the hepatic LDL receptors and reduce the plasma CETP levels; and (iv) these receptors are induced by GH treatment (30).

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Abbreviations: apoB, apolipoprotein B; CE, esterified cholesterol; CER, cholesterol esterification rate; CETA, cholesteryl ester transfer activity; CETP, cholesteryl ester transfer protein; DTNB, 5,5-dithio-bis-[2-nitrobenzoic acid]; EST, cholesterol esterification; GH, growth hormone; HDL, high density lipoprotein; HDL-C, HDL-cholesterol; hrGH, human recombinant growth hormone; IGF-1, insulin growth factor-1; LCAT, lecithin cholesterol acyltransferase; LDL, low density lipoprotein; LDL-C, LDL-cholesterol; Lp(a), lipoprotein(a); PLTP, phospholipid transfer protein; RCT, reverse cholesterol transport; TLC, thin-layer chromatography; UC, unesterified cholesterol; VLDL, very low density lipoprotein.

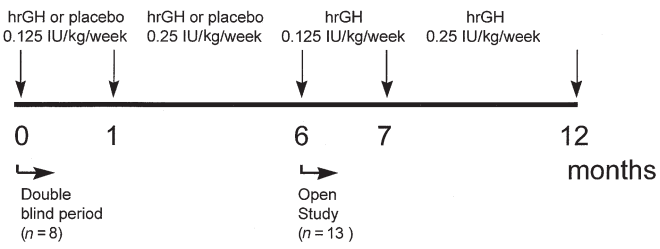
Tan *et al*. (31) showed that plasma CETP activity is increased in acromegalic patients. CETP concentration and activity in GH-deficient subjects have never been studied. Moreover, according to Rudling *et al*. (30), GH also lowers plasma LDL-C by raising the hepatic LDL receptor expression, which is an analogous mechanism to that of simvastatin (32) and of cholestyramine (33). Although CETP activity has recently been shown to diminish on GH replacement therapy (8), CETP plasma concentration has never been measured and its relation to the plasma lipoprotein concentration in this therapy is unknown.

## MATERIALS AND METHODS

*Subjects.* Thirteen (8 male, 5 female; mean age ± SD: 39 ± 9 yr) GH-deficient adults recruited for the protocol completed the study. The patients presented either GH deficiency or hypopituitarism resulting from pituitary adenoma, craniopharyngioma, or hypophysitis. GH deficiency was defined as a maximum stimulated serum GH level below 5 mU/L in response to insulin-induced hypoglycemia (blood glucose < 2.2 mmol/L) or to oral clonidine (100 µg). Patients with multiple pituitary deficiencies had been on stable replacement with thyroxin, cortisol, sex hormones, and with desmopressin when necessary, and maintained for at least 6 mon prior to entering the protocol. No subjects had received GH during the previous 12 mon or suffered from diabetes mellitus, renal, hepatic, or other secondary causes of hyperlipidemia. Informed consent was obtained from all patients, and the Ethics Committee of the Hospital das Clínicas of University of São Paulo Medical School approved the study.

*Study protocol.* Eight patients were submitted in the initial 6 mon to a double-blind treatment with either hrGH (Genotropin, Kabi-Pharmacia, Stockholm, Sweden) or an identically presented placebo. Thereafter, all of them ($n$ = 8) received hrGH in an open study for a further 6 mon. Another group consisted of five additional subjects that participated only in the open study, receiving hrGH for 6 mon. Therefore, 13 patients were on hrGH for at least 6 mon.

The dose of GH was 0.125 IU/kg/wk for the first month, and thereafter 0.25 IU/kg/wk, maximum of 4 IU/d. Because the study remained blinded throughout in the first group, the GH dose for all eight subjects was lowered during the seventh month, and reverted to the full dose thereafter (Scheme 1). GH or placebo was self-administered using the KabiPen (Kabi-Pharmacia) from 16 IU/mL vials as single subcutaneous injections 7 nights/wk at bedtime, and compliance was checked by vial counting. All analyses were carried out at the beginning of the study and then every 3 mon. The GH replacement effectiveness was properly assessed measuring the serum levels of insulin growth factor (IGF-1).




**SCHEME 1**

*Laboratory procedures.* After an overnight fasting period venous blood samples were drawn over 0.1% EDTA, and plasma was obtained after low-speed centrifugation. The following preservatives were added per milliliter of plasma: 2 mmol/L benzamidine (5 µL), 0.5% gentamycin and 0.25% chloramphenicol (20 µL), 25 mg phenylmethylsulfonylfluoride/28.7 mL dimethyl sulfoxide (0.5 µL), and aprotinin (10 µL). The LCAT inhibitor 5,5-dithio-bis-[2-nitrobenzoic acid] 0.1% (DTNB, 5.5 µL/mL) was added to samples used for cholesteryl ester transfer activity (CETA) and CETP measurements. An aliquot was used for precipitation of apoB-containing lipoproteins with dextran sulfate and magnesium chloride (34), and all samples were stored at –70°C until further analysis. All chemicals were purchased from Sigma (St. Louis, MO). Total and unesterified cholesterol and triglyceride levels were determined in duplicate using enzymatic diagnostic kits (Boehringer Mannheim, Buenos Aires, Argentina; and Merck, Darmstadt, Germany, respectively); lipoprotein cholesterol fractions were calculated by the Friedewald formula (35). Plasma apoB, apoA-1 and Lp(a) levels were measured by radioimmunoassay kits (Mercodia, Uppsala, Sweden), and plasma IGF-1 concentrations by the commercially available DSL kit (Diagnostic Systems Labs, Webster, TX).

*HDL-cholesterol esterification and transfer rates.* The HDL-cholesterol esterification rate (CER) and the cholesteryl ester transfer activity (CETA) were determined by the methods described elsewhere (36–39). Briefly, $^{14}$C-cholesterol (0.18 µCi; New England Nuclear, Boston, MA) diluted in ethanol (5 µL) was added to small Whatman-1 filter-paper disks in triplicate. The latter were then incubated at 4°C for 18 h with 30 µL of apoB-LP-free plasma containing HDL mixed with 70 µL of a buffer solution (Tris, 10 µmol/L; NaCl, 150 µmol/L; EDTA, 0.01%; $NaN_3$, 0.03%). Thereafter, incubations occurred in a water bath at 37°C for 30 min for the CER measurement. Aliquots were immediately submitted to thin-layer chromatography (TLC) on silica gel G and developed with hexane/ethyl ether/acetic acid (70:30:1, by vol). Both unesterified (UC) and esterified (CE) cholesterol spots were scraped into counting vials containing toluene/Triton X-100 solution with 1,4-bis [2-(5-phenyloxazole)] benzene and 2,5-diphenyloxazole (0.5:5, w/w), and radioactivity was measured in a beta scintillation counter (LS6000, Beckman Instruments, Fullerton, CA). LCAT activity (CER) was expressed as percent activity and calculated from the 30-min radioactivity values as $[^{14}\text{C-CE}/(^{14}\text{C-CE} + ^{14}\text{C-UC})] \times 100$.

Incubations identical to those employed for CER were also carried for 24 h; 80 to 90% of $^{14}$C-UC in HDL was then present in the esterified form ($^{14}$C-CE HDL) and was utilized for the endogenous CETP activity (CETA) analysis. Whole plasma from each patient (400 µL) with added DTNB was then admixed to the tube containing $^{14}$C-CE HDL. Aliquots in triplicate were then incubated at both 4 and 37°C for 4 h,

and apoB-LP was precipitated as previously described. The supernatant containing $^{14}$C-CE HDL was utilized for separation of $^{14}$C-CE by TLC as described above. The percentage of radioactive cholesteryl ester transferred from HDL to VLDL and LDL (CETA) was calculated, as [1 − ($^{14}$C-CE at 37°C/$^{14}$C-CE at 4°C)] × 100.

*Measurement of CETP concentration.* Plasma CETP concentration was measured at the Lipoprotein and Atherosclerosis Group, University of Ottawa Heart Institute, Ontario, Canada, using a solid-phase radioimmunoassay method (40).

*Lipoprotein composition.* Lipoprotein composition was determined only in the second group of patients ($n = 5$) that participated in the open protocol, on admission and after 3 and 6 mon of therapy. Fresh plasma density was adjusted to 1.21 g/mL with addition of KBr and submitted to discontinuous gradient ultracentrifugation (41) in a SW41 rotor, Beckman L8 ultracentrifuge (Beckman Instruments, Palo Alto, CA) at 196,000 × *g*, 4°C for 24 h. The VLDL ($d < 1.006$ g/mL), intermediate density lipoprotein ($1.006 < d < 1.019$ g/mL), LDL ($1.019 < d < 1.063$ g/mL), $HDL_2$ ($1.063 < d < 1.125$ g/mL), and $HDL_3$ ($1.125 < d < 1.21$ g/mL) obtained were stored at −70°C until further analysis. Total cholesterol, UC, and triglycerides were enzymatically measured, as described above. Protein was measured by the method of Lowry *et al.* (42) and phospholipids by the Bartlett method (43).

*Statistical analysis*. All data are presented as mean ± SD or median (range). Comparisons between baseline, placebo, and hrGH treatment values were done using analysis of variance for repeated measurements, followed by the Student-Neuman-Keuls multiple-range test or Friedman's test for repeated measurements, followed by Dunn's multiple comparisons test. All correlations were analyzed by the Spearman test.

## RESULTS

During the double-blind period hrGH was administered to 5 patients and placebo to 3 patients; thereafter, hrGH was administered to all 8 individuals. Consequently, hrGH was administered to 5 patients for 12 months. However, because 5 additional cases were added at the sixth month of the protocol, 13 subjects were on hrGH for 6 mon. None of the parameters analyzed differed between the basal and the placebo phase, and consequently, basal and placebo data are representative of the period before treatment. Several of the biochemical plasma parameters analyzed (Table 1), namely, total plasma cholesterol, HDL-C, apoB-LP and apoA1-LP, did not differ between the periods before and during hrGH treatment, and body weight remained stable throughout the study. Triglyceride levels rose transiently at 3 mon on hrGH therapy. The plasma level of Lp(a) was permanently raised by hrGH, and, confirming the efficacy of treatment, the IGF-1 levels were increased as well.

Plasma CETP and LDL-C concentrations fell significantly at 3 but not at 6 and 12 mon on hrGH replacement; total cholesterol and apoB lipoprotein followed the same trend, although not reaching significant differences.

As shown elsewhere (28), the CETA method is not sensitive for detecting small variations in the plasma CETP mass. Nonetheless, as expected (25) the endogenous CETA and CETP values correlated with each other ($r = 0.29$, $P < 0.05$). Endogenous CETA likely represents the CE content transferred from HDL to the triglyceride-containing particles because there is a strong inverse correlation between CETA, HDL-C level ($r = -0.68$; $P < 0.001$), and apoA1 concentration ($r = -0.64$; $P < 0.001$). This plasma triglyceride-depen-

**TABLE 1**
**Plasma Lipids and Lipoprotein Concentrations[a]**

| | Before hrGH (basal and placebo) | During hrGH | | |
|---|---|---|---|---|
| | | 3 mon ($n = 13$) | 6 mon ($n = 13$) | 12 mon ($n = 5$) |
| Total cholesterol (mg/dL) | 191 ± 37 | 180 ± 39 | 187 ± 34 | 202 ± 30 |
| LDL cholesterol (mg/dL) | 122 ± 33 | 101 ± 29* | 114 ± 32 | 130 ± 25 |
| HDL cholesterol (mg/dL) | 42 ± 3 | 42 ± 3 | 42 ± 4 | 41 ± 5 |
| Triglycerides (mg/dL) | 133 ± 51 | 180 ± 91* | 151 ± 73 | 154 ± 72 |
| ApoB (mg/dL) | 107 ± 23 | 94 ± 24 | 98 ± 19 | 98 ± 16 |
| ApoA1 (mg/dL) | 92 ± 29 | 80 ± 27 | 92 ± 55 | 63 ± 9 |
| Lp(a) | 255 | 259* | 268** | 344 |
| (U/L) | (21–625) | (17–756) | (25–808) | (84–735) |
| CETP (μg/mL) | 2.33 ± 0.49 | 1.80 ± 0.53* | 2.10 ± 0.45 | 2.30 ± 0.58 |
| CETA | 46 | 50 | 52 | 57 |
| (%) | (30–63) | (34–69) | (30–64) | (46–74) |
| CER | 9.3 | 10.0 | 9.8 | 8.7 |
| (%) | (6.6–16) | (7.7–18.7) | (6.8–13.7) | (6.9–13.2) |
| IGF-1 (ng/mL) | 56 ± 40 | 293 ± 190* | 313 ± 193* | 361 ± 215 |

[a]Calculated by the Friedewald formula, plasma biochemistry values, and parameters of cholesteryl ester metabolism before and during human recombinant growth hormone (hrGH) replacement treatment: 3–6 mon ($n = 13$) and 12 mon ($n = 5$). Because the latter was a small group, statistical analysis was not provided. Data as mean ± SD or as median (range).
[b]Statistically significant difference between the periods before and during hrGH: *$P < 0.05$ mean values compared by analysis of variance as measured by the Student-Newman-Keuls test; **$P < 0.05$ median values compared by Friedman, as measured by Dunn's test. LDL, low density lipoprotein; HDL, high density lipoprotein; apoB, apolipoprotein B; apoA1, apolipoprotein A1; Lp(a), lipoprotein(a); CETP, cholesteryl ester transfer protein; CETA, cholesteryl ester transfer activity; CER, cholesteryl esterification rate; IGF-1, insulin growth factor-1.

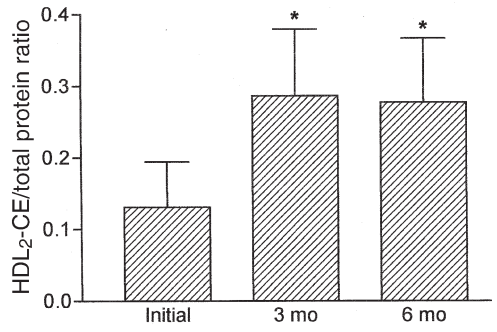



**FIG. 1.** Plasma lipoprotein composition ($HDL_2$-CE/total protein, mg/mg, mean ± SD as bar heights) obtained by ultracentrifugation from five patients. Initial values before human recombinant growth hormone (hrGH) compared by the Friedman test to 3 and 6 mon on hrGH therapy: $*P < 0.05$. None of the other lipoprotein fractions differed between control and treatment periods (data not shown). $HDL_2$, high density lipoprotein$_2$; CE, esterified cholesterol.

dent transfer process facilitates the LCAT-dependent rate of esterification of HDL-C because, as expected, CETA and CER values correlate with each other ($r = 0.35$; $P < 0.01$). According to this interpretation an inverse correlation between the HDL-C concentration and the CER value ($r = –0.33$; $P < 0.01$) was expected.

Although the *in vitro* measured endogenous CETA and CER were not modified on the hrGH therapy, the cholesteryl ester content of $HDL_2$ isolated by ultracentrifugation increased during hrGH (Fig. 1). Data on the composition of all other lipoprotein fractions separated by ultracentrifugation ($n = 5$) are not shown because they were not modified by the hrGH treatment.

## DISCUSSION

LDL-C concentration is known to increase in GH-deficient adults when compared to normal controls (3,4), however, the concentration of LDL-C does not differ between patients with active and controlled acromegaly (44). On the other hand, previous investigations on hrGH treatment of GH-deficient patients had shown LDL-C concentration as permanently lowered (4,7–10), not modified (4,11), or only transiently lowered (12). No clear explanations are found for these discrepant results since hrGH treatment lowered LDL-C permanently when administered either at high (7,10) or at low doses (8,9). In addition, LDL-C level failed to permanently respond to hrGH administration utilizing similarly low doses (11), as in the present report, or high hrGH dose (12).

Interestingly, the simultaneous, although transient, decrease of LDL-C and of CETP concentrations elicited by hrGH supports other studies that have suggested a close relationship between LDL metabolism and the regulation of the CETP gene expression: (i) plasma CETP levels are high in primary hypercholesterolemic subjects (26,27), and diminish upon the administration of cholestyramine (29), a nonabsorbable drug that stimulates the gene expression of the hepatic LDL receptors; (ii) cholesterol feeding increases both the transcription of the human CETP gene (23) and the plasma CETP concentration (24) and diminishes the number of hepatic LDL receptors (25); (iii) these receptors are induced by GH treatment (30).

Plasma triglycerides did not vary in the majority of the trials with hrGH replacement administered to GH-deficient adults. However, measurements were usually made at baseline and after 6 (4) or 12 mon of therapy (8). When measurements of triglyceride levels are carried out earlier along the treatment course, a transient rise has been observed (45). Furthermore, since GH replacement brings on a faster hepatic triglyceride synthesis rate (46), a transient rise in the plasma triglyceride concentration could represent a greater number of VLDL particles. However, along the treatment course a greater number of hepatic LDL receptors that recognize VLDL might have offset the faster plasma VLDL-triglyceride synthesis rate.

CETP concentrations measured by radioimmunoassay were also modified at 3 but not at 6 and 12 mon on hrGH. Endogenously measured CETA was not modified but this procedure is simultaneously dependent on the availability of VLDL-triglyceride, which takes up cholesteryl ester from HDL (16–18,47) and on the CETP concentration. Seemingly, the higher concentration of plasma triglycerides failed to raise the endogenous CETA value, possibly owing to the lower CETP concentration. In a recent study by Beentjes *et al.* (8), GH replacement lowered the exogenous CETA value after 12 mon, but CETP concentration was not measured. Interestingly, these authors also showed that the LCAT activity as well as the rate of cholesterol esterification (EST) was lowered by GH replacement. Incidentally, in their work phospholipid transfer protein (PLTP) activity was not modified although CETP is known to have substantial PLTP activity (17,48).

In view of our finding that the CETP concentration returned to normal after 6 mon, and remained stable in five patients at 12 mon (mean μg/mL = 2.30 ± 0.58), although statistical treatment of the latter data is not provided because it was a small group, we suggest that some other factor may have lowered the exogenous CETA at 12 mon in the Beentjes *et al*. study (8). Free fatty acids nonetheless could not be responsible for this effect because they increase in plasma on GH treatment (49) and are known to raise the CETA either directly (50) or indirectly, owing to their described role of suppressing a lipid transfer protein inhibitor (51,52).

Although the concentrations of plasma CETP and of $HDL_2$-CE are known to be reciprocally related (17), the level of the latter was not related to the concentration of CETP because CETP decreased transiently only, whereas $HDL_2$-CE remained elevated throughout the whole span of the hrGH replacement. Therefore, the concentration of $HDL_2$-CE is independently modified by hrGH by unknown mechanisms. The modification of the $HDL_2$-CE/protein ratio elicited by hrGH replacement shown in the ultracentrifuged plasma samples ($n = 5$) does not reflect the total plasma HDL-C concentration as measured after the precipitation of the apoB-containing lipoproteins reported in this and in another study (8), possibly because $HDL_2$ is a lesser fraction of the total HDL.

In the study by Beentjes *et al.* (8), lower rates of plasma EST were associated with diminished LCAT and CETA activities after 12 mon but not after 6 mon on a similar mean dose of hrGH replacement (2 U/d) as that utilized in our study (2.4 U/d). Interestingly, Lp(a) concentration was not modified in their study but has been reported as increased by GH treatment in our study and in most reports (4,9,10,13,14), including in active acromegalics (44). No obvious explanations are afforded for the discrepancy between all these reports and the data of Beentjes *et al.* (8).

In conclusion, this study shows, for the first time, that hrGH administration to GH-deficient subjects transiently reduces the plasma CETP concentrations and permanently raises the plasma $HDL_2$-CE/protein ratio. Confirming several other studies, we also demonstrated that hrGH permanently brings on a higher plasma Lp(a) concentration but only transiently lowers the plasma LDL-C level. Therefore, the relevance to atherosclerosis of hrGH replacement therapy cannot be predicted in the long run because, although a high Lp(a) plasma level is potentially harmful, the elevated $HDL_2$-CE/protein ratio could be beneficial.

## ACKNOWLEDGMENTS

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# Influence of Formulas with Borage Oil or Borage Oil Plus Fish Oil on the Arachidonic Acid Status in Premature Infants

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**ABSTRACT:** Several studies have reported that feeding γ-linolenic acid (GLA) has resulted in no increase in arachidonic acid (AA) in newborns. This result was ascribed to the eicosapentaenoic acid (EPA)-rich fish oil used in these formulas. Docosahexaenoic acid (DHA) sources with only minor amounts of EPA are now available, thus the addition of GLA to infant formulas might be considered an alternative to AA supplementation. Sixty-six premature infants were randomized to feeding one of four formulas [ST: no GLA, no long-chain polyunsaturated fatty acids; BO: 0.6% GLA (borage oil); BO + FOLOW: 0.6% GLA, 0.3% DHA, 0.06% EPA; BO + FOHIGH: 0.6% GLA, 0.3% DHA, 0.2% EPA] or human milk (HM, nonrandomized) for 4 wk. Anthropometric measures and blood samples were obtained at study entry and after 14 and 28 d. There were no significant differences between groups in anthropometric measures, tocopherol, and retinol status at any of the studied time points. The AA content of plasma phospholipids was similar between groups at study start and decreased significantly until day 28 in all formula-fed groups, but not in the breast-fed infants [ST: 6.6 ± 0.2%, BO: 6.9 ± 0.3%, BO + FOLOW: 6.9 ± 0.4%, BO + FOHIGH: 6.7 ± 0.2%, HM: 8.6 ± 0.5%, where values are reported as mean ± standard error; all formulas significantly different ($P \leq 0.05$) from HM]. There was no significant influence of GLA or fish oil addition to the diet. GLA had only a very limited effect on AA status which was too small to obtain satisfactory concentrations (concentrations similar to breast-fed babies) under the circumstances tested. The effect of GLA on AA is independent of the EPA and DHA content in the diet within the dose ranges studied.

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The addition of suitable amounts of long-chain polyunsaturated fatty acids (LC-PUFA) to infant formulas, especially arachidonic (AA) and docosahexaenoic (DHA) acids, establishes concentrations of these fatty acids in infant plasma phospholipids equivalent to those found in breast-fed infants (1,2). Evidence is accumulating that the availability of appreciable LC-PUFA concentrations is associated with the neurological development of preterm (3) and term babies (4,5). This is ascribed to the importance of these fatty acids for membrane properties, as there are high relative concentrations of DHA in retina and brain (6). AA and other 20-carbon atom fatty acids [dihomo-γ-linolenic (DGLA) and eicosapentaenoic (EPA)] are important precursors for eicosanoids (7). Although functional effects of pure AA supplementation have not been investigated in humans, a stable supply of AA, as achieved by breast feeding (8), seems desirable for formula-fed babies. In rats negative effects of low AA plasma concentrations, induced by fish oil feeding, on postnatal development have been shown (9). Furthermore, AA status has been associated with infant growth (10,11).

An exogenous supply of LC-PUFA is required to achieve plasma concentrations equivalent to those found in breast-fed babies, although newborns already synthesize LC-PUFA endogenously from the essential precursors linoleic (LA) and α-linolenic acid during the first days after birth (12–14). Although the pathways of conversion have been elucidated, the question whether the low plasma and tissue concentrations found without dietary supplement are due to the high metabolic demand of LC-PUFA or to low conversion has not been answered (15). The pathways of n-3 and n-6 essential fatty acid conversion to AA and DHA share the enzymes for Δ-6, Δ-5 desaturation and chain elongation, but it is not yet known whether there are different variants of these enzymes (16). The initial step is a Δ-6 desaturation, which converts LA to γ-linolenic acid (GLA). There are strong indications that this is the rate-limiting step for the endogenous synthesis of AA (17). Based on this concept, an exogenous supply of GLA might increase the plasma concentration of n-6 LC-PUFA in infants, as was reported to occur in adults (18).

Many manufacturers produce infant formulas containing from 0.4 to 1.0% of fatty acids as LC-PUFA (19). Although fish oils are readily available as a source for n-3 LC-PUFA, the introduction of AA is a greater challenge. Egg phospholipids and single-cell oils are the primary AA sources used in infant formulas, but they are comparatively expensive or have not been approved for formula use in all countries. Alternatively, readily available sources of GLA-containing oils, e.g., borage, black currant seed or evening primrose oil, could be

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Abbreviations: AA, arachidonic acid (20:4n-6); BO, borage oil; Chol, cholesterol; DGLA, dihomo-γ-linolenic acid (20:3n-6); DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3); GLA, γ-linolenic acid (18:3n-6); HM, human milk; LA, linoleic acid (18:2n-6); LC-PUFA, long-chain polyunsaturated fatty acids (≥ 20 C atoms, ≥ 2 double bonds); MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SAT, saturated fatty acid; ST, standard formula; TG, triglycerides.

added to the formulas (20). These oils have been considered safe in older children even in pharmacological doses (21).

Some studies investigated the influence of a GLA and n-3 LC-PUFA supply to term infants and found no positive effects on AA percentage in red blood cell phospholipids (22–24). Since formulas tested in these studies also contained fish oils rich in EPA, it appeared possible that the lack of a positive effect of the GLA supplement might have been due to an inhibiting effect of high EPA and DHA supply on AA synthesis (8,20). This is based on the observation that feeding n-3 LC-PUFA-rich marine oil caused increased concentrations of n-3 LC-PUFA and decreased AA concentrations in infants and adults (25,26). However, in term infants a formula containing 0.5% GLA only marginally increased the AA level, although EPA contributed only 0.07% to dietary fatty acids (22). DHA sources that contain only minor amounts of EPA are now readily available, thus we investigated the inclusion of a slightly higher dose of GLA into a preterm formula as an alternative to AA supplementation.

We compared four different formulas for preterm infants containing: (i) no GLA and no LC-PUFA, (ii) 0.6% GLA and no LC-PUFA, (iii) 0.6% GLA and 0.3% DHA combined with 0.06% EPA, and (iv) 0.2% EPA and 0.3% DHA plus 0.6% GLA. A nonrandomized reference group of breast-fed infants was also followed. While the majority of the previous infant studies analyzed red blood cells, which might have an attenuated and delayed response to dietary changes, we chose to analyze plasma phospholipids.

Since LC-PUFA supplementation of the diet decreased the tocopherol/lipid ratio in the erythrocytes of infants and the plasma vitamin E concentrations in adults (27,28) and since in newborns serious health risks have been associated with low vitamin E levels (29), we also analyzed plasma concentrations of lipid-soluble vitamins to detect potential influences of the various fatty acid supplies on the infants' antioxidative status (30).

## SUBJECTS AND METHODS

Premature infants were recruited for the study at the Children's Hospital Gyor (Hungary) from December 1994 to May 1997. Inclusion criteria were a birth weight below 1800 g and full enteral feeding with more than 120 mL milk/kg/d. Excluded from the study were any infants with serious metabolic or congenital anomalies; infants on artificial ventilation; infants with septic infections, gastrointestinal problems or metabolic diseases; and infants receiving any parenteral lipids during the study period. Systemic corticosteroid therapy during the study was only allowed for up to 3 d, otherwise subjects were excluded. According to the standard hospital protocol for parenteral nutrition, all participating infants were fed a 20% lipid emulsion based on soybean oil. Up to 10% of total energy intake from sources other than the corresponding study diet were permitted.

The study protocol was approved by the Ethical Committee of the Hungarian Medical Association. After explanation of the study protocol, written parental consent for participation was obtained for all infants before enrollment. If mothers provided HM, the infants were fed with breast milk. If mothers chose not to provide breast milk, the infants were randomized double blind to one of the four formula groups without further stratification. Randomization numbers were computer-generated (Roche, Basel, Switzerland) and allocated sequentially in the order of enrollment to the infants. Double blinding was achieved, as the identity of the dispensed formula was provided in a sealed envelope for each infant. Only in case of an immediately reportable adverse event was this envelope to be opened.

The study formulas were produced by Nutricia (Zoetermeer, The Netherlands) based on the low birth weight infant formula "Nenatal." The formulas delivered 80 kcal per 100 mL, and contained 11% (of energy) protein (whey/casein = 3:2), 40% carbohydrates (mainly lactose and polysaccharides), and 49% fat. The dietary fat (4.4 g/100 mL) was based on a blend of milk fat, coconut oil, soy oil, sunflower oil, and canola oil. These were the only fat components of the standard formula (ST), whereas the GLA content was increased by the addition of borage oil (BO). The two other formulas (BO + FOLOW, BO + FOHIGH) additionally contained fish oils differing in their EPA/DHA ratio (Hoffmann-La Roche Ltd., Basel, Switzerland). Both were composed to deliver 0.3% DHA but contained either 0.06% (BO + FOLOW) or 0.2% EPA (BO + FOHIGH). Table 1 shows the detailed fatty acid composition of the different formulas and of the breast milk fed (mean and standard error of individual samples), as analyzed in our laboratory. In the case of breast milk feeding, a 5-mL sample of breast milk was collected on days 0 and 14. All formulas contained 0.1 mg vitamin A and 1.3 mg vitamin E per 100 mL.

On the day of enrollment (study day 0), 2 wk later (study day 14), and 4 wk later (study day 28) anthropometric measures were recorded and blood samples were taken. At all time points a deviation of maximally 1 d from the date determined by the protocol was tolerated. Weight, length, and head circumference were measured according to standard hospital procedures. Blood samples (0.5 mL) were taken by venipuncture and immediately transferred into EDTA-containing tubes. Blood cells and plasma were separated by centrifugation at $1000 \times g$ for 5 min. A plasma aliquot of at least 200 μL was frozen immediately at −80°C for later analysis. Red blood cells were washed three times in 0.9% aqueous NaCl solution, hemolyzed, and stored at −80°C until analysis (data not shown). The remaining portion of the sample was used for the determination of routine laboratory parameters: alanine-amino-transferase, γ-glutamyltranspeptidase, total bilirubin, creatinine, total protein, glucose, carbamide-N, sodium, potassium, and calcium. For further assessment of possible adverse effects a series of hematological measures were performed, e.g., red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, hemoglobin E, white blood cell count, platelet count, and hemogram. Pulse rate and blood pressure were also recorded.

As differences of 20% or greater in the primary study parameter, AA concentration in plasma phospholipids, were considered clinically important, the sample size was planned on the data of Decsi and Koletzko (31) who reported a rela-

**TABLE 1**
**Fatty Acid Composition (%w/w) of the Formulas Used in the Study (results of our own analyses) and from the Individual Human Milk Samples on Day 0 and Day 14[a]**

| | | | | | HM | |
|---|---|---|---|---|---|---|
| Fatty acid | ST ($n$ = 2) | BO ($n$ = 4) (mean ± SE) | BO + FOLOW ($n$ = 2) | BO + FOHIGH ($n$ = 2) | Day 0 ($n$ = 12) (mean ± SE) | Day 14 ($n$ = 13) (mean ± SE) |
| ∑SAT | 41.10/41.34 | 41.53 ± 0.07 | 40.87/40.96 | 40.44/40.64 | 46.32 ± 1.76 | 47.47 ± 1.17 |
| ∑MUFA | 44.05/43.88 | 43.75 ± 0.11 | 44.02/44.09 | 44.07/43.88 | 34.68 ± 1.12 | 34.19 ± 1.09 |
| ∑TRANS | 0.05/0.05 | 0.03 ± 0.01 | 0.05/0.06 | 0.07/0.07 | 0.72 ± 0.14 | 0.85 ± 0.14 |
| 20:3n-9 | ND | ND | ND | ND | 0.01 ± 0.01 | 0.01 ± 0.01 |
| 18:2n-6 | 13.47/13.40 | 12.88 ± 0.03 | 12.73/12.74 | 12.97/12.97 | 15.65 ± 1.25 | 15.15 ± 1.14 |
| 18:3n-6 | 0.01/0.01 | 0.65 ± 0.02 | 0.60/0.60 | 0.64/0.64 | 0.15 ± 0.01 | 0.17 ± 0.01 |
| 20:2n-6 | ND | 0.01 ± 0.00 | 0.01/0.01 | 0.02/ND | 0.40 ± 0.04 | 0.32 ± 0.03 |
| 20:3n-6 | 0.01/0.01 | 0.01 ± 0.00 | 0.01/0.01 | 0.01/0.01 | 0.43 ± 0.03 | 0.41 ± 0.03 |
| 20:4n-6 | 0.01/0.01 | 0.01 ± 0.00 | 0.03/0.03 | 0.02/0.02 | 0.51 ± 0.03 | 0.47 ± 0.03 |
| 22:2n-6 | 0.06/0.06 | 0.06 ± 0.00 | 0.06/0.06 | 0.05/0.05 | 0.05 ± 0.01 | 0.04 ± 0.01 |
| 22:4n-6 | ND | ND | ND | ND | 0.14 ± 0.01 | 0.12 ± 0.01 |
| 18:3n-3 | 1.21/1.20 | 1.06 ± 0.09 | 1.20/1.20 | 1.26/1.26 | 0.51 ± 0.05 | 0.43 ± 0.04 |
| 18:4n-3 | 0.02/0.02 | 0.02 ± 0.01 | 0.02/0.02 | 0.04/0.04 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| 20:3n-3 | ND | ND | ND | ND | 0.02 ± 0.01 | 0.01 ± 0.01 |
| 20:5n-3 | 0.01/0.01 | ND | 0.06/0.06 | 0.15/0.15 | 0.03 ± 0.01 | 0.02 ± 0.01 |
| 22:5n-3 | 0.01/0.01 | 0.01 ± 0.00 | 0.02/0.02 | 0.02/0.02 | 0.11 ± 0.01 | 0.10 ± 0.01 |
| 22:6n-3 | ND | ND | 0.24/0.25 | 0.25/0.25 | 0.22 ± 0.03 | 0.20 ± 0.03 |
| LC-PUFA | 0.10/0.10 | 0.10 ± 0.00 | 0.42/0.42 | 0.52/0.51 | 1.95 ± 0.12 | 1.73 ± 0.12 |
| 18:2n-6/18:3n-3 | 11.10/11.12 | 12.39 ± 1.06 | 10.61/10.63 | 10.30/10.33 | 33.92 ± 3.87 | 38.64 ± 4.56 |

[a]ST, standard formula; BO, borage oil; BO + FOLOW, borage oil + 0.3% DHA and 0.06% EPA; BO + FOHIGH, borage oil + 0.3% DHA and 0.2% EPA; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SAT, saturated fatty acids; MUFA, monounsaturated fatty acids; TRANS, *trans* fatty acids; ND, not detected; LC-PUFA, long-chain polyunsaturated fatty acids.

tive standard deviation of 24% for AA. With 13 infants per group, the considered difference between groups was to be detected with 90% probability and a 5% risk of type I error.

*Analysis of plasma phospholipids*. After the addition of dipentadecanoyl-phosphatidylcholine as an internal standard, total lipids from 100 µL plasma were extracted into 2 mL hexane/isopropanol (4:1) and subsequently twice into 2 mL of hexane (32). Extracts were combined and dried under a stream of $N_2$. For application on silica gel plates (Merck KG, Darmstadt, Germany), the residue was taken up in chloroform/methanol (1:1). Phospholipids were isolated by development of the plates in *n*-heptane/diisopropylether/glacial acetic acid (60:40:3, by vol) (33). After visualization with 2,7-dichlorofluorescein, phospholipid-containing bands were scraped off and transferred into reaction vials. Synthesis of fatty acid methyl esters was performed by heating the phospholipids to 85°C for 45 min after dissolution in 1.5 mL 3M methanolic hydrochloric acid. After neutralization of the reaction mixture with carbonate buffer, methyl esters were extracted twice into 1 mL hexane and the combined extracts taken to dryness under nitrogen. For storage (–80°C) until gas chromatographic analysis, the samples were dissolved in 40 µL hexane (containing 2 g/L butylhydroxytoluene) and transferred into microvials.

Gas chromatography was performed on an HP5890 series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a split/splitless injector (250°C, split ratio = 1:20, column head pressure 1.20 bar) and a flame-ionization detector (300°C). Separation of individual fatty acid methyl esters was achieved with a BPX70 column (SGE, Weiterstadt, Germany) with 60 m length and 0.32 mm inner diameter. The temperature program started at 130°C, increased at a rate of 3°C/min up to 200°C, and continued without delay up to 210°C at a rate of 1°C/min. For identification and determination of response factors, commercially available standards were used [Nu-Chek-Prep, Elysian, MN; Sigma, Deisenhofen, Germany; docosapentaenoic (n-6) acid methyl ester was a gift from Omega Tech, Boulder, CO]. Chromatographic data were recorded and evaluated using EZChrom Elite Ver. 2.1 software (Scientific Software Inc., Pleasanton, CA). Because of the small plasma volumes, samples were weighed in addition to volumetric dosage, and absolute plasma concentrations are given as mg fatty acid methyl ester per kg plasma.

*Analysis of milk lipids*. Milk samples were analyzed as previously described (34). Briefly, after the addition of 100 µL potassium oxalate (3.5%) solution, lipids from 1 mL milk were extracted into 3 mL of a mixture of ethanol/*tert*-butylmethylether/petrolether (1:1:1). To obtain quantitative recovery, a second extraction with 2 mL *tert*-butyl-methylether/petrolether (1:1) was performed. Extracts were combined and transferred into a preweighed vial; solvents were evaporated under nitrogen, and the remainder was dried in a desiccator overnight. Total lipid content could now be determined gravimetrically. For transesterification at 90°C for 60 min, the lipids were dissolved in 2 mL 1.5 M methanolic hydrochloric acid

and 1 mL hexane. After the addition of 2 mL water, the methyl esters were extracted into 2 mL of hexane and analyzed by gas chromatography as described for plasma samples.

*Analyses of vitamins A and E*. These analyses were performed as previously described from 100 µL plasma samples by high-performance liquid chromatography (35). After the addition of 100 µL ethanol to the samples, vitamins were extracted three times into 1 mL hexane and the combined extracts taken to dryness. For chromatography the extract was reconstituted in 100 µL mobile phase [acetonitrile/tetrahydrofuran/methanol/ammonium acetate (1%) = 684:220:68:28] containing tocol as standard for quantification. A high-performance liquid chromatography system (L6200 pump, AS 2000 autosampler, L4250 ultraviolet-visible detector, F-1050 fluorescence detector; Merck), equipped with a reversed-phase column (LiChrospher 100, RP-18, 25 cm long, Merck) was used for chromatography. With a flow rate of 0.65 mL/min, retinol, α-tocopherol and δ-tocopherol could be separated, while β- and γ-tocopherol coeluted. For retinol detection the photometer was set to 325 nm, and tocopherols were detected by their fluorescence at 330 nm after excitation at 298 nm. Data were recorded and evaluated with EZChrom Elite Ver. 2.1 software (Scientific Software Inc., Pleasanton, CA).

Total triglycerides (TG) and total cholesterol (Chol) were determined using a Vitros 250 autoanalyzer (Johnson & Johnson, Neckargmünd, Germany).

*Statistical analysis*. Data characterizing subjects are given as means ± standard deviations. One-way analysis of variance was used to detect statistically significant differences ($P \leq 0.05$) between groups. Analytical results for fatty acids and lipid-soluble vitamins are presented as means ± standard errors of the mean. Statistical evaluation of the results at the end of the study was performed by analysis of variance, including the corresponding values at study start as covariates. Differences between group means were taken as statistically significant if the 95% confidence interval (Scheffé method for correction of multiple testing) of differences between groups did not include 0. Coefficients of correlation were calculated according to Pearson and were taken as significant if $P$ was equal to or less than 0.05. Individual changes with time were investigated by paired $t$-tests (significance level 0.05). Calculations were performed using S Plus, Ver. 4.5 (Mathsoft Inc., Seattle, WA).

## RESULTS

Sixty-six infants with gestational ages between 25 and 37 wk were enrolled into the study. One infant from group ST was excluded from the study because of infectious disease. Thus the results from 65 infants who completed the study were evaluated. There were no statistically significant differences between the five dietary groups in clinical characteristics, body weight, length, head circumference, and postnatal age (Table 2). Although infants in groups BO and BO + FOHIGH tended to be older, there were no significant differences at study start and study end.

Infants presented very similar fatty acid compositions of plasma phospholipids across all groups at study start (Table 3). Saturated fatty acids (SAT) contributed almost one-half and *cis*-monounsaturated about 20% of the total. The only major difference at study start was a lower GLA content in the plasma phospholipids of the breast-fed group, although 0.15% GLA was detected in HM fat.

Tendencies indicating the statistically analyzed values at study end could already be observed for all investigated parameters at study day 14; thus there seemed to be a continuous trend during the whole study period.

**TABLE 2**
**Clinical and Anthropometric Description of the Infants (mean ± SD) Studied at Birth, Day 0 (study start), Day 14, and Day 28 (study end)**

| | ST | BO | BO + FOLOW | BO + FOHIGH | HM |
|---|---|---|---|---|---|
| Number of infants | 13 | 13 | 13 | 14 | 13 |
| Birth weight (g) | 1494 ± 269 | 1302 ± 229.3 | 1500 ± 199 | 1362 ± 284 | 1404 ± 290 |
| Age at study start (d) | 21.2 ± 10.4 | 32.4 ± 18.2 | 22.6 ± 10.9 | 33.9 ± 15.1 | 29.5 ± 13.3 |
| | | | Body weight[a] (g) | | |
| Day 0 | 1522 ± 222 | 1422 ± 188 | 1535 ± 198 | 1516 ± 223 | 1532 ± 250 |
| Day 14 | 1840 ± 275 | 1790 ± 249 | 1888 ± 223 | 1865 ± 319 | 1822 ± 294 |
| Day 28 | 2315 ± 325* | 2239 ± 306* | 2388 ± 250* | 2391 ± 377* | 2239 ± 380* |
| | | | Length[a] (cm) | | |
| Day 0 | 43.2 ± 2.9 | 42.2 ± 2.1 | 42.9 ± 3.4 | 42.8 ± 1.7 | 42.7 ± 2.5 |
| Day 14 | 44.0 ± 2.4 | 44.2 ±1.9 | 44.1 ± 2.7 | 44.6 ± 1.9 | 44.0 ± 2.2 |
| Day 28 | 46.6 ± 2.9* | 46.0 ± 2.3* | 46.9 ± 1.7* | 46.2 ± 1.9* | 46.3 ± 2.5* |
| | | | Head circumference[a] (cm) | | |
| Day 0 | 30.0 ± 2.0 | 28.2 ± 2.8 | 29.2 ± 1.7 | 29.9 ± 1.6 | 28.9 ± 2.0 |
| Day 14 | 31.3 ± 1.8 | 30.7 ± 1.7 | 31.5 ± 1.7 | 31.5 ± 1.7 | 30.6 ± 1.1 |
| Day 28 | 32.9 ± 1.5* | 32.2 ± 1.5* | 33.0 ± 1.1* | 32.86 ± 1.6* | 32.2 ± 0.9* |

[a]Asterisk indicates a significant change ($P \leq 0.05$) from day 0 to day 28. For abbreviations for formulas used see Table 1.

**TABLE 3**
**Fatty Acid Composition (%w/w, mean ± SE) of Infant Plasma Phospholipids at Study Start (day 0), After 2 wk on Study Diet (day 14), and at Study End After 4 wk (day 28)**

| | ST | BO | BO + FOLOW | BO + FOHIGH | HM |
|---|---|---|---|---|---|
| | | | Day 0 | | |
| ∑SAT | 48.01 ± 0.87 | 47.53 ± 0.53 | 47.72 ± 0.71 | 48.21 ± 0.32 | 48.28 ± 0.46 |
| ∑MUFA | 20.69 ± 0.78 | 21.64 ± 1.13 | 20.11 ± 0.78 | 19.61 ± 0.86 | 19.44 ± 0.45 |
| ∑TRANS | 0.86 ± 0.07 | 0.89 ± 0.06 | 0.83 ± 0.07 | 0.87 ± 0.04 | 0.94 ± 0.07 |
| 20:3n-9 | 0.78 ± 0.22 | 0.96 ± 0.31 | 0.48 ± 0.09 | 0.81 ± 0.21 | 0.47 ± 0.15 |
| 18:2n-6 | 13.94 ± 0.94 | 13.73 ± 1.20 | 14.49 ± 0.90 | 14.57 ± 1.08 | 14.72 ± 0.44 |
| 18:3n-6 | 0.11 ± 0.03 | 0.12 ± 0.02 | 0.10 ± 0.03 | 0.12 ± 0.03 | 0.01 ± 0.01 |
| 20:2n-6 | 0.39 ± 0.04 | 0.47 ± 0.03 | 0.44 ± 0.02 | 0.46 ± 0.02 | 0.46 ± 0.02 |
| 20:3n-6 | 2.71 ± 0.17 | 2.68 ± 0.12 | 2.69 ± 0.11 | 2.83 ± 0.17 | 2.83 ± 0.17 |
| 20:4n-6 | 9.37 ± 0.52 | 8.55 ± 0.41 | 9.80 ± 0.36 | 9.18 ± 0.42 | 9.47 ± 0.35 |
| 22:2n-6 | 0.55 ± 0.04 | 0.50 ± 0.04 | 0.49 ± 0.04 | 0.56 ± 0.05 | 0.59 ± 0.04 |
| 22:4n-6 | 0.50 ± 0.05 | 0.55 ± 0.03 | 0.53 ± 0.03 | 0.55 ± 0.03 | 0.51 ± 0.02 |
| 20:3/20:4 | 0.30 ± 0.02 | 0.32 ± 0.02 | 0.28 ± 0.02 | 0.32 ± 0.03 | 0.30 ± 0.02 |
| ∑n-6 LC-PUFA | 13.51 ± 0.62 | 12.74 ± 0.50 | 13.95 ± 0.35 | 13.57 ± 0.49 | 13.85 ± 0.42 |
| 18:3n-3 | ND | 0.03 ± 0.01 | 0.01 ± 0.01 | 0.02 ± 0.01 | ND |
| 20:5n-3 | 0.06 ± 0.03 | 0.15 ± 0.02 | 0.14 ± 0.05 | 0.09 ± 0.04 | 0.04 ± 0.02 |
| 22:5n-3 | 0.19 ± 0.04 | 0.27 ± 0.05 | 0.18 ± 0.04 | 0.25 ± 0.04 | 0.24 ± 0.03 |
| 22:6n-3 | 1.85 ± 0.09 | 1.93 ± 0.14 | 1.97 ± 0.08 | 1.87 ± 0.10 | 2.01 ± 0.14 |
| ∑n-3 LC-PUFA | 2.10 ± 0.13 | 2.35 ± 0.18 | 2.30 ± 0.12 | 2.22 ± 0.13 | 2.29 ± 0.16 |
| ∑PUFA | 30.43 ± 1.02 | 29.94 ± 1 .40 | 31.33 ± 0.80 | 31.30 ± 1.06 | 31.33 ± 0.71 |
| ∑PUFA/∑SAT | 0.64 ± 0.03 | 0.63 ± 0.03 | 0.66 ± 0.02 | 0.65 ± 0.02 | 0.65 ± 0.02 |
| ∑LC-PUFA | 16.39 ± 0.72 | 16.05 ± 0.46 | 16.72 ± 0.40 | 16.60 ± 0.51 | 16.61 ± 0.54 |
| | | | Day 14 | | |
| ∑SAT | 43.86 ± 0.44 | 43.91 ± 0.46 | 44.36 ± 0.68 | 44.56 ± 0.46 | 47.98 ± 0.52 |
| ∑MUFA | 23.77 ± 0.37 | 23.60 ± 0.38 | 23.33 ± 0.56 | 22.99 ± 0.54 | 18.94 ± 0.43 |
| ∑TRANS | 0.55 ± 0.03 | 0.54 ± 0.03 | 0.52 ± 0.02 | 0.53 ± 0.05 | 0.97 ± 0.05 |
| 20:3n-9 | 0.19 ± 0.04 | 0.20 ± 0.03 | 0.17 ± 0.03 | 0.13 ± 0.04 | 0.39 ± 0.11 |
| 18:2n-6 | 18.38 ± 0.60 | 17.81 ± 0.53 | 16.59 ± 0.38 | 16.93 ± 0.38 | 16.31 ± 0.60 |
| 18:3n-6 | 0.06 ± 0.02 | 0.15 ± 0.02 | 0.16 ± 0.02 | 0.13 ± 0.02 | 0.08 ± 0.03 |
| 20:2n-6 | 0.42 ± 0.02 | 0.43 ± 0.02 | 0.40 ± 0.02 | 0.41 ± 0.02 | 0.49 ± 0.02 |
| 20:3n-6 | 2.51 ± 0.17 | 3.17 ± 0.11 | 3.25 ± 0.18 | 3.06 ± 0.11 | 2.88 ± 0.14 |
| 20:4n-6 | 7.37 ± 0.38 | 7.33 ± 0.22 | 7.57 ± 0.24 | 7.43 ± 0.28 | 8.57 ± 0.33 |
| 22:2n-6 | 0.52 ± 0.05 | 0.52 ± 0.05 | 0.51 ± 0.06 | 0.63 ± 0.04 | 0.57 ± 0.03 |
| 22:4n-6 | 0.36 ± 0.03 | 0.38 ± 0.02 | 0.32 ± 0.03 | 0.32 ± 0.03 | 0.54 ± 0.03 |
| 20:3/20:4 | 0.35 ± 0.03 | 0.44 ± 0.02 | 0.43 ± 0.03 | 0.42 ± 0.03 | 0.34 ± 0.02 |
| ∑n-6 LC-PUFA | 11.18 ± 0.42 | 11.83 ± 0.22 | 12.05 ± 0.26 | 11.85 ± 0.24 | 13.05 ± 0.37 |
| 18:3n-3 | 0.05 ± 0.02 | 0.09 ± 0.02 | 0.03 ± 0.01 | 0.05 ± 0.02 | 0.01 ± 0.01 |
| 20:5n-3 | 0.14 ± 0.03 | 0.13 ± 0.04 | 0.36 ± 0.02 | 0.42 ± 0.04 | 0.08 ± 0.03 |
| 22:5n-3 | 0.16 ± 0.03 | 0.20 ± 0.03 | 0.16 ± 0.04 | 0.22 ± 0.02 | 0.32 ± 0.02 |
| 22:6n-3 | 1.66 ± 0.13 | 1.55 ± 0.12 | 2.28 ± 0.12 | 2.17 ± 0.09 | 1.89 ± 0.14 |
| ∑n-3 LC-PUFA | 1.95 ± 0.15 | 1.87 ± 0.15 | 2.80 ± 0.14 | 2.82 ± 0.11 | 2.29 ± 0.15 |
| ∑PUFA | 31.82 ± 0.26 | 31.96 ± 0.48 | 31.79 ± 0.44 | 31.91 ± 0.28 | 32.11 ± 0.68 |
| ∑PUFA/SAT | 0.73 ± 0.01 | 0.73 ± 0.02 | 0.72 ± 0.02 | 0.72 ± 0.01 | 0.67 ± 0.02 |
| ∑LC-PUFA | 13.33 ± 0.52 | 13.91 ± 0.28 | 15.01 ± 0.40 | 14.80 ± 0.26 | 15.72 ± 0.44 |
| | | | Day 28 | | |
| ∑SAT | 44.08 ± 0.45$^{a,*}$ | 43.95 ± 0.47$^{b,*}$ | 40.95 ± 3.45$^{c,*}$ | 44.52 ± 0.40$^{d,*}$ | 47.54 ± 0.63$^{a,b,c,d}$ |
| ∑MUFA | 24.00± 0.42$^{a,*}$ | 23.63 ± 0.31$^{b}$ | 21.35 ± 1.87$^{c,*}$ | 23.05 ± 0.43$^{d,*}$ | 19.30 ± 0.63$^{a,b,c,d}$ |
| ∑TRANS | 0.49 ± 0.06$^{a,*}$ | 0.54 ± 0.03$^{b,*}$ | 0.54 ± 0.07$^{c,*}$ | 0.58 ± 0.04$^{d,*}$ | 0.90 ± 0.06$^{a,b,c,d}$ |
| 20:3n-9 | 0.29 ± 0.03* | 0.16 ± 0.02$^{a,*}$ | 0.19 ± 0.03* | 0.14 ± 0.03$^{b,*}$ | 0.50 ± 0.15$^{a,b}$ |
| 18:2n-6 | 18.66 ± 0.34$^{a,*}$ | 18.41 ± 0.34* | 17.31 ± 0.51* | 17.64 ± 0.27* | 16.51 ± 0.84$^{a,*}$ |
| 18:3n-6 | 0.09 ± 0.02 | 0.15 ± 0.01 | 0.15 ± 0.03 | 0.14 ± 0.02 | 0.09 ± 0.03* |
| 20:2n-6 | 0.45 ± 0.02 | 0.42 ± 0.02 | 0.44 ± 0.02 | 0.39 ± 0.02* | 0.46 ± 0.02 |
| 20:3n-6 | 2.69 ± 0.13 | 3.11 ± 0.17 | 3.29 ± 0.18* | 3.00 ± 0.09 | 2.86 ± 0.19 |
| 20:4n-6 | 6.64 ± 0.21$^{a,*}$ | 6.94 ± 0.27$^{b,*}$ | 6.87 ± 0.38$^{c,*}$ | 6.71 ± 0.20$^{d,*}$ | 8.57 ± 0.47$^{a,b,c,d}$ |
| 22:2n-6 | 0.59 ± 0.05 | 0.58 ± 0.05 | 0.48 ± 0.04 | 0.62 ± 0.05 | 0.63 ± 0.04 |
| 22:4n-6 | 0.31 ± 0.01$^{a,*}$ | 0.35 ± 0.03$^{b,*}$ | 0.32 ± 0.03$^{c,*}$ | 0.25 ± 0.02$^{d,*}$ | 0.52 ± 0.04$^{a,b,c,d}$ |
| 20:3/20:4 | 0.41 ± 0.02* | 0.46 ± 0.03* | 0.50 ± 0.04$^{a,*}$ | 0.45 ± 0.02* | 0.34 ± 0.02$^{a}$ |
| ∑n-6 LC-PUFA | 10.69 ± 0.27$^{a,*}$ | 11.40 ± 0.32* | 10.52 ± 0.95$^{b,*}$ | 10.99 ± 0.23$^{c,*}$ | 13.05 ± 0.55$^{a,b,c}$ |
| 18:3n-3 | 0.05 ± 0.02* | 0.10 ± 0.02$^{a,*}$ | 0.06 ± 0.02* | 0.06 ± 0.02* | 0.01 ± 0.01$^{a}$ |
| 20:5n-3 | 0.12 ± 0.03$^{a}$ | 0.13 ± 0.04$^{b,c}$ | 0.35 ± 0.06$^{b,d,*}$ | 0.41 ± 0.05$^{a,c,e,*}$ | 0.06 ± 0.03$^{d,e}$ |
| 22:5n-3 | 0.15 ± 0.03$^{a,b}$ | 0.20 ± 0.03$^{c}$ | 0.26 ± 0.02$^{a,*}$ | 0.20 ± 0.03$^{d}$ | 0.32 ± 0.02$^{b,c,d,*}$ |
| 22:6n-3 | 1.37 ± 0.07$^{a,b,*}$ | 1.33 ± 0.13$^{c,d,*}$ | 2.20 ± 0.12$^{a,c,e}$ | 2.27 ± 0.09$^{b,d,f,*}$ | 1.72 ± 0.14$^{e,f,*}$ |
| ∑n-3 LC-PUFA | 1.64 ± 0.09$^{a,b,*}$ | 1.66 ± 0.16$^{c,d,*}$ | 2.60 ± 0.27$^{a,c,e,*}$ | 2.88 ± 0.13$^{b,d,f,*}$ | 2.10 ± 0.16$^{e,f}$ |
| ∑PUFA | 31.42 ± 0.28 | 31.87 ± 0.45 | 29.48 ± 2.50 | 31.85 ± 0.22 | 32.26 ± 0.48 |
| ∑PUFA/∑SAT | 0.71 ± 0.01* | 0.73 ± 0.02* | 0.72 ± 0.02* | 0.72 ± 0.01* | 0.68 ± 0.02 |
| ∑LC-PUFA | 12.62 ± 0.35$^{a,*}$ | 13.22 ± 0.42* | 13.30 ± 1.20* | 14.01 ± 0.27* | 15.65 ± 0.68$^{a}$ |

[a]Common roman letter superscripts within a row indicate a significant difference ($P \leq 0.05$) between groups. For abbreviations see Table 1.

After 4 wk on the study diet, the percentage of SAT fatty acids decreased, while *cis*-monounsaturated fatty acids (MUFA) increased in all formula groups (Tables 3,4). In contrast, there were no changes in the breast-fed group, reflecting a higher percentage of SAT and a lower percentage of *cis*-MUFA in breast milk compared to the formula diets (Tables 3,4). The *trans* fatty acid content in the plasma lipids of breast-fed babies was almost twice as high as in all formula-fed groups, which corresponds to about 0.8% *trans* fatty acids in breast milk and no detectable *trans*-MUFA in formulas based on plant oil. Mead acid (20:3n-9) percentage decreased significantly over time in all formula groups, but not in HM-fed infants. In the diet, the percentage of the precursor oleic acid was higher in the formulas than in breast milk (43 vs. 31%), but traces of Mead acid were detectable only in breast milk.

At day 28, on the diets with 0.6% GLA there was no marked difference in phospholipid GLA content (0.1% in group ST vs. 0.15% in the other formula groups, not significant); only the breast-fed group showed a significant increase with time. No obvious difference was caused by additional dietary supply of n-3 LC-PUFA in the diet. The same trend was observed for the absolute concentrations, with all mean values below 2.5 mg/kg plasma. There were no significant differences in the content (absolute and relative) of DGLA, which is obtained by chain elongation of GLA. LA content increased significantly with time in all groups, but only the differences between groups ST and HM evolved to significance at day 28. The concentration of the elongation product of LA, 20:2n-6, like LA, showed no significant group differences, although only HM contained 20:2n-6. The content of the major n-6 LC-PUFA AA, which was very similar in all groups on day 0, decreased significantly until day 28 in all formula groups but not in the breast milk (0.5% AA) group, indicating that supplementation with GLA did not increase the AA content (Table 3). After 4 wk on the study diets, the AA percentage was significantly higher in the HM group, whereas no other group differences approached statistical significance. Absolute concentrations were only different between ST and HM (Table 4). The chain elongation product of AA (22:4n-6) was significantly lower in the formula-fed groups than in HM, but this cannot be ascribed to elongase activity since only breast milk contained 22:4n-6 (0.14%).

Addition of fish oil that was either high or low in EPA content did not further decrease n-6 LC-PUFA but markedly increased the DHA content in phospholipids (Tables 3,4). Mean EPA percentage was about twice as high in these groups as in all other groups, although EPA contributed only 0.06 and 0.15% to dietary fatty acids. Absolute concentrations of EPA were more than sixfold higher in the fish oil groups than in the HM group and twofold higher than in the other formula groups, respectively (Table 4). In contrast to EPA and DHA, the levels of the intermediate 22:5n-3 tended to be higher in HM infants, who received an exogenous supply (0.1%) of 22:5n-3. There was no significant difference between groups BO + FOLOW and BO + FOHIGH in any fatty acid concentration.

There was a marked influence of fish oil supply on the ratio of n-6 LC-PUFA to n-3 LC-PUFA. The ratios at day 28 were similar in breast-fed infants and in infants from groups ST and BO, whereas the n-6/n-3 LC-PUFA ratio dropped significantly ($P \leq 0.05$) from 6.27 ± 0.33 (M ± SE) to 4.23 ± 0.30 in BO + FOLOW and from 6.36 ± 0.36 to 3.97 ± 0.27 in BO + FOHIGH, respectively, and in BO it increased significantly from 5.72 ± 0.39 to 7.36 ± 0.48.

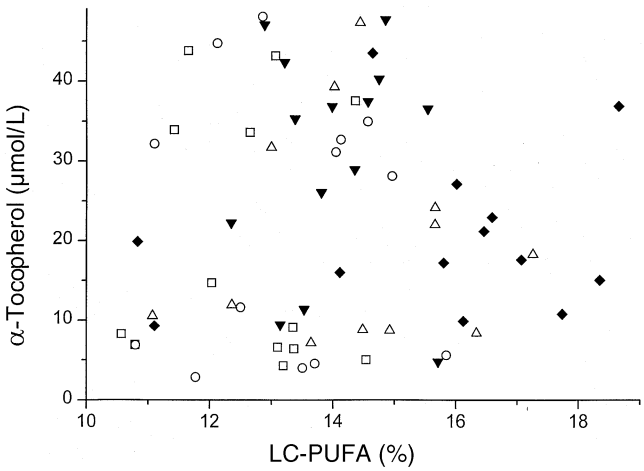


**FIG. 1.** Individual α-tocopherol concentrations (µmol/L) in relation to the percentage of LC-PUFA in plasma phospholipids (□ ST, ○ BO, △ BO + FOLOW, ▼ BO + FOHIGH, ◆ HM) showing neither a significant difference between groups nor a significant correlation for any of the groups. Abbreviations: LC-PUFA, long-chain polyunsaturated fatty acids; ST, standard formula; BO, borage oil; BO + FOLOW, borage oil + 0.3% docosahexaenoic acid (DHA) and 0.06% eicosapentaenoic acid (EPA); BO + FOHIGH, BO + 0.3% DHA + 0.2% EPA; HM, human milk.

The amount of antioxidative vitamins in the formulas (0.1 mg retinol and 1.3 mg tocopherol per 100 mL) resulted in plasma concentrations of retinol and α-tocopherol that were not different from breast-fed infants (Table 5). There was no detectable influence of LC-PUFA addition to the formulas on this parameter. In addition, there was no significant correlation between LC-PUFA percentage and α-tocopherol concentration (Fig. 1). Only for δ-tocopherol, which showed low concentrations in all infants and which is an isomer with low biological activity (36), could significant differences be observed between BO and HM as well as BO + FOHIGH and HM. The adjustment of α-tocopherol and retinol concentrations for Chol and TG by multiple regression according to Jordan *et al.* (37) did not reveal significant differences between groups (Table 5). In all groups there was a trend for α-tocopherol to increase with time, but it reached significance at day 28 only in groups BO and BO + FOHIGH.

## DISCUSSION

This study was designed to evaluate the influence of added GLA in preterm infant formula on the composition of n-6 LC-PUFA in infant plasma phospholipids. The added GLA resulted in dietary intakes of at least 32–34 mg/kg/d in groups BO, BO + FOLOW and BO + FOHIGH, which is close to the sum of GLA, DGLA, and AA typically ingested with breast

**TABLE 4**
**Absolute Concentrations of Fatty Acids in Plasma Phospholipids (mg/kg plasma, mean ± SE) for the Three Time Points Investigated[a]**

| | ST | BO | BO + FOLOW | BO + FOHIGH | HM |
|---|---|---|---|---|---|
| | | | Day 0 | | |
| Total fatty acids | 1042.7 ± 59.7 | 1171.5 ± 51.8 | 1113.5 ± 41.2 | 1029.9 ± 43.4 | 963.2 ± 46.7 |
| ∑SAT | 500.1 ± 28.6 | 555.7 ± 22.9 | 530.9 ± 20.2 | 496.3 ± 20.4 | 465.0 ± 23.3 |
| ∑MUFA | 217.6 ± 17.7 | 253.2 ± 16.1 | 222.3 ± 8.7 | 201.0 ± 10.8 | 186.4 ± 9.0 |
| ∑TRANS | 9.0 ± 0.9 | 10.3 ± 0.7 | 9.1 ± 0.8 | 9.0 ± 0.7 | 9.0 ± 0.6 |
| 20:3n-9 | 9.2 ± 2.9 | 11.2 ± 3.7 | 5.3 ± 1.0 | 8.3 ± 2.3 | 4.9 ± 1.8 |
| 18:2n-6 | 141.0 ± 7.7 | 161.9 ± 17.1 | 164.0 ± 15.3 | 150.9 ± 14.0 | 141.2 ± 6.8 |
| 18:3n-6 | 1.2 ± 0.4 | 1.4 ± 0.3 | 1.1 ± 0.3 | 1.2 ± 0.3 | 0.1 ± 0.1 |
| 20:2n-6 | 4.1 ± 0.4 | 5.5 ± 0.5 | 4.9 ± 0.4 | 4.7 ± 0.2 | 4.4 ± 0.3 |
| 20:3n-6 | 28.5 ± 2.5 | 31.3 ± 2.0 | 29.9 ± 1.6 | 29.2 ± 2.3 | 27.4 ± 2.3 |
| 20:4n-6 | 98.9 ± 9.3 | 100.7 ± 7.6 | 108.9 ± 5.4 | 94.8 ± 6.3 | 92.1 ± 6.4 |
| 22:2n-6 | 5.7 ± 0.5 | 5.7 ± 0.4 | 5.4 ± 0.4 | 5.7 ± 0.6 | 5.6 ± 0.4 |
| 22:4n-6 | 5.4 ± 0.7 | 6.4 ± 0.5 | 5.9 ± 0.4 | 5.6 ± 0.6 | 4.9 ± 0.3 |
| ∑n-6 LC-PUFA | 142.6 ± 12.4 | 149.7 ± 10.0 | 155.1 ± 6.6 | 140.1 ± 8.3 | 134.3 ± 8.6 |
| 18:3n-3 | ND | 0.4 ± 0.2 | 0.1 ± 0.1 | 0.2 ± 0.1 | ND |
| 20:5n-3 | 0.7 ± 0.3 | 1.8 ± 0.3 | 1.5 ± 0.5 | 0.9 ± 0.36 | 0.4 ± 0.2 |
| 22:5n-3 | 1.9 ± 0.4 | 3.2 ± 0.6 | 2.03 ± 0.5 | 2.6 ± 0.4 | 2.4 ± 0.3 |
| 22:6n-3 | 19.5 ± 1.7 | 22.8 ± 2.2 | 22.0 ± 1.2 | 19.5 ± 1.6 | 19.5 ± 1.8 |
| ∑n-3 LC-PUFA | 22.0 ± 2.0 | 27.8 ± 2.7 | 25.5 ± 1.6 | 23.1 ± 2.0 | 22.3 ± 2.0 |
| ∑PUFA | 316.0 ± 19.5 | 352.3 ± 25.8 | 351.0 ± 19.7 | 323.7 ± 19.9 | 302.8 ± 16.7 |
| ∑LC-PUFA | 173.8 ± 15.4 | 188.7 ± 11.3 | 185.8 ± 7.6 | 171.4 ± 9.8 | 161.5 ± 11.0 |
| | | | Day 14 | | |
| Total fatty acids | 1142.7 ± 68.9 | 1210.2 ± 65.8 | 1136.4 ± 54.4 | 1124.3 ± 43.4 | 1063.7 ± 66.6 |
| ∑SAT | 501.2 ± 30.6 | 530.2 ± 27.6 | 503.5 ± 24.7 | 501.9 ± 22.3 | 509.9 ± 32.6 |
| ∑MUFA | 271.7 ± 17.0 | 286.9 ± 18.2 | 264.5 ± 12.8 | 257.1 ± 8.7 | 201.8 ± 14.4 |
| ∑TRANS | 6.4 ± 0.7 | 6.5 ± 0.4 | 5.9 ± 0.4 | 6.126 ± 0.7 | 10.4 ± 0.9 |
| 20:3n-9 | 2.4 ± 0.5 | 2.5 ± 0.4 | 2.0 ± 0.4 | 1.5 ± 0.5 | 4.5 ± 1.8 |
| 18:2n-6 | 207.0 ± 9.8 | 216.1 ± 13.9 | 188.8 ± 11.8 | 190.8 ± 9.4 | 171.8 ± 10.1 |
| 18:3n-6 | 0.7 ± 0.2 | 1.8 ± 0.3 | 1.7 ± 0.3 | 1.5 ± 0.3 | 0.9 ± 0.4 |
| 20:2n-6 | 4.8 ± 0.4 | 5.2 ± 0.4 | 4.6 ± 0.3 | 4.6 ± 0.3 | 5.2 ± 0.4 |
| 20:3n-6 | 28.8 ± 2.6 | 38.5 ± 2.7 | 36.7 ± 2.4 | 34.3 ± 1.6 | 30.6 ± 2.6 |
| 20:4n-6 | 86.4 ± 9.2 | 88.1 ± 4.5 | 87.0 ± 6.1 | 83.7 ± 4.8 | 92.0 ± 7.2 |
| 22:2n-6 | 5.83 ± 0.6 | 6.1 ± 0.5 | 5.6 ± 0.5 | 7.2 ± 0.6 | 6.2 ± 0.6 |
| 22:4n-6 | 4.3 ± 0.7 | 4.5 ± 0.3 | 3.8 ± 0.4 | 3.6 ± 0.3 | 5.7 ± 0.5 |
| ∑n-6 LC-PUFA | 130.2 ± 12.0 | 142.3 ± 7.0 | 137.6 ± 7.9 | 133.3 ± 6.0 | 139.7 ± 10.5 |
| 18:3n-3 | 0.6 ± 0.2 | 1.1 ± 0.2 | 0.4 ± 0.2 | 0.6 ± 0.2 | 0.1 ± 0.1 |
| 20:5n-3 | 1.6 ± 0.4 | 1.7 ± 0.6 | 4.0 ± 0.3 | 4.7 ± 0.5 | 0.9 ± 0.4 |
| 22:5n-3 | 1.8 ± 0.3 | 2.6 ± 0.4 | 1.8 ± 0.4 | 2.5 ± 0.2 | 3.4 ± 0.2 |
| 22:6n-3 | 19.2 ± 1.9 | 18.7 ± 1.9 | 26.3 ± 2.2 | 24.2 ± 1.0 | 20.4 ± 2.2 |
| ∑n-3 LC-PUFA | 22.6 ± 2.3 | 22.9 ± 2.4 | 32.1 ± 2.6 | 31.4 ± 1.3 | 24.7 ± 2.5 |
| ∑PUFA | 363.4 ± 21.8 | 386.69 ± 21.6 | 362.6 ± 20.0 | 359.1 ± 14.7 | 341.7 ± 21.6 |
| ∑LC-PUFA | 155.2 ± 1.0 | 167.74 ± 8.9 | 171.6 ± 10.5 | 166.2 ± 6.8 | 168.9 ± 13.4 |
| | | | Day 28 | | |
| Total fatty acids | 1139.0 ± 44.1 | 1195.9 ± 65.0 | 1202.2 ± 47.2 | 1154.0 ± 57.0 | 1077.1 ± 67.7 |
| ∑SAT | 501.4 ± 18.3 | 523.7 ± 26.3 | 532.5 ± 20.1* | 514.3 ± 26.7* | 510.2 ± 29.7* |
| ∑MUFA | 274.2 ± 13.2* | 282.8 ± 15.6 | 279.3 ± 15.2* | 265.1 ± 12.3* | 209.7 ± 18.0 |
| ∑TRANS | 5.7 ± 0.8$^{a,*}$ | 6.4 ± 0.4$^{b,*}$ | 6.9 ± 0.6* | 6.8 ± 0.7$^{c,*}$ | 9.6 ± 0.7$^{a,b,c}$ |
| 20:3n-9 | 3.4 ± 0.4 | 2.0 ± 0.4$^{a,*}$ | 2.4 ± 0.5* | 1.6 ± 0.3$^{b,*}$ | 5.7 ± 1.9$^{a,b}$ |
| 18:2n-6 | 212.3 ± 8.9* | 221.4 ± 14.6* | 208.0 ± 9.5* | 203.2 ± 9.6* | 176.3 ± 12.3* |
| 18:3n-6 | 1.1 ± 0.3 | 1.8 ± 0.2 | 1.9 ± 0.4 | 1.7 ± 0.2 | 1.0 ± 0.3* |
| 20:2n-6 | 5.1 ± 0.3* | 5.1 ± 0.4 | 5.3 ± 0.3 | 4.5 ± 0.2 | 4.9 ± 0.3 |
| 20:3n-6 | 30.5 ± 1.7 | 37.8 ± 3.5 | 39.7 ± 3.1* | 34.8 ± 2.2* | 30.4 ± 2.5 |
| 20:4n-6 | 75.6 ± 3.8$^{a,*}$ | 82.5 ± 4.7 | 82.1 ± 4.7* | 77.7 ± 5.0* | 93.8 ± 10.2$^{a}$ |
| 22:2n-6 | 6.7 ± 0.6 | 6.8 ± 0.6 | 5.7 ± 0.5 | 7.4 ± 0.8* | 6.8 ± 0.6* |
| 22:4n-6 | 3.6 ± 0.2$^{a,*}$ | 4.2 ± 0.4$^{b,*}$ | 3.9 ± 0.3$^{c,*}$ | 3.0 ± 0.4$^{d,*}$ | 5.6 ± 0.6$^{a,b,c,d}$ |
| ∑n-6 LC-PUFA | 121.5 ± 5.3 | 136.5 ± 8.3 | 136.7 ± 6.7* | 127.4 ± 7.9 | 141.6 ± 12.5 |
| 18:3n-3 | 0.6 ± 0.2* | 1.2 ± 0.2* | 0.8 ± 0.2* | 0.8 ± 0.2* | 0.1 ± 0.1 |
| 20:5n-3 | 1.4 ± 0.4$^{a,b}$ | 1.7 ± 0.5$^{c,d}$ | 4.2 ± 0.8$^{a,c,e,*}$ | 4.6 ± 0.5$^{b,d,f,*}$ | 0.7 ± 0.4$^{e,f}$ |
| 22:5n-3 | 1.8 ± 0.4$^{a,b}$ | 2.5 ± 0.4 | 3.2 ± 0.2$^{a,*}$ | 2.3 ± 0.3 | 3.4 ± 0.3$^{b,*}$ |
| 22:6n-3 | 15.7 ± 1.1$^{a,b}$ | 16.1 ± 1.8$^{c,d,*}$ | 26.5 ± 1.9$^{a,c,*}$ | 26.3 ± 1.8$^{b,d,e,*}$ | 18.7 ± 2.1$^{e}$ |
| ∑n-3 LC-PUFA | 18.9 ± 1.4$^{a,b}$ | 20.2 ± 2.4$^{c,d,*}$ | 33.9 ± 2.6$^{a,c,e,*}$ | 33.3 ± 2.2$^{b,d,f,*}$ | 22.8 ± 2.7$^{e,f}$ |
| ∑PUFA | 357.7 ± 14.0 | 383.0 ± 24.1 | 383.6 ± 15.6 | 367.9 ± 18.9 | 347.5 ± 22.8* |
| ∑LC-PUFA | 143.7 ± 6.9 | 158.7 ± 10.4 | 172.9 ± 9.1 | 162.3 ± 9.8 | 170.1 ± 15.4 |

[a]Asterisk indicates a significant change ($P \leq 0.05$) from Day 0 to Day 28; common roman letter superscripts within a row indicate a significant difference ($P \leq 0.05$) between groups. For abbreviations see Table 1.

**TABLE 5**
**Concentration (μmol/L, mean ± SE) of Retinol and Tocopherol Isomers in Infant Plasma at Study Start (day 0), After 2 wk on Study Diets (day 14), and After 4 wk on Study Diets (day 28)[a]**

| | ST | BO | BO + FOLOW | BO + FOHIGH | HM |
|---|---|---|---|---|---|
| | | | Day 0 | | |
| Retinol | 0.49 ± 0.08 | 0.38 ± 0.03 | 0.39 ± 0.05 | 0.36 ± 0.04 | 0.43 ± 0.05 |
| α-Tocopherol | 13.03 ± 1.52 | 12.08 ± 1.86 | 12.18 ± 1.66 | 14.48 ± 1.92 | 13.80 ± 1.90 |
| δ-Tocopherol | 0.11 ± 0.03 | 0.09 ± 0.02 | 0.1 ± 0.02 | 0.11 ± 0.03 | 0.08 ± 0.01 |
| β,γ-Tocopherol | 0.61 ± 0.18 | 0.59 ± 0.14 | 0.48 ± 0.11 | 0.67 ± 0.21 | 0.49 ± 0.12 |
| α-Tocopherol adj. | 13.78 ± 1.33 | 11.13 ± 1.41 | 12.88 ± 1.63 | 15.20 ± 1.94 | 14.18 ± 1.87 |
| Retinol adj. | 0.51 ± 0.06 | 0.37 ± 0.03 | 0.41 ± 0.05 | 0.41 ± 0.04 | 0.43 ± 0.05 |
| Chol (mg/dL) | 101.3 ± 9.3 | 112.5 ± 8.6 | 101.9 ± 5.2 | 89.5 ± 6.6 | 104.7 ± 11.2 |
| TG (mg/dL) | 81.3 ± 8.9 | 116.6 ± 17.4 | 82.0 ± 8.6 | 78.6 ± 7.2 | 86.8 ± 7.4 |
| | | | Day 14 | | |
| Retinol | 0.34 ± 0.03 | 0.35 ± 0.03 | 0.37 ± 0.03 | 0.37 ± 0.03 | 0.35 ± 0.03 |
| α-Tocopherol | 18.15 ± 3.7 | 19.26 ± 4.02 | 14.53 ± 2.95 | 26.24 ± 4.15 | 16.66 ± 1.84 |
| δ-Tocopherol | 0.27 ± 0.06 | 0.37 ± 0.09 | 0.22 ± 0.04 | 0.39 ± 0.06 | 0.08 ± 0.01 |
| β,γ-Tocopherol | 1.52 ± 0.44 | 1.5 ± 0.38 | 0.89 ± 0.17 | 1.68 ± 0.24 | 0.51 ± 0.06 |
| α-Tocopherol adj. | 17.78 ± 3.97 | 18.17 ± 3.60 | 14.81 ± 3.09 | 26.95 ± 3.86 | 17.26 ± 2.01 |
| Retinol adj. | 0.34 ± 0.03 | 0.33 ± 0.03 | 0.37 ± 0.03 | 0.39 ± 0.03 | 0.34 ± 0.03 |
| Chol (mg/dL) | 106.8 ± 8.6 | 117.5 ± 10.4 | 108.3 ± 5.4 | 95.0 ± 4.5 | 113.0 ± 11.5 |
| TG (mg/dL) | 99.4 ± 11.8 | 109.2 ± 12.6 | 87.7 ± 8.1 | 83.5 ± 11.1 | 81.2 ± 7.7 |
| | | | Day 28 | | |
| Retinol | 0.44 ± 0.04 | 0.39 ± 0.04 | 0.41 ± 0.03 | 0.46 ± 0.04 | 0.42 ± 0.05 |
| α-Tocopherol | 19.47 ± 4.44 | 22.1 ± 4.6* | 19.84 ± 3.88 | 30.44 ± 3.72* | 20.54 ± 2.83* |
| δ-Tocopherol | 0.25 ± 0.05 | 0.37 ± 0.08[a,*] | 0.28 ± 0.05* | 0.43 ± 0.05[b,*] | 0.12 ± 0.03[a,b] |
| β,γ-Tocopherol | 1.36 ± 0.31 | 1.43 ± 0.31* | 1.19 ± 0.2* | 1.69 ± 0.18[a,*] | 0.62 ± 0.13[a] |
| α-Tocopherol adj. | 18.75 ± 4.54 | 20.58 ± 4.69* | 20.16 ± 3.81 | 30.14 ± 3.70* | 20.73 ± 2.99 |
| Retinol adj. | 0.43 ± 0.04 | 0.37 ± 0.04 | 0.39 ± 0.02 | 0.48 ± 0.03 | 0.40 ± 0.05 |
| Chol (mg/dL) | 110.5 ± 6.3 | 115.8 ± 9.5 | 116.7 ± 5.5* | 100.0 ± 5.4 | 116.6 ± 11.0 |
| TG (mg/dL) | 104.4 ± 13.1 | 108.2 ± 8.2 | 85.1 ± 11.7 | 99.7 ± 16.0 | 87.4 ± 10.2 |

[a]Common roman letter superscripts within a row indicate a significant difference between groups; asterisk indicates a significant change ($P \leq 0.05$) from day 0 to day 28. Chol, cholesterol; TG, triglycerides; for other abbreviations see Table 1.

milk (38) and agrees reasonably well with the observed intake of about 43 mg/kg/d in the studied breast-fed infants.

The formulas showed a LA/α-linolenic acid ratio of about 11, which was markedly different from the mean ratio of 36 in human milk lipids, caused by the low α-linolenic acid content of human milk. The slightly higher LA content of 15.4% in breast milk compared to 13% in the formulas was not reflected by higher LA concentrations in the plasma phospholipids and is not assumed to cause an increase in AA, as the addition of LA to formulas showed only small effects on circulating AA (39).

There were no significant differences between groups with respect to plasma TG and total Chol concentrations, even though breast milk contains considerably more Chol than formulas for infants based on plant oil (40). This lack of a difference might be due to the fact that the infants were studied in the period when enteral feeding had just started. However, the concentrations were similar to values observed previously in newborn infants (41,42).

Total fatty acids in plasma phospholipids did not differ between groups or change with time and were well within the range of previously observed values in preterm infants of this age (36). AA levels were similar in all groups at study start, while there were clear differences between the formula groups and the breast milk group at the later time points, reaching significance at day 28. The decrease with time in infants not supplemented with AA is in agreement with earlier reports (36,43,44). The addition of GLA either alone or in combination with fish oil did not significantly improve the AA status as compared to the unsupplemented group. This confirms previous observations in studies where infant formulas had been supplemented with 0.5 or 0.3% GLA in addition to EPA and DHA (0.4 and 0.3%) or (0.6 and 0.4%), respectively, and either an unchanged or a decreased AA content in red blood cell lipids was observed (23,24). Thus, even in the absence of dietary n-3 LC-PUFA, an intake of up to 0.6% GLA failed to produce a significant increase of AA status. On the other hand, GLA supplementation in children and adults showed differing effects on AA status (18,45). Johnson *et al*. (18) reported a significant increase of total plasma AA percentage after 3 wk of GLA (1.5–6 g/d) intake, which corresponded to intakes ≥20 mg/kg/d assuming a body weight of 70 kg. Animal studies clearly showed that GLA is converted to AA (16), but whether there is an increase of AA concentrations in humans seems to depend on further factors. Stable isotope studies in term infants identified Δ-6 desaturation as the limiting step in the endogenous conversion of dietary LA to AA (13). The principal activity of the conversion of LA *via* GLA and DGLA to AA in neonates has been demonstrated *in vitro* (46) and *in vivo* (12–14), but the addition of the intermediate GLA to the diet does not seem to im-

prove the yield of AA. As it can be assumed that GLA has been efficiently absorbed (47), there must be some other physiological reason.

Although DGLA was delivered only in trace amounts with all tested formulas, DGLA showed plasma phospholipid concentrations in the GLA-containing formula groups that were higher than in the HM group, but lower values were present in the ST group, although differences were not significant (Table 3). It appears that 0.6% of GLA in the diet may well replace about 0.4% of DGLA, as provided with breast milk lipids (22), thus pointing toward rapid elongation. This is in accordance with other findings in neonates, healthy adults, and patients with atopic dermatitis (18,24,48). A further indication for an active fatty acid elongase in the studied preterm neonates is the finding that 20:2n-6, the elongation product of LA, was found in similar concentrations in all feeding groups, although only breast milk contained appreciable amounts of this fatty acid.

Phospholipid GLA concentrations did not respond to dietary GLA supply, which may be explained by the low affinity of GLA toward incorporation into phospholipids (49). Moreover, lipoprotein lipase preferentially hydrolyzes fatty acids with 16 or 18 carbon atoms from chylomicrons as compared to polyenoic fatty acids with 20 carbon atoms (50). There appears to be more uptake of the shorter fatty acids by tissues for oxidation, while a lower proportion is available for conversion to LC-PUFA or direct incorporation into phospholipids in the liver. This is in agreement with the observation of Leyton *et al*. (51) who reported that 27% of orally administered GLA in rats was oxidized to $CO_2$ within 24 h, while only 14% each of DGLA or AA were recovered in breath. Brouwer *et al.* (52) and Woltil *et al.* (53) reported that dietary GLA is preferentially incorporated into Chol esters in adults and that a significantly higher GLA content in plasma Chol esters of low birth weight infants was found when they were fed a formula with 0.3% GLA than when they were fed HM.

Obviously, dietary GLA was efficiently elongated to DGLA, possibly already in the intestine (54). Elongation products and remaining precursors reached the circulation after incorporation into chylomicrons, and it is possible that from these lipoproteins GLA was preferentially eliminated and oxidized. Thus only a negligible portion of GLA might have been available in the liver for conversion into AA and incorporation into phospholipids. Owing to the active elongation, the ingested GLA appears in the liver converted to DGLA, and might be handled similarly to dietary DGLA. After feeding deuterated DGLA to adults and measuring the concentration of deuterated AA in plasma, it was estimated that only a very low percentage of exogenous DGLA is converted to AA (55,56). The study by Emken *et al*. (56) offers a possible explanation for an inconsistent effect of GLA feeding on AA concentrations, as they noted that in subjects with an AA intake of 0.2 g/d, only about 2% of DGLA was desaturated, while this percentage increased to 18% with an AA intake of 1.7 g/d. In a larger group of subjects it was demonstrated that the high AA intakes were followed by elevated AA concentrations in all plasma lipid fractions and blood cells (57). Hence, the effect of GLA supplementation might depend on the current AA status. As there was no AA intake in the formula groups studied and AA concentrations were lower than those observed by Emken *et al.*, in combination with a possible immaturity of the desaturating enzyme system, these findings could explain the absence of an increase of AA after GLA supplementation.

This study also investigated the influence of fish oil supplementation on infant plasma phospholipid composition. The addition of 0.3% DHA established phospholipid DHA values similar to those found in the HM-fed group, and even exceeded them in a population with rather low HM contents of DHA. These findings are in accordance with published studies in term infants; e.g., Horby-Jorgensen *et al*. (24) reported 1.9% DHA in red blood cell phosphatidylcholine after feeding a formula with 0.3% DHA for 1 mon compared to 1.25% in an unsupplemented group. In preterm infants 0.2% DHA compared to 0% DHA in dietary fatty acids caused a concentration difference of about 5 mg/L in plasma total phospholipid DHA after 4 wk (25). In previous studies, dietary fish oil without simultaneous addition of preformed AA into the formula had led to AA concentrations below those of groups with LC-PUFA unsupplemented (25), which was explained by the competition of EPA and DHA for the incorporation into phospholipids (58) and an inhibitory effect of n-3 fatty acids on desaturase activities (59,60). It has been postulated that DHA and not EPA is the potent desaturase inhibitor in humans and that it mainly affects Δ-6 desaturation (61). The absence of a further AA decrease with DHA intake may be explained by the GLA supply in both fish oil-supplemented groups, thereby circumventing the Δ-6 desaturation step. Effects similar to those seen here were reported by Ward *et al*. (62) in brain phospholipids of artificially reared rat pups on diets varying in their GLA content from 0.5 to 3% and with either 0.5 or 2.5% DHA (62). They demonstrated that even 3% GLA in dietary lipids did not significantly increase AA content compared to 0.5% GLA. In contrast to our results, Makrides *et al*. (23) found a lower AA value of 10.2 ± 1.0% (mean ± SD) of total fatty acids from erythrocytes in term infants fed for 16 wk a formula with 0.58% EPA, 0.36% DHA, and 0.27% GLA compared to an unsupplemented control group (12.9 ± 1.3%), but they did not study a group fed the same dose of n-3 LC-PUFA but no GLA.

Tocopherol and retinol did not differ between groups on day 28 in either the absolute concentrations or in α-tocopherol and retinol concentrations after adjustment by multiple regression. At study day 0, 50% of the infants showed α-tocopherol concentrations below a level of 12.4 μmol/L, considered as the threshold for vitamin E sufficiency (63). This number decreased to 38% at day 28 with mean values for all groups from day 14 on above the threshold of sufficiency. Although there are suggestions to aim at much higher vitamin E concentrations, the intake in the formula-fed groups was 1.6 mg vitamin E/100 kcal (1.9 mg vitamin E/g PUFA), which is well above recommendations of the European Society for Pediatric Gastroenterology Hepatology and Nutrition (64).

In conclusion, our results are in line with previous findings (22,24,53,65) and show that addition of 0.6% GLA to formula diets does not increase AA content in plasma phospholipids of preterm infants and does not achieve values similar to comparable HM-fed infants. This effect does not seem to depend on the n-3 LC-PUFA content of the diet with the dose ranges studied. Although AA in the diet cannot be substituted by equimolar amounts of GLA, its addition may have other beneficial effects, e.g., increased high density lipoproteins (66). For the achievement of a LC-PUFA status similar to breast-fed infants, the supplementation of AA and n-3 LC-PUFA seems necessary.

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# Effects of Δ5 Polyunsaturated Fatty Acids of Maritime Pine *(Pinus pinaster)* Seed Oil on the Fatty Acid Profile of the Developing Brain of Rats

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**ABSTRACT:** Conifer (pine) seeds are a potential source of dietary oils, but their safety and nutritional properties are not well established. Conifer seed oils differ from common edible vegetable oils in having a series of unusual polyunsaturated fatty acids (PUFA) with a polymethylene-interrupted (PMI) double bond system and a double bond at the Δ5 position. A rat study was conducted to assess whether Δ5 PMI-PUFA of conifer seeds could alter the levels of n-6 and n-3 long-chain polyunsaturated fatty acids (LC-PUFA) in mothers' milk and the developing brain of fetuses and pups. Feeding maritime pine *(Pinus pinaster)* seed oil (MPO) diet with a Δ5 PMI-PUFA content of 1.4 g/100 g throughout pregnancy and lactation resulted in a large incorporation of Δ5 PMI-PUFA in mothers' milk (5.1 ± 0.5% of total fatty acids). The fetus (17 d old) and pup (22 d) brains, however, accumulated very little (0.6 and 0.4% of total fatty acids, respectively) Δ5 PMI-PUFA. Mother's milk and pup's brain of the MPO group contained normal levels of 20:4n-6, 22:4n-6, and 20:5n-3 compared to a reference group of rats fed a fat blend of sunflower, high-oleic sunflower, and canola oils. The level of 22:6n-3, however, was slightly but significantly ($P < 0.05$) higher in milk and pup brain of the MPO group. These results show that Δ5 PMI-PUFA of MPO exert no negative effect on the levels of n-6 and n-3 LC-PUFA in rat brain during its early development.

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Wolff *et al.* (1) recently proposed that seeds from conifer (pine) plants should be considered as a potential source of dietary oil for humans. Conifers are the most common naturally growing plant in the Northern Hemisphere, and their seeds contain about 65% triacylglycerol (TAG) oil. Currently, conifer seed oils are not directly used as dietary oils, but the seeds are consumed in some countries as condiments in food preparations. In China, seeds from *Biota orientalis* (a conifer species) are used for pharmaceutical purposes (2). Several animal studies have shown that conifer seed oils have a substantial lipid-lowering potential (3–5). These reports might imply that conifer seed oils are safe and provide health benefits to humans. However, more studies are needed to further explore the biological and nutritional effects of conifer seed oils before considering them as a safe dietary oil.

Conifer seed oils have a very unusual series of $C_{18}$- and $C_{20}$-polyunsaturated fatty acids (PUFA), in which the first double bond is in the Δ5 position and the next immediate double bond is at Δ9 or Δ11 (1), so that the first two double bonds are separated by two or four methylene ($CH_2$) units. The other subsequent bonds, however, are separated by one $CH_2$ unit. This pattern is in contrast to the usual PUFA of common vegetable oils and animal fats, in which the neighboring double bonds are always separated from each other by a single $CH_2$ unit. Since the first two double bonds of the unusual PUFA of conifer seed oils are separated by two or more $CH_2$ units, it is appropriate to name them as Δ5 polymethylene-interrupted PUFA and to abbreviate them as Δ5 PMI-PUFA. The Δ5 PMI-PUFA so far identified in conifer seed oils included 5,9-18:2 (taxoleic acid), 5,11-18:2 (ephedrenic acid), 5,9,12-18:3 (pinolenic acid), 5,9,12,15-18:4 (coniferonic acid), 5,11-20:2; 5,11,14-20:3 (sciadonic acid), and 5,11,14,17-20:4 (juniperonic acid) (1). The totals of these acids in different varieties of conifer seeds usually range from 5 to 35% of the total fatty acids, of which the major ones are sciadonic and pinolenic acids.

Results from the few studies on the biological effects of conifer seed oils suggest that sciadonic acid has an enormous potential to replace arachidonic acid (20:4n-6, or AA) in tissues and cells (6–9). Studies by Berger and coworkers (7,8) have shown that sciadonic acid is primarily incorporated in cardiac and hepatic phosphatidylinositol (PI) in the mouse. In hepatic PI, sciadonic acid replaced AA, resulting in a 50% reduction of the level of AA. Another study reported that sciadonic acid reduced the AA content of the PI fraction from a control value of 15.9 to 7% in a cultured cell system (9). This replacement of AA by sciadonic acid should be a concern in infant nutrition because AA, along with other $C_{20}$ and $C_{22}$ n-6 as well n-3 PUFA, particularly docosahexaenoic acid (22:6n-3, or DHA), is recognized as essential for the development of brain and retina (10). Insufficient supply of these

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Abbreviations: AA, arachidonic acid; amu, atomic mass units; DHA, docosahexaenoic acid; DMOX, 2-alkalenyl-4,4-dimethyloxazoline; FAME, fatty acid methyl esters; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; HOSF/SF/CO, high oleic sunflower/sunflower/canola oils; LC-PUFA, long chain-polyunsaturated fatty acids; MPO, maritime pine seed oil; MUFA, monounsaturated fatty acids; PMI-PUFA, polymethylene interrupted polyunsaturated fatty acids; PI, phosphatidylinositol; PL, phospholipid; PUFA, polyunsaturated fatty acids, SFA, saturated fatty acids; TAG, triacylglycerol; TLC, thin-layer chromatography.

fatty acids during the rapid development phase of the central nervous system, which occurs primarily during embryogenesis and neonatal periods, might result in poor visual acuity and cognitive function in later life (10).

The main objective of this study was to determine, using the rat as the experimental model, whether mothers' intake of sciadonic acid and other Δ5 PMI-PUFA from conifer seed oils during pregnancy and lactation has an effect on the proportions of AA, DHA and other $C_{20}$ and $C_{22}$ n-6 and n-3 PUFA in mothers' milk and the developing brain of fetuses and pups. In this study we utilized oil extracted from *Pinus pinaster* (maritime pine) seeds as the representative conifer seed oil. *Pinus pinaster* is one of the common conifer varieties growing in the Northern Hemisphere.

## EXPERIMENTAL PROCEDURES

*Animals and diets*. Twenty-day-old female Wistar rats (Charles River, Québec, Canada) were randomly assigned according to body weight (259 ± 11 g) to two dietary groups (A and B) with 10 females in each group. They were housed individually in metal cages in an air-conditioned room maintained at 22°C and 60% relative humidity with a 12 h day/12 h night cycle. The rats in group A were fed a semipurified diet containing a blend of high-oleic sunflower, sunflower, and canola oils (35:55:10) (HOSF/SF/CO) in the proportion of 10 g/100 g. The rats in group B were fed a similar diet but containing maritime pine seed oil (MPO, provided by Société Bertin, Lagny-le-Sec, France) in the same proportion. The fatty acid composition of the two diets (determined by gas–liquid chromatography, see below) is given in Table 1. Both diets contained similar levels of linoleic acid (45% of total fatty acids) and α-linolenic acid (1.3%). The Δ5 PMI-PUFA occurring in MPO were replaced by oleic acid in the HOSF/SF/CO diet. The diets were prepared according to the AIN-93 recommendations (American Institute of Nutrition) for rodent diets (11). Other dietary components were (g/100 g diet): casein, 22; cornstarch, 47.95; granulated sugar, 10; fiber (Alfa-floc), 5; mineral mixture AIN-93G, 3.50; vitamin mixture AIN-93-VX, 1; L-cystine, 0.30; and choline bitartrate, 0.25. Rats were allowed free access to water and food. Amounts of food consumed were recorded, and fresh food and water were provided once in 4 d. The animals were weighed weekly.

**TABLE 1**
**Fatty Acid Composition (wt% of total fatty acids) of HOSF/SF/CO and MPO Diets Fed to Pregnant ($n$ = 5) and Lactating Rats ($n$ = 5)[a]**

| Fatty acid | HOSF/SF/CO | MPO |
|---|---|---|
| 16:0 | 6.2 | 5.3 |
| 18:0 | 3.8 | 2.8 |
| Total SFA | 11.2 | 8.8 |
| 18:1n-9 | 41.9 | 27.9 |
| Total MUFA | 42.8 | 29.0 |
| 18:2n-6 | 44.7 | 45.8 |
| 18:3n-3 | 1.4 | 1.2 |
| 5,9-18:2 | ND | 0.8 |
| 5,9,12-18:3 | ND | 6.6 |
| 5,11-20:2 | ND | 0.7 |
| 5,11,14-20:3 | ND | 6.0 |
| Total Δ5 PMI-PUFA | ND | 14.1 |
| 7,11,14-20:3 | ND | TR |

[a]HOSF, high-oleic sunflower oil; SF, sunflower oil; CO, canola oil; MPO, maritime pine seed oil; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; Δ5 PMI-PUFA, polymethylene-interrupted polyunsaturated fatty acids; TR, trace amounts (≤0.05% of total fatty acids); ND, not detected.

After 16 d on the diet, the rats were allowed to mate by overnight pairing. At the 17th day of pregnancy, five rats from each group were killed by exsanguination while under 3% isoflurane anesthesia. Blood, liver, and breast tissues from the mother, and brains and fetuses were collected for lipid analysis. Blood (about 7 mL/rat) was withdrawn immediately from the aorta and stored in EDTA tubes. Tissue samples were frozen immediately in liquid nitrogen and stored at −70°C until analyses. All of the other rats were allowed to give birth, and the number of pups per mother and the birth weights of the neonates were recorded. A milk sample was collected on the 21st day after delivery. All the animals were killed on day 22 of lactation, and blood, liver, brain, and breast tissues were collected. Health Canada's guide for the care and use of laboratory animals was followed and the study protocol was approved by the Animal Care Committee of Health Canada.

*Fatty acid analysis.* Lipids from the tissue samples were extracted according to the Bligh and Dyer procedure (12). A small portion of the extracted lipid was separated into the various lipid classes by thin-layer chromatography (TLC) and the phospholipid (PL) and TAG fractions were collected. The dietary oils, tissue total lipids, PL, and TAG fractions were converted to fatty acid methyl esters (FAME) using $BF_3$/MeOH reagent according to the procedure of Morrison and Smith (13). The FAME were analyzed using a gas–liquid chromatograph equipped with a flame-ionization detector (Hewlett-Packard 5890 Series II, Avondale, PA) and a CP-Sil 88 capillary column (100 m × 0.25 mm i.d.; Chrompack, Middelburg, The Netherlands). The gas chromatography (GC) peaks for the FAME were identified by their relative retention times in comparison with FAME standards and with published data (14). The identifications were confirmed by calculating equivalent chain length and, for *P. pinaster* oil FAME, by argentation-TLC and GC–mass spectrometry (GC–MS) analyses of 2-alkalenyl-4,4-dimethyloxazoline (DMOX) derivatives of fatty acids (15–17). The GC–MS analyses were executed using a VG Analytical MS system, Model 7070 EQ (VG Analytical, Manchester, England), equipped with a 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. GC separation of the DMOX derivatives was performed on the same CP Sil 88 column described for the FAME analysis.

*Hematologic measurements.* The complete blood count was measured using a Coulter Counter S-PLUS IV system (Coulter Electronics, Hialeah, FL) on whole blood samples collected in EDTA tubes.

*Statistical analysis*. Statistica software, version 6.0 (Statsoft, Tulsa, OK) was used to analyze the data. For each dietary group, means and standard deviations were calculated and the significant differences were assessed by Tukey's honest significant test. Differences were considered significant when $P$-value <0.05. All data in tables are reported as means and standard deviations.

## RESULTS

*General health parameters*. The adult female rats grew normally, and there were no significant differences in the body weights between the two dietary groups, measured at regular intervals during the feeding phase of the experiment (Table 2). The ratios of liver weight to total body weight also were similar. The effect of the diet on pup brain weight was assessed by calculating the pup brain weight to pup total body weight ratio. The values were not different between the two dietary groups. Daily food intakes by mothers in both groups were high but normal for pregnant and lactating mothers, and there was no difference between the two groups. Visual inspection at necropsy indicated no apparent tissue and organ abnormalities of both mothers and their pups. Litter sizes of HOSF/SF/CO (12.7 ± 4.6 pups) and MPO (16.0 ± 2.1 pups) groups were high but within the normal range for Wistar rats (3–18 pups), and the difference between the two groups was not significant.

*Hematologic indices*. The lactating mothers of the MPO group at 21 d postpartum had a leukocyte count of 7.42 ± 1.86 $\times 10^9$/L , which was significantly ($P = 0.0044$) higher than that of the lactating mothers in the HOSF/SF/CO group (3.95 ± 1.17 $\times 10^9$/L ). Mature female rats (125 d old) normally range between 6.8 and 15.7 $\times 10^9$ leukocytes/L (18). This suggests that the leukocyte count was normal in the MPO group but too low in the HOSF/SF/CO group. Platelet count was also significantly ($P = 0.0384$) lower in the HOSF/SF/CO group (6.98 ± 0.25 $\times 10^{10}$/L vs. 7.12 ± 0.29 $\times 10^{10}$/L for MPO). There were no significant differences ($P > 0.05$) in the other hematology indices (erythrocyte count, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, red cell distribution width, and mean platelet volume) between the two dietary groups (data not shown).

*Fatty acid composition of blood, liver, and breast tissue from 17-d-pregnant rats*. All the dietary Δ5 PMI-PUFA were detected in blood, liver, and breast tissue samples from the 17-d pregnant rats fed MPO (Tables 3–5). As expected, none of these Δ5 fatty acids was detected in the pregnant rats fed HOSF/SF/CO. The proportion of total Δ5 PMI-PUFA of the MPO group was higher in blood (6.8%) (Table 3) than in the liver total lipids (3.4%), liver PL (2.6%) (Table 4), and breast total lipids (3.9%) (Table 5). In all tissues 5,11,14-20:3 and 5,9,12-18:3 were the major Δ5 PMI-PUFA, whereas 5,9-18:2 and 5,11-20:2 were present in relatively lower proportions. In addition to these fatty acids, the MPO-fed rats showed the presence of 7,11,14-20:3. The proportion of this PMI-PUFA in blood and tissues was higher than in the PMO diet. This unusual PMI-PUFA was not detected in rats fed HOSF/SF/CO.

There were no notable differences found in the blood and tissue levels of n-3 and n-6 long-chain-PUFA (LC-PUFA) between the two dietary groups.

*Fatty acid composition of blood and liver of lactating mothers at day 22 of lactation*. Blood and liver of lactating mothers of the MPO group on day 22 after delivery (i.e., 63 d of feeding) also contained Δ5 PMI-PUFA (Tables 6 and 7). Here also, 5,9,12-18:3 and 5,11,14-20:3 were the major Δ5 PMI-PUFA. The PL and TAG fractions of liver contained similar proportions of total Δ5 PMI-PUFA (Table 7). However, 5,11,14-20:3 was primarily in the PL fraction, whereas 5,9,12-18:3 was mainly in the TAG fraction. As in pregnant rats, 7,11,14-20:3 acid was also detected in maternal blood and liver samples, but at considerably lower levels. As expected, none of these fatty acids was detected in the lactating mothers of the HOSF/SF/CO group. Blood and liver of the mothers fed the HOSF/SF/CO diet, compared to those of the

**TABLE 2**
**Body and Organ Weights of Mothers ($n = 5$) and 22-d-old Pups and Food Intakes by Mothers**

| | HOSF/SF/CO (g) | MPO (g) |
|---|---|---|
| Maternal body weight on day 1 | 254.1 ± 11.4 | 262.1 ± 12.3 |
| Maternal body weight on day 22 | 298.8 ± 13.9 | 309.1 ± 18.9 |
| Maternal body weight on day 34 | 362.4 ± 22.3 | 374.7 ± 21.5 |
| Maternal body weight on day 62 | 336.7 ± 16.3 | 347.2 ± 18.7 |
| Maternal[a] liver weight/body weight $\times 10^2$ | 45.7 ± 4.5 | 45.3 ± 5.4 |
| Pup brain weight/body weight $\times 10^3$ | 26.8 ± 4.1 | 30.9 ± 4.0 |
| Average daily food intakes by mothers (g) | 36.3 ± 6.5 | 30.9 ± 3.6 |

[a]Lactating mothers killed on day 22 of lactation. There was no significant difference ($P > 0.05$) in the body and liver weights of dams, brain weights of pups, and mothers' daily food intake between the two dietary groups. For abbreviations see Table 1.

**TABLE 3**
**Fatty Acid Composition (wt% of total fatty acids) of Blood Total Lipids of 17-d-Pregnant Rats ($n = 5$) Fed HOSF/SF/CO or MPO Diets[a]**

| Fatty acid | HOSF/SF/CO group | MPO group |
|---|---|---|
| Total SFA | 28.6 ± 2.9[a] | 34.4 ± 3.4[b] |
| Total MUFA | 26.2 ± 6.3 | 21.5 ± 4.0 |
| 18:2n-6 | 19.7 ± 2.8 | 20.8 ± 2.1 |
| 20:4n-6 | 16.4 ± 5.7 | 16.3 ± 4.6 |
| 22:4n-6 | 0.9 ± 0.2 | 0.7 ± 0.2 |
| 22:5n-6 | 3.5 ± 1.6 | 2.4 ± 0.7 |
| Total n-6 LC-PUFA | 21.4 ± 6.6 | 20.0 ± 5.0 |
| 20:5n-3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 22:5n-3 | 0.2 ± 0.1 | 0.2 ± 0.0 |
| 22:6n-3 | 3.4 ± 1.2 | 2.3 ± 0.7 |
| Total n-3 LC-PUFA | 3.7 ± 1.3 | 2.5 ± 0.7 |
| 5,9-18:2 | 0.0 ± 0.0[a] | 0.4 ± 0.1[b] |
| 5,9,12-18:3 | 0.0 ± 0.0[a] | 3.1 ± 0.5[b] |
| 5,11-20:2 | 0.0 ± 0.0[a] | 0.2 ± 0.1[b] |
| 5,11,14-20:3 | 0.0 ± 0.0[a] | 3.1 ± 0.4[b] |
| Total Δ5 PMI-PUFA | 0.0 ± 0.0[a] | 6.8 ± 0.9[b] |
| 7,11,14-20:3 | 0.0 ± 0.0[a] | 0.3 ± 0.1[b] |

[a]Values in a row with a different roman superscript are significantly ($P < 0.05$) different. LC-PUFA, long-chain ($C_{20}$ and $C_{22}$) polyunsaturated fatty acids; for other abbreviations see Table 1.

**TABLE 4**
**Liver Total Fat and Phospholipid Fatty Acid Composition (wt% of total fatty acids) of 17-d-Pregnant Rats (*n* = 5) Fed HOSF/SF/CO or MPO Diet[a]**

| | Liver total fat | | Liver phospholipids | |
|---|---|---|---|---|
| Fatty acid | HOSF/SF/CO | MPO | HOSF/SF/CO | MPO |
| Total SFA | 38.8 ± 0.6 | 39.1 ± 3.2 | 45.2 ± 6.9 | 47.6 ± 4.3 |
| Total MUFA | 16.8 ± 1.4 | 16.4 ± 1.8 | 5.2 ± 0.6 | 4.8 ± 0.2 |
| 18:2n-6 | 12.3 ± 0.3 | 14.0 ± 2.3 | 8.5 ± 0.6 | 8.8 ± 0.2 |
| 20:4n-6 | 16.9 ± 1.4 | 17.0 ± 1.5 | 21.9 ± 4.4 | 21.8 ± 3.3 |
| 22:4n-6 | 0.6 ± 0.1 | 0.6 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 |
| 22:5n-6 | 7.0 ± 2.5 | 5.6 ± 1.1 | 8.8 ± 2.7 | 7.2 ± 1.3 |
| Total n-6 LC-PUFA | 24.0 ± 1.8 | 25.1 ± 1.7 | 33.1 ± 4.1 | 30.8 ± 3.7 |
| 20:5n-3 | 0.0 ± 0.2 | 0.0 ± 0.2 | 0.0 ± 0.2 | 0.0 ± 0.2 |
| 22:5n-3 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.0 | 0.2 ± 0.0 |
| 22:6n-3 | 6.4 ± 0.6 | 5.8 ± 0.5 | 8.6 ± 1.9 | 7.8 ± 1.0 |
| Total n-3 LC-PUFA | 7.0 ± 0.5 | 6.6 ± 0.7 | 8.8 ± 2.0 | 8.0 ± 1.0 |
| 5,9-18:2 | 0.0 ± 0.0[a] | 0.2 ± 0.0[b] | 0.0 ± 0.0[a] | 0.1 ± 0.0[a] |
| 5,9,12-18:3 | 0.0 ± 0.0[a] | 1.7 ± 0.4[b] | 0.0 ± 0.0[a] | 1.2 ± 0.4[b] |
| 5,11-20:2 | 0.0 ± 0.0[a] | 0.0 ± 0.1[b] | 0.0 ± 0.0[a] | 0.0 ± 0.0[a] |
| 5,11,14-20:3 | 0.0 ± 0.0[a] | 1.4 ± 0.2[b] | 0.0 ± 0.0[a] | 1.3 ± 0.4[b] |
| Total Δ5 PMI-PUFA | 0.0 ± 0.0[a] | 3.4 ± 0.7[b] | 0.0 ± 0.0[a] | 2.6 ± 0.8[b] |
| 7,11,14-20:3 | 0.0 ± 0.0[a] | 0.2 ± 0.0[b] | 0.0 ± 0.0[a] | 0.1 ± 0.0[b] |

[a]Values in a row with different roman superscripts between HOSF/SF/CO and MPO groups are significantly ($P < 0.05$) different. For abbreviations see Tables 1 and 3.

MPO-fed mothers, contained significantly higher proportions of MUFA (primarily oleic acid). The proportion of 18:2n-6 in blood lipids was similar in both groups (Table 6); however, the liver TAG of the MPO rats showed a higher proportion of 18:2n-6 than those of the HOSF/SF/CO rats. In contrast, there was no difference in the 18:2 proportion in liver PL between the two dietary groups. The proportion of AA in blood and liver lipids was similar between the two groups. With regard to other LC-PUFA, 22:4n-6, 20:5n-3, 22:5n-3, and 22:6n-3 proportions were slightly higher in the MPO rats, whereas 22:5n-6 was slightly lower.

*Fatty acid composition of whole body and brain of 17-d-old fetus.* Significant accumulations of the Δ5 PMI-PUFA were observed in the fetal body and brain during pregnancy of the MPO group (Table 8). The proportion of total Δ5 PMI-PUFA in brain PL was 0.6% of total fatty acids, whereas the body total fat contained slightly higher proportions (1.4%). As in the pregnant and lactating mothers, 5,9,12-18:3 and 5,11,14-20:3 were the main Δ5 PMI-PUFA. The compounds 5,9-18:2 and 5,11-20:2 were almost absent in both the total body fat and brain. The 7,11,14-20:3 was present in proportions similar to those in maternal blood fat.

**TABLE 5**
**Fatty Acid Composition (wt% of total fatty acids) of Breast Total Lipids of 17-d-Pregnant Rats (*n* = 5) Fed HOSF/SF/CO or MPO Diets[a]**

| Fatty acid | HOSF/SF/CO | MPO |
|---|---|---|
| Total SFA | 36.9 ± 11.3 | 30.0 ± 5.0 |
| Total MUFA | 37.6 ± 6.3 | 38.5 ± 3.4 |
| 18:2n-6 | 20.6 ± 3.5[a] | 25.9 ± 1.6[b] |
| 20:4n-6 | 2.1 ± 0.8 | 2.3 ± 0.3 |
| 22:4n-6 | 0.8 ± 0.6 | 0.8 ± 0.3 |
| 22:5n-6 | 0.2 ± 0.1 | 0.2 ± 0.1 |
| Total n-6 LC-PUFA | 3.7 ± 1.7 | 4.0 ± 1.1 |
| 20:5n-3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 22:5n-3 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| 22:6n-3 | 0.3 ± 0.1 | 0.4 ± 0.1 |
| Total n-3 LC-PUFA | 0.5 ± 0.2 | 0.5 ± 0.1 |
| 5,9-18:2 | 0.0 ± 0.0[a] | 0.3 ± 0.0[b] |
| 5,9,12-18:3 | 0.0 ± 0.0[a] | 1.9 ± 0.1[b] |
| 5,11-20:2 | 0.0 ± 0.0[a] | 0.2 ± 0.1[b] |
| 5,11,14-20:3 | 0.0 ± 0.0[a] | 1.6 ± 0.1[b] |
| Total Δ5 PMI-PUFA | 0.0 ± 0.0[a] | 3.9 ± 0.2[b] |
| 7,11,14-20:3 | 0.0 ± 0.0[a] | 0.5 ± 0.1[b] |

[a]Values in a row with different roman superscripts are significantly ($P < 0.05$) different. For abbreviations see Tables 1 and 3.

**TABLE 6**
**Fatty Acid Composition (wt% of total fatty acids) of Blood Total Lipids of Lactating Rats (on day 22 of lactation) (*n* = 5) Fed HOSF/SF/CO or MPO Diets[a]**

| Fatty acid | HOSF/SF/CO | MPO |
|---|---|---|
| Total SFA | 35.4 ± 0.9 | 36.6 ± 0.9 |
| Total MUFA | 17.8 ± 1.6[a] | 13.0 ± 1.6[b] |
| 18:2n-6 | 16.6 ± 2.1 | 14.0 ± 2.5 |
| 20:4n-6 | 23.7 ± 3.5 | 22.6 ± 3.5 |
| 22:4n-6 | 1.4 ± 0.1[a] | 1.7 ± 0.1[b] |
| 22:5n-6 | 1.3 ± 0.1[b] | 0.9 ± 0.2[b] |
| Total n-6 LC-PUFA | 27.7 ± 3.4 | 26.0 ± 3.7 |
| 20:5n-3 | 0.0 ± 0.0 | 0.6 ± 0.6 |
| 22:5n-3 | 0.4 ± 0.2 | 0.6 ± 0.3 |
| 22:6n-3 | 1.8 ± 0.3[a] | 2.8 ± 0.5[b] |
| Total n-3 LC-PUFA | 2.2 ± 0.4[a] | 3.9 ± 0.8[b] |
| 5,9-18:2 | 0.0 ± 0.0[a] | 0.1 ± 0.1[b] |
| 5,9,12-18:3 | 0.0 ± 0.0[a] | 2.4 ± 0.4[b] |
| 5,11-20:2 | 0.0 ± 0.0[a] | 0.1 ± 0.0[b] |
| 5,11,14-20:3 | 0.0 ± 0.0[a] | 3.1 ± 0.3[b] |
| Total Δ5 PMI-PUFA | 0.0 ± 0.0[a] | 5.7 ± 0.6[b] |
| 7,11,14-20:3 | 0.0 ± 0.0[a] | 0.1 ± 0.1[b] |

[a]Values in a row with different roman superscripts are significantly ($P < 0.05$) different. For abbreviations see Tables 1 and 3.

**TABLE 7**
**Fatty Acid Composition (wt% of total fatty acids) of Phospholipids and Triacylglycerols of Liver of Lactating Rats (at day 22 of lactation) ($n$ = 5) Fed HOSF/SF/CO or MPO Diets[a]**

| | Phospholipids | | Triacylglycerol | |
|---|---|---|---|---|
| Fatty acid | HOSF/SF/CO | MPO | HOSF/SF/CO | MPO |
| Total SFA | 39.4 ± 0.4 | 39.2 ± 2.0 | 31.4 ± 3.3 | 32.1 ± 3.5 |
| Total MUFA | 10.0 ± 1.3[a] | 6.3 ± 0.8[b] | 51.9 ± 1.6[a] | 34.1 ± 3.5[b] |
| 18:2n-6 | 12.9 ± 1.8 | 12.8 ± 2.0 | 14.1 ± 1.7[a] | 25.5 ± 1.1[b] |
| 20:4n-6 | 25.9 ± 2.4 | 23.1 ± 2.1 | 0.3 ± 0.1[a] | 0.5 ± 0.2[b] |
| 22:4n-6 | 0.6 ± 0.2 | 0.4 ± 0.2 | 0.0 ± 0.0[a] | 0.1 ± 0.0[b] |
| 22:5n-6 | 3.3 ± 0.2[a] | 0.9 ± 0.4[b] | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Total n-6 LC-PUFA | 31.7 ± 1.9[a] | 25.7 ± 2.2[b] | 2.3 ± 0.3 | 2.7 ± 0.5 |
| 20:5n-3 | 0.1 ± 0.0[a] | 0.3 ± 0.1[b] | 0.0 ± 0.0[a] | 0.3 ± 0.1[b] |
| 22:5n-3 | 0.5 ± 0.1[a] | 1.1 ± 0.3[b] | 0.0 ± 0.0[a] | 0.1 ± 0.0[b] |
| 22:6n-3 | 5.1 ± 1.3[a] | 10.0 ± 1.7[b] | 0.0 ± 0.0[a] | 0.1 ± 0.0[b] |
| Total n-3 LC-PUFA | 5.7 ± 1.2[a] | 11.4 ± 1.5[b] | 0.0 ± 0.0[a] | 0.2 ± 0.1[b] |
| 5,9-18:2 | 0.0 ± 0.0[a] | 0.1 ± 0.0[b] | 0.0 ± 0.0[a] | 0.7 ± 0.0[b] |
| 5,9,12-18:2 | 0.0 ± 0.0[a] | 1.7 ± 0.4[b] | 0.0 ± 0.0[a] | 3.1 ± 0.5[b] |
| 5.11-20:2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0[a] | 0.1 ± 0.0[b] |
| 5,11,14-20:3 | 0.0 ± 0.0[a] | 2.2 ± 0.3[b] | 0.0 ± 0.0[a] | 0.5 ± 0.1[b] |
| Total Δ5 PMI-PUFA | 0.0 ± 0.0[a] | 4.0 ± 0.8[b] | 0.0 ± 0.0[a] | 4.4 ± 0.6[b] |
| 7,11,14-20:3 | 0.0 ± 0.0[a] | 0.1 ± 0.0[b] | 0.0 ± 0.0[a] | 0.1 ± 0.0[b] |

[a]Values in a row with different roman superscripts between HOSF/SF/CO and MPO groups are significantly ($P < 0.05$) different. For abbreviations see Tables 1 and 3.

The brain PL fraction of the MPO group showed a lower proportion of saturated fatty acids (SFA) than that of the HOSF/SF/CO group. This low proportion was compensated by large increases in total monounsaturated fatty acids (MUFA) (primarily oleic acid), 20:4n-6, and slight, but significant, increases of 22:4n-6 and 22:5n-6 acids in the MPO group. However, in fetal body total fat, there were no significant differences in these fatty acids between the two groups.

*Fatty acid composition of milk.* All of the dietary Δ5 PMI-PUFA were found in mothers' milk of the MPO group withdrawn on day 21 of lactation (Table 9). Compared with maternal blood fatty acid composition, there was a large incorporation of Δ5 PMI-PUFA with 18 carbon atoms into milk fat. The 7,11,14-20:3 was also incorporated, and its proportion reached 0.3% of total milk fatty acids. Major differences in the other fatty acids were also seen between dietary groups. The proportion of total MUFA was lower, whereas the proportions of total SFA and 18:2n-6 were higher in the MPO group than in the HOSF/SF/CO group. In addition, the MPO group contained slightly but significantly higher proportions

**TABLE 8**
**Fatty Acid Composition (wt% of total fatty acids) of Brain Phospholipids and Whole Body Total Fat of 17-d-old Rat Fetuses ($n$ = 5) of Mothers Fed HOSF/SF/CO or MPO Diets[a]**

| | Fetal brain phospholipids | | Fetal body total fat | |
|---|---|---|---|---|
| Fatty acid | HOSF/SF/CO | MPO | HOSF/SF/CO | MPO |
| Total SFA | 54.6 ± 1.9[a] | 47.7 ± 2.4[b] | 42.0 ± 5.3 | 48.1 ± 8.5 |
| Total MUFA | 18.6 ± 1.6[a] | 21.1 ± 2.0[b] | 24.1 ± 2.2 | 21.7 ± 4.2 |
| 18:2n-6 | 1.0 ± 0.2 | 1.1 ± 0.2 | 5.5 ± 1.1 | 4.6 ± 1.1 |
| 20:4n-6 | 12.0 ± 1.0[a] | 14.1 ± 1.3[b] | 16.0 ± 1.1 | 14.7 ± 1.9 |
| 22:4n-6 | 3.3 ± 0.3[a] | 3.8 ± 0.3[b] | 3.9 ± 0.4 | 3.7 ± 0.5 |
| 22:5n-6 | 4.5 ± 0.4[a] | 5.7 ± 0.8[b] | 3.0 ± 0.5 | 2.8 ± 0.3 |
| Total n-6 LC-PUFA | 20.5 ± 0.7[a] | 24.6 ± 2.6[b] | 23.8 ± 1.6 | 21.8 ± 2.8 |
| 20:5n-3 | 0.3 ± 0.0 | 0.4 ± 0.1 | 0.3 ± 0.0 | 0.3 ± 0.1 |
| 22:5n-3 | 0.2 ± 0.3 | 0.1 ± 0.1 | 0.2 ± 0.0 | 0.1 ± 0.1 |
| 22:6n-3 | 4.5 ± 0.9[a] | 5.2 ± 0.8[b] | 3.7 ± 0.5 | 3.0 ± 0.5 |
| Total n-3 LC-PUFA | 4.9 ± 0.9[a] | 5.7 ± 0.9[b] | 4.1 ± 0.6 | 3.4 ± 0.6 |
| 5,9-18:2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 5,9,12-18:2 | 0.0 ± 0.0[a] | 0.2 ± 0.0[b] | 0.0 ± 0.0[a] | 0.6 ± 0.1[b] |
| 5.11-20:2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 5,11,14-20:3 | 0.0 ± 0.0[a] | 0.4 ± 0.1[b] | 0.0 ± 0.0[a] | 0.8 ± 0.2[b] |
| Total Δ5 PMI-PUFA | 0.0 ± 0.0[a] | 0.6 ± 0.1[b] | 0.0 ± 0.0[a] | 1.4 ± 0.3[b] |
| 7,11,14-20:3 | 0.0 ± 0.0[a] | 0.3 ± 0.1[b] | 0.0 ± 0.0[a] | 0.7 ± 0.1[b] |

[a]Values in a row with different roman superscripts between HOSF/SF/CO and MPO groups are significantly ($P < 0.05$) different. For abbreviations see Tables 1 and 3.

**TABLE 9**
**Fatty Acid Composition (wt% of total fatty acids) of Milk Total Fat of Lactating Rats Fed HOSF/SF/CO or MPO Diets[a] (milk samples withdrawn on day 21 of lactation)**

| Fatty acid | HOSF/SF/CO | MPO |
|---|---|---|
| Total SFA | 37.9 ± 3.1[a] | 50.3 ± 5.0[b] |
| Total MUFA | 34.9 ± 1.7[a] | 17.2 ± 2.7[b] |
| 18:2n-6 | 23.4 ± 1.5[a] | 27.1 ± 2.0[b] |
| 20:4n-6 | 1.5 ± 0.2 | 1.2 ± 0.2 |
| 22:4n-6 | 0.3 ± 0.1 | 0.3 ± 0.1 |
| 22:5n-6 | 0.1 ± 0.0[a] | 0.0 ± 0.0[a] |
| Total n-6 LC-PUFA | 2.6 ± 0.3 | 2.2 ± 0.2 |
| 20:5n-3 | 0.0 ± 0.0 | 0.1 ± 0.1 |
| 22:5n-3 | 0.1 ± 0.0[a] | 0.1 ± 0.0[b] |
| 22:6n-3 | 0.2 ± 0.0[a] | 0.3 ± 0.0[b] |
| Total n-3 LC-PUFA | 0.3 ± 0.1[a] | 0.5 ± 0.1[b] |
| 5,9-18:2 | 0.0 ± 0.0[a] | 0.4 ± 0.0[b] |
| 5,9,12-18:3 | 0.0 ± 0.0[a] | 2.6 ± 0.2[b] |
| 5,11-20:2 | 0.0 ± 0.0[a] | 0.2 ± 0.0[b] |
| 5,11,14-20:3 | 0.0 ± 0.0[a] | 1.9 ± 0.3[b] |
| Total Δ5 PMI-PUFA | 0.0 ± 0.0[a] | 5.1 ± 0.5[b] |
| 7,11,14-20:3 | 0.0 ± 0.0[a] | 0.3 ± 0.1[b] |

[a]Values in a row with different roman superscripts are significantly ($P < 0.05$) different. For abbreviations see Tables 1 and 3.

of 20:5n-3, 22:5n-3, and 22:6n-3. The proportions of 20:4n-6 and other n-6 LC-PUFA, however, were similar between the two groups. Consequently, the ratio of total n-6 LC-PUFA to total n-3 LC-PUFA was significantly higher for the HOSFO/SFO/CO group (1.70 ± 0.15) than for the MPO group (1.44 ± 0.06).

*Fatty acid composition of brain PL and blood total fat of 22-d-old pups*. All of the dietary Δ5 PMI-PUFA were found in blood of the pups from the MPO group, and they accounted for 9% of the total fatty acids (Table 10). In contrast, pup brain contained very little Δ5 PMI-PUFA (0.4% of total fatty acids). In pup blood, both 5,9,12-18:3 and 5,11,14-20:3 were found in important proportions but only 5,9-18:2 and 5,11,14-20:3 were detected in brain. The brain of the MPO group compared to the HOSF/SF/CO contained significantly higher proportions of 22:6n-3 and total n-3 LC-PUFA.

## DISCUSSION

The measurement of routine indicators of nutritional adequacy such as body and organ weights, and hematologic measurements demonstrated that consumption of MPO had no adverse effects on mothers' and pups' health.

*Δ5 PMI-PUFA levels in pregnant and lactating rats*. The fatty acid data show that feeding of pregnant and lactating rats with MPO results in the incorporation of Δ5 PMI-PUFA into blood followed by deposition into various tissues including breast and milk. The 5,9,12-18:3 and 5,11,14- 20:3 were the primary Δ5 PMI-PUFA detected in blood and tissues of pregnant and lactating rats. Of these two, 5,11,14-20:3 was more concentrated in the liver PL fraction, whereas 5,9,12-18:3 was concentrated in the liver TAG fraction. The specific incorporation of 5,11,14-20:3 in the PL fraction may indicate a special affinity, perhaps due to its structural similarity with AA. Nevertheless, no significant replacement of AA was observed in the liver PL of both pregnant and lactating rats, but we cannot exclude that this may have occurred in PI, which is quantitatively a minor lipid class in the liver (9). This point deserves further investigation.

*Δ5 PMI-PUFA in fetus and pups*. The 17-d-old fetal brain and whole body contained detectable levels of Δ5 PMI-PUFA, which shows that there is a transfer of these fatty acids from the mother to fetus during pregnancy. However, the lev-

**TABLE 10**
**Fatty Acid Composition (wt% of total fatty acids) of Blood Total Fat and Brain Phospholipids of 22-d-old Pups ($n = 5$) from Mothers Fed HOSF/SF/CO or MPO Diets[a]**

| | Pup blood total fat | | Pup brain phospholipids | |
|---|---|---|---|---|
| Fatty acid | HOSF/SF/CO | MPO | HOSF/SF/CO | MPO |
| Total SFA | 40.6 ± 9.4 | 36.8 ± 6.8 | 43.1 ± 3.7 | 43.6 ± 2.0 |
| Total MUFA | 22.3 ± 1.5[a] | 15.6 ± 1.4[b] | 18.3 ± 0.2 | 18.4 ± 0.3 |
| 18:2n-6 | 18.5 ± 4.3 | 20.0 ± 2.2 | 1.2 ± 0.1 | 1.2 ± 0.1 |
| 20:4n-6 | 13.6 ± 4.1 | 12.3 ± 3.0 | 13.9 ± 1.0 | 13.4 ± 0.8 |
| 22:4n-6 | 1.1 ± 0.4 | 1.0 ± 0.3 | 4.8 ± 0.5 | 4.4 ± 0.3 |
| 22:5n-6 | 1.1 ± 0.5 | 0.7 ± 0.3 | 3.8 ± 0.7[a] | 2.9 ± 0.2[b] |
| Total n-6 LC-PUFA | 16.5 ± 5.0 | 14.8 ± 3.5 | 23.2 ± 2.0 | 21.4 ± 1.1 |
| 20:5n-3 | 0.1 ± 0.1 | 0.0 ± 0.1 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 22:5n-3 | 0.4 ± 0.1[a] | 0.7 ± 0.2[b] | 0.5 ± 0.5 | 0.4 ± 0.3 |
| 22:6n-3 | 1.1 ± 0.5 | 1.3 ± 0.6 | 13.2 ± 1.6[a] | 14.4 ± 1.0[b] |
| Total n-3 LC-PUFA | 1.6 ± 0.6 | 2.2 ± 0.8 | 13.8 ± 2.0[a] | 14.9 ± 1.0[b] |
| 5,9-18:2 | 0.0 ± 0.0[a] | 0.4 ± 0.1[b] | 0.0 ± 0.0 | 0.1 ± 0.0 |
| 5,9,12-18:2 | 0.0 ± 0.0[a] | 3.3 ± 0.4[b] | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 5.11-20:2 | 0.0 ± 0.0[a] | 0.3 ± 0.0[b] | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 5,11,14-20:3 | 0.0 ± 0.0[a] | 4.6 ± 0.6[b] | 0.0 ± 0.0[a] | 0.4 ± 0.1[b] |
| Total Δ5 PMI-PUFA | 0.0 ± 0.0[a] | 9.0 ± 0.9[b] | 0.0 ± 0.0[a] | 0.4 ± 0.2[b] |
| 7,11,14-20:3 | 0.0 ± 0.0[a] | 0.4 ± 0.1[b] | 0.0 ± 0.0[a] | 0.1 ± 0.1[b] |

[a]Values in a row with different roman superscripts between HOSF/SF/CO and MPO groups are significantly ($P < 0.05$) different. For abbreviations see Tables 1 and 3.

els detected in the fetus were considerably lower than those in mothers' blood. This indicates that there is a mechanism in the placenta for selectively slowing the passage of Δ5 PMI-PUFA from maternal blood to the fetus. An alternative explanation is that there is a preferential passage through the placenta for normal fatty acids particularly oleic acid and, n-6 and n-3 LC-PUFA. The proportions of these fatty acids were considerably higher in fetal than in maternal blood. These data would also suggest that Δ5 PMI-PUFA are not recognized as usual PUFA by the fetus during fatty acid transfer from the placenta, and therefore, Δ5 PMI-PUFA appear not to interfere with LC-PUFA during transport.

Among the different Δ5 PMI-PUFA, 5,9,12-18:3 and 5,11,14-20:3 acids were primarily incorporated into fetal tissues, and 5,9-18:2 and 5,11-20:2 acids were poorly incorporated. Between 5,9,12-18:3 and 5,11,14-20:3, the latter was more efficiently incorporated. For example, the ratio of 5,11,14-20:3 to 5,9,12-18:3 in pregnant mothers' blood was 1.12, and this ratio was increased to 1.80 and 1.31 in brain PL and whole body fat, respectively, of the 17-d-old fetus. This preferential incorporation might be explained by its structural closeness to 8,11,14-20:3 and 5,8,11,14-20:4. The primary difference between these three $C_{20}$-PUFA is the absence of a Δ8 bond in 5,11,14-20:3. This similarity might give 5,11,14-20:3 a slight advantage over 5,9,12-18:3 during placental transfer to the fetus. An alternative possibility concerning the lower incorporation of 5,9,12-18:3 in fetus may be related to the surprisingly high level of 7,11,14-20:3 in the same tissue; 7,11,14-20:3 is the two-carbon chain elongation product of 5,9,12-18:3, and its proportion in fetal whole body fat was twice that found in maternal blood. Whereas the ratio of 7,11,14-20:3 to 5,9,12-18:3 was 0.10 in the maternal blood, this ratio reached 1.57 and 1.11 in brain PL and whole body fat, respectively. This may indicate that 5,9,12-18:3 is elongated inside the fetal milieu to 7,11,14-20:3, and this would result in low levels of 5,9,12-18:3 in tissues. The fetal whole body fat compared to the fetal brain PL contained a higher proportion of Δ5 PMI-PUFA (1.4 vs. 0.6%). This may indicate that brain is relatively protected against incorporation of Δ5 PMI-PUFA. It may be that the blood–brain barrier restricts the passage of Δ5 PMI-PUFA.

The fatty acid data suggest that transfer of Δ5 PMI-PUFA from milk into pups was more efficient than the transfer of these fatty acids from pregnant mothers to the fetus. For example, the ratio of the proportion of Δ5 PMI-PUFA between fetal whole body fat and pregnant rats' blood total fat was 0.21, whereas it was 1.56 between pups' blood total fat and milk fat. This increased transfer of Δ5 PMI-PUFA from milk to pup is not surprising as digestion in the newborn relies mainly on lingual lipase because the bile salt levels fall below critical micellar concentration (19); under these conditions pancreative lipase is inactive. Lingual lipase preferentially cleaves the *sn*-3 position of TAG fatty acids present on dietary TAG, giving high free fatty acid levels. Free fatty acids are more readily absorbed than monoacylglycerol under conditions of low intestinal intraluminal levels of bile salts. Therefore, the location of LC-PUFA at the *sn*-3 position of milk TAG leads to their preferential release during gastric lipolysis (20). The Δ5 PMI-PUFA, particularly 5,9,12-18:3 and 5,11,14-20:3, because of their structural similarity to n-6 PUFA, should also be concentrated in the *sn*-3 position of milk TAG and, consequently, preferentially cleaved and well absorbed. It is necessary to study the Δ5 PMI-PUFA distribution in milk TAG to confirm the site of esterification as the *sn*-3 position, as was reported for conifer seed oil TAG structure (21).

Although pups' blood of the MPO group contained a large proportion of total Δ5 PMI-PUFA (9% of total fatty acids), pups' brain PL contained very little Δ5 PMI-PUFA (0.4%), and this level was slightly lower than in fetal brain PL (0.6%). This shows that the blood–brain barrier effectively inhibited the transfer of Δ5 PMI-PUFA to the brain during its development. A comparison between the Δ5 PMI-PUFA distribution of fetal and pups' brain PL presents some interesting biochemical features related to the metabolism of these fatty acids. The 5,11,14-20:3 is present at similar proportions in both fetal body total fat and brain PL, and 5,9-18:2 and 5,11-20:2 are nearly absent. But a noticeable difference is seen in the proportions of 5,9,12-18:3. This Δ5 PMI-PUFA is found in fetal brain PL (0.2% of total fatty acids), but it is almost absent in pups' brain PL. Nevertheless, pups' brain PL contained 7,11,14-20:3 at two times the level found in fetal brain PL (0.6 vs. 0.3% of total fatty acids). This might indicate that the process of chain elongation of 5,9,12-18:3 to 7,11,14-20:3 in brain is active during its development from the fetus to pup stage.

*Effect of diet on tissue levels of essential fatty acids*. The fatty acid data show that the consumption of Δ5 PMI-PUFA from MPO during pregnancy and lactation produces no drastic changes in the proportions of AA, DHA, and other n-6 and n-3 LC-PUFA in the various tissues of both pregnant rats and fetuses, including the brain. Moreover, the possible direct replacement of AA by 5,11,14-20:3 (sciadonic acid), which was suspected because this was reported in several previous studies (6–9) and because of the close structural similarity between AA and sciadonic acid, was not observed in this study. Previous studies had suggested that replacement of AA by sciadonic acid occurs mainly in PL fraction, but this was not evident from the fatty acid data of the present study. In contrast, PL from various tissues of this study showed no change in the AA level; and in some tissue PL, particularly from 17-d-old fetal brain, there was a slight but significant elevation of the proportion of AA as well as other n-6 LC-PUFA and DHA. The DHA proportion was also increased in 22-d-old pup brains from mothers treated with MPO. These increases in the proportions of n-3 and n-6 LC-PUFA may be beneficial for pups in developing their visual acuity and cognitive functions. The mechanism by which the dietary Δ5 PMI-PUFA can modify LC-PUFA proportions in brain, liver, and other tissues, however, remains unclear.

In summary, our study demonstrated that intake of sciadonic acid and other Δ5 PMI-PUFA from MPO by rats during pregnancy and lactation presents no adverse effects on the proportions of AA, DHA, and other $C_{20}$ and $C_{22}$ n-6 and n-3

PUFA in the developing brain of fetuses and infants. Moreover, the present study suggests that MPO would be helpful in increasing the n-3 and n-6 LC-PUFA levels in brain of fetus and infants and therefore their consumption might be beneficial for pregnant and lactating women.

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# The Effect of Conjugated Linoleic Acid Isomers on Fatty Acid Profiles of Liver and Adipose Tissues and Their Conversion to Isomers of 16:2 and 18:3 Conjugated Fatty Acids in Rats

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**ABSTRACT:** Conjugated linoleic acid (CLA) is a collective term that describes different isomers of linoleic acid with conjugated double bonds. Although the main dietary isomer is 9*cis*,11*trans*-18:2, which is present in dairy products and ruminant fat, the biological effects of CLA generally have been studied using mixtures in which the 9*cis*,11*trans*- and the 10*trans*,12*cis*-18:2 were present at similar levels. In the present work, we have studied the impact of each isomer (9*cis*,11*trans*- and 10*trans*,12*cis*-18:2) given separately in the diet of rats for 6 wk. The 10*trans*,12*cis*-18:2 decreased the triacylglycerol content of the liver (–32%) and increased the 18:0 content at the expense of 18:1n-9, suggesting an alteration of the Δ9 desaturase activity, as was already demonstrated *in vitro*. This was not observed when the 9*cis*,11*trans*-18:2 was given in the diet. Moreover, the 10*trans*,12*cis*-18:2 induced an increase in the $C_{22}$ polyunsaturated fatty acids in the liver lipids. The 10*trans*,12*cis*-18:2 was mainly metabolized into conjugated 16:2 and 18:3, which have been identified. The 9*cis*,11*trans* isomer was preferentially metabolized into a conjugated 20:3 isomer. Thus, the 9*cis*,11*trans*- and the 10*trans*,12*cis*-CLA isomers are metabolized differently and have distinct effects on the metabolism of polyunsaturated fatty acids in rat liver while altering liver triglyceride levels differentially.

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Conjugated linoleic acid (CLA) is a collective term that describes several conjugated isomers of linoleic acid (1,2). CLA isomers have been reported to have beneficial properties with respect to some experimentally induced cancers (3–6) and to some parameters that are considered as cardiovascular risk factors (7,8). However, a recent paper by Munday *et al.* (9) showed that, even if feeding CLA resulted in a serum lipoprotein profile considered to be less atherogenic, the presence of CLA in the diet increased the development of aortic fatty streak. CLA have also been reported to be effective in preventing the catabolic effects of immune stimulation and to have beneficial effects on diabetic rats (10).

Moreover, CLA can induce a reduction of body fat in mice, chicks, and pigs (11–14). Feeding 4.2 g/d of CLA to humans (men and women) for 3 mon resulted in a reduction of body fat (15), but an increase in lipid peroxidation (16). On the contrary, a recent study by Zambell *et al.* (17) showed that feeding CLA capsules (3 g/d) for 64 d to healthy adult women did not result in any change in fat-free mass, fat mass or energy expenditure which contrasts with the findings in animals. However, the CLA isomers used in the different studies were different.

CLA are present in human food. They mainly occur in dairy products (18–24) but also in meat from ruminants (25), hydrogenated fats (26), and used frying oils (27). The main dietary CLA isomer is 9*cis*,11*trans*-18:2, whereas synthetic CLA mixtures contain many other 18:2 isomers with conjugated double bonds (1,2). These synthetic samples contain 9,11 dienes as well as 8,10, 10,12, and 11,13 *cis-trans*/*trans-cis* isomers, which are accompanied by some all-*cis* and all-*trans* isomers. Most of the experimental studies were carried out using such synthetic CLA mixtures, and the role of each isomer has not yet been clearly identified. A recent study (28) suggested a specific effect of the 10*trans*,12*cis*-18:2 isomer on body composition. Furthermore, Ip *et al.* (5) also showed that an enriched CLA butterfat, containing mainly the 9*cis*,11*trans* isomer, had a similar activity as a potent cancer-preventive agent to the commercial CLA mixture frequently used in animal models of chemically induced carcinogenesis.

The antioxidant role of CLA, which was a hypothesis of the mechanism of its action, has been reevaluated (29). Some studies suggest that dietary CLA may alter eicosanoid biosynthesis (30). Such data could also explain their action, but the mechanism is not clearly understood (21). It has been suggested that CLA alter the fatty acid composition of animal tissues when given in the diet (31). However, these studies were carried out using a mixture of CLA isomers and the precise effect of each major isomer has not been studied yet. In the present paper, we report data on the lipid profile and the fatty acid composition of liver lipids and the fatty acid composition of the adipose tissue from rats fed for 6 wk a diet containing 1% of either 9*cis*,11*trans*- or 10*trans*,12*cis*-18:2, which are the major isomers present in synthetic CLA mixtures utilized so far for animal studies. Metabolites of the CLA isomers were also identified in liver lipids and adipose tissue.

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Abbreviations: CE, cholesteryl ester; CL, cardiolipid; CLA, conjugated linoleic acid; DAG, diacylglycerols; DMOX, dimethyloxazoline; FFA, free fatty acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; MTAD, 4-methyl-1,2,4-triazoline-3,5-dione; PC, phosphatidylcholine; PL, phospholipid; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; TLC–FID, thin-layer chromatography–flame-ionization detection.

## MATERIALS AND METHODS

*Animals*. Twenty weanling male Wistar rats (IFFA Credo, L'Arbresle, France) were housed in individual stainless steel cages in an animal house maintained at 26 ± 1°C (light/dark: 12 h/12 h). They were randomly divided into four experimental groups of five animals which were fed a semisynthetic diet containing 6% (by weight) of lipids. The composition of the diet, in terms of dry weight (g/1000 g), was as follows: casein, 180; corn starch, 460; sucrose, 220; cellulose, 20; mineral mixture, 50 [The mineral mixture contained: $CaCO_3$, 12 g; $K_2HPO_4$, 10.75 g; $CaHPO_4$, 10.75 g; $MgSO_4 \cdot 7H_2O$, 5 g; NaCl, 3 g; MgO, 2 g; $FeSO_4 \cdot 7H_2O$, 400 mg; $ZnSO_4 \cdot 7H_2O$, 350 mg; $MnSO_4 \cdot H_2O$, 100 mg; $CuSO_4 \cdot 5H_2O$, 50 mg; $Na_2SiO_7 \cdot 3H_2O$, 25 mg, $AlK(SO_4)_2 \cdot 12H_2O$, 10 mg; $K_2CrO_4$, 7.5 mg; NaF, 5 mg; $NiSO_4 \cdot 6H_2O$, 5 mg; $H_3BO_3$, 5 mg; $CoSO_4 \cdot 7H_2O$, 2.5 mg, $KIO_3$, 2 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 mg; LiCl, 0.75 mg; $Na_2SeO_3$, 0.75 mg; $NH_4VO_3$, 0.5 mg, sucrose, 5.5 g]; vitamin mixture, 10 (The vitamin mixture contained: retinol acetate, 5000 IU; cholecalciferol, 1250 IU; DL-α-tocopherol acetate, 100 IU; phylloquinone, 1 mg; thiamine chlorhydrate, 10 mg; riboflavin, 10 mg; nicotinic acid, 50 mg; Ca-pantothenate, 25 mg, pyridoxin chlorhydrate, 10 mg; D-biotin, 0.2 mg; folic acid, 2 mg; cyanocobalamin, 25 µg; choline chlorhydrate, 1 g; DL-methionine, 2 g; *p*-aminobenzoic acid, 50 mg; inositol, 100 mg; sucrose, 5.5 g); oil, 50; free fatty acids, 10. Dietary lipids were made up of 5% of a mixture of high-oleic sunflower and linseed oils (98:2, w/w) and 1% of conjugated CLA as free fatty acid (FFA: experimental groups) or 1% of oleic acid (control group). The different experimental groups were fed either 1% of 9*cis*,11*trans*-18:2 (group 9*cis*,11*trans*), 1% of previously synthetized 10*trans*,12*cis*-18:2 (32,33) (group 10*trans*,12*cis*), or a synthetic commercial mixture of conjugated-18:2 isomers (group CLA mixture). The detailed fatty acid composition of each FFA moiety of the diets is presented in Table 1. The animals were fed for 6 wk *ad libitum* with free access to tap water. This experiment was carried out according to the French guidelines for animal studies.

*Lipid analyses.* At the end of the 6-wk experimental period, the animals were anesthetized with diethyl ether, exsanguinated, and the liver and adipose tissue were quickly excised, blotted on filter paper, and weighed. Liver and adipose tissue lipids were extracted using a mixture of chloroform and methanol (2:1, vol/vol) (34).

Quantitative lipid class analyses were carried out by the Iatroscan (Iatron, Tokyo, Japan) thin-layer chromatography–flame-ionization detection (TLC–FID) system using a mixture of hexane/diethyl ether/acetic acid (97:3:1, by vol) as solvent (35).

Liver lipid classes were fractionated into phospholipids (PL), cholesteryl esters (CE), and triacylglycerols (TAG) using $NH_2$ cartridges (Phase Separation Products, Saint Quentin en Yvelines, France) (36). The purity of each fraction was assessed by the TLC–FID method (35). PL were separated into classes by high-performance liquid chromatography (HPLC) as previously described (37).

Fatty acids from the total PL, PL classes, CE, and TAG of the liver as well as total lipids from adipose tissue were esterified using sodium methylate (1 M) followed by boron trifluoride in methanol (14%) according to Glass (38). Sphingomyelin was transformed into methyl esters as described by Kramer *et al.* (39)

The resulting fatty acid methyl esters were then analyzed by gas–liquid chromatography (GLC) using an HP 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA), equipped with an automatic HP 6890 injector, a BPX 70 fused-silica capillary column (50 m × 0.33 mm i.d., 0.25 µm film thickness; SGE, Melbourne, Australia) and a flame-ionization detector as previously described (40). Quantitative data were obtained using the WOW software (Thermo Separation Products, Les Ulis, France).

*Isolation of CLA metabolites.* The isolation of CLA metabolites was carried out using HPLC on FFA. Free fatty acids were obtained by a mild saponification as described by Banni *et al.* (41). Briefly, small aliquots (4 mg) of lipids extracted from the adipose tissue of rats fed the 10*trans*,12*cis*-18:2 isomer were dissolved in 5 mL of ethanol. Then 100 µL of EGTA (14.5 mg/mL $H_2O$), 1 mL of a 25% water solution of ascorbic acid, and 0.5 mL of 10 N KOH, were added. The solutions were left in the dark at room temperature for 16 h. The FFA were extracted by phase partition with 10 mL of *n*-hexane, 7 mL of $H_2O$, 350 µL HCl 36% (pH samples 3–4) and by centrifugation at 2000 rpm for 1 h. Hexane was evaporated, and the FFA were dissolved in 1 mL of $CH_3CN/CH_3COOH$ (100:0.14, vol/vol).

HPLC was carried out on a Waters system (Waters Co., Milford, MA) equipped with a model 600 pump and a model 996 photodiode array detector. FFA separations were carried out using a Nucleosil column (C18 5 µm, 250 × 4.6 mm; Interchim, Montluçon, France) and a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (570:30:0.12, by vol) at a flow rate of 1.5 mL/min. The spectrum from 190 to 300 nm was used to detect conjugated fatty acids (234 nm) and nonconjugated fatty acids (200 nm). Data were processed by the Waters Millenium 2010 software. Peaks showing an absorption at 234 nm were collected for further GC–mass spectrometry (MS) analysis.

*Identification of conjugated fatty acid metabolites by GC–MS.* The identification of the metabolites of the 18:2 con-

**TABLE 1**
**Fatty Acid Composition (%) of the Free Fatty Acid Fractions of the Diets**

| | Control | 9*cis*,11*trans* | 10*trans*,12*cis* | CLA mixture |
|---|---|---|---|---|
| 18:1 | 89.4 | 0.7 | 0.2 | 6.4 |
| 18:2n-6 | 3.7 | 1.0 | 0.1 | 0.5 |
| 9*cis*,11*trans*-18:2 | — | 87.0 | 5.8 | 43.0 |
| 10*trans*,12*cis*-18:2 | — | 1.5 | 92.0 | 44.5 |
| Others conjugated 18:2 | — | 8.4 | 1.9 | 2.6 |
| Others[a] | 6.9 | 1.4 | — | 2.9 |

[a]Mainly include 16:0. CLA, conjugated linoleic acid.

jugated isomers was carried out on their 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) adducts following the method of Dobson (42). Briefly, conjugated methyl esters (100 µg) and MTAD (200 µg) in dichloromethane (300 µL) were mixed in a test tube at 0°C by agitating for less than 10 s. The reaction was stopped by addition of 1,3-hexadiene. The mixture was evaporated to dryness and redissolved in dichloromethane for GC–MS analyses. GC–MS analyses were performed with an HP 5870 gas chromatograph coupled with an HP 5970 (Hewlett-Packard) quadrupole mass spectrometer. Fatty acid methyl ester MTAD adducts were analyzed on a HP5 bonded fused-silica capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness; Hewlett-Packard) using a splitless/split injector (290°C). The transfer line temperature was 290°C. Helium was used as carrier gas at a velocity of 35 cm/s. The oven temperature was 50°C (1 min)—20°C/min—260°C—3°C/min—290°C. Electron impact mass spectra were recorded at 70 ev between 100 and 500 amu in 700 ms.

An aliquot of the methyl ester of the HPLC fraction containing the 18:3 isomers collected from the adipose tissue of rats fed the 10*trans*,12*cis*-18:2 was converted into dimethyloxazoline derivatives (DMOX) and analyzed by GC–MS as previously described by Juaneda and Sébédio (43).

Briefly, 100 µL of 2-amino-2-methyl-1-propanol was added to the fatty acid methyl ester (up to 1 mg) in a screw-capped tube, purged with $N_2$, and sealed using a polytetrafluoroethylene cap. The reaction mixture was maintained at 170°C. The DMOX derivatives were analyzed on a BPX70 column using an HP 5890 coupled to an HP 5970 mass spectrometer.

*Statistical analysis*. Results are expressed as means ± standard deviation (SD). The values were compared using the PROC ANOVA and the Student-Newmann-Keuls or Dunnet test from SAS software (Cary, NC). *P* values of less than 0.05 were considered as significant.

## RESULTS

*Animals.* The CLA intake ranged from 179 mg/d for the 10*trans*,12*cis*-18:2 group to 186 mg/d for the animals fed the CLA mixture.

At the end of the experimental feeding period, the weights of the animals were not significantly different, 300 ± 10, 309 ± 15, 316 ± 9, 318 ± 6 g (means ± SEM) in groups fed the control, 9*cis*,11*trans*, 10*trans*,12*cis,* and the CLA mixture diets, respectively.

*Liver lipid classes.* The lipid content ranged from 48.2 mg/g of liver for the animals fed the 10*trans*,12*cis*-18:2 diet to 55.8 mg/g of liver for the animals fed the 9*cis*,11*trans* diet. No significant differences were observed between the control and the experimental groups.

The lipid composition of the livers from rats fed the 6-wk experimental diet is presented in Figure 1: the diacylglycerol (DAG), cholesterol, and FFA contents were not affected by the diet while the TAG content was decreased when the animals were fed the 10*trans*,12*cis* isomer (17.6%) compared to the control group (26.1%). This decrease was balanced by the

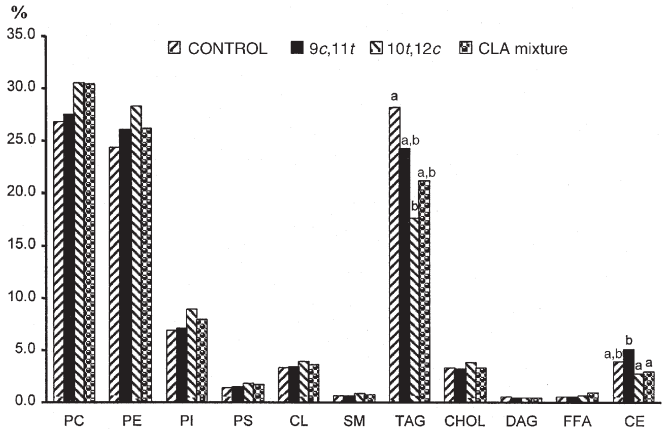


**FIG. 1.** Composition (%) of liver lipid classes. Bars having different superscripts are significantly different ($P < 0.05$, Newmann-Keuls test). Values are means of five determinations. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipid; SM, sphingomyelin; TAG, triacylglycerol; CHOL, cholesterol; DAG, diacylglycerol; FFA, free fatty acid; CE, cholesteryl ester; CLA, conjugated linoleic acid.

PL content. The three CLA treatments did not induce any changes in the partition of the different PL classes.

*Fatty acid composition of the liver lipid classes*. Figure 2 shows the major fatty acid changes induced by feeding the three CLA diets compared to the control diet. In the TAG, the levels of 18:1n-9 were lower in the animals fed CLA compared to control animals. The presence of 10*trans*,12*cis*-18:2 in the diet resulted in a significant decrease of 16:1n-7 and 18:1n-9 in the TAG of the animals of this group. The CLA mixture had a similar effect as the 10*trans*,12*cis* isomer except for 18:0, which did not change, and 16:1n-7, which increased.

In the CE, the 10*trans*,12*cis* isomer induced a decrease in 16:1n-7 and an increase in 18:0. The presence of 9*cis*,11*trans*-18:2 in the diet resulted in a decrease in 20:4n-6. The CLA mixture raised the quantities of 16:0 and 18:0.

The major changes were observed in the PL, where the intake of 10*trans*,12*cis*-18:2 decreased the amounts of 16:0, both 18:1 isomers, and 20:4n-6 but increased 18:0 and all the $C_{22}$ polyunsaturated isomers. The presence of 9*cis*,11*trans*-18:2 in the diet resulted in an increase in the 16:0 and a decrease in the 20:4n-6 contents. The CLA mixture had the same effects as the 10*trans*,12*cis*-18:2 except for most of the $C_{22}$ polyunsaturated fatty acids (PUFA).

The CLA treatment did not induce any changes in the partition of the different PL classes (Fig. 1). A significant increase in the $C_{22}$ PUFA content after the intake of 10*trans*,12*cis*-18:2 (especially for 22:5n-6 and 22:5n-3) was only observed in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Table 2). In PE, feeding the 10*trans*,12*cis* isomer or the CLA mixture resulted in a decrease of arachidonic acid.

*Conjugated fatty acids in liver lipid classes*. The highest incorporation of 9*cis*,11*trans*- and of 10*trans*,12*cis*-18:2 was observed in TAG and in CE (Table 3). Moreover, more 9*cis*,11*trans*- than 10*trans*,12*cis*-18:2 was observed in both

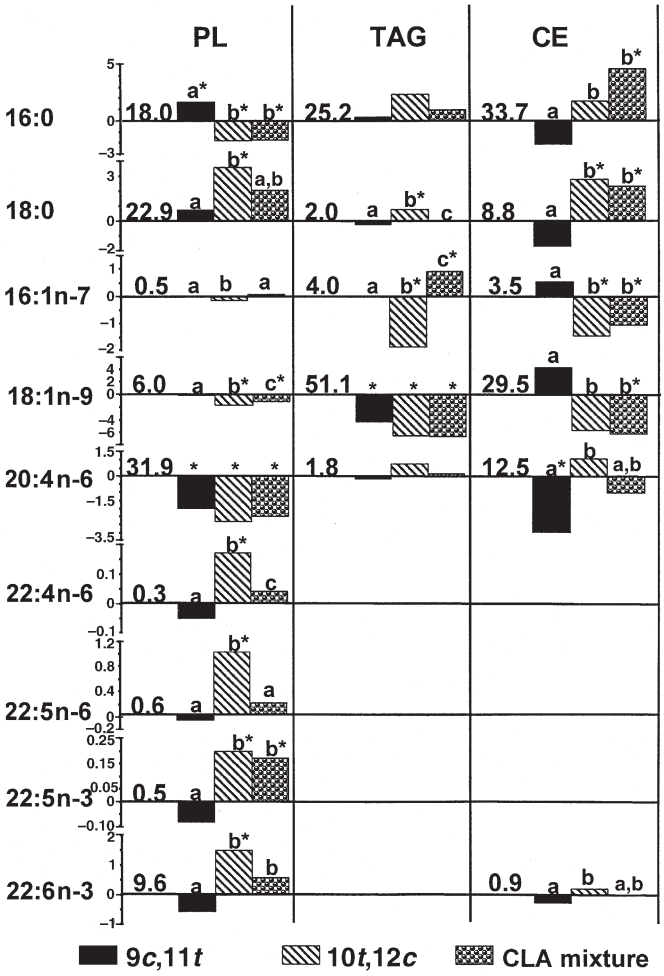


**FIG. 2.** Changes in main fatty acids compared to control in PL, TAG, and CE in liver from rats fed different CLA isomers. Bars having different superscripts are significantly different ($P < 0.05$, Newman-Keuls test). Asterisk (*) indicates statistically different from the control group ($P < 0.05$, Dunnett test). For abbreviations see Figure 1. Data reported as Δ%.

neutral lipid classes. The 18:3 and 20:3 conjugated isomers were only detected in the TAG, the main metabolite from the 10*trans*,12*cis*-18:2 being 6,10,12-18:3, whereas the 9*cis*,11*trans* isomer produced mainly the 8*cis*,11*cis*,13*trans*-20:3 conjugated isomer (see below for the identifications). The occurrence of conjugated 16:2 was only detected in the TAG from the animals fed the 10*trans*,12*cis*-18:2 diet. Detailed fatty acid analyses of the PL classes revealed that PC, PE, and cardiolipids (CL) contained appreciable amounts of CLA isomers (from 0.3 to 0.6% of the total fatty acids), whereas only trace amounts (less than 0.05%) were incorporated in phosphatidylinositol and phosphatidylserine. In PE and CL, the incorporation of the 9*cis*,11*trans* isomer was about twice that of 10*trans*,12*cis*-18:2, whereas similar quantities were found in PC (data not shown).

*Adipose tissue fatty acids*. The major fatty acids found in adipose tissue are reported in Table 4. Feeding the 10*trans*,12*cis*-18:2 isomer resulted in an increase in saturated fatty acids (14:0, 16:0, and 18:0) and 16:1n-9. The CLA mixture only increased the quantity of 14:0 and 16:1n-9. The 9*cis*,11*trans*-18:2 only decreased the quantity of 18:0. The extent of incorporation of 9*cis*,11*trans*-18:2 was twice that of the 10*trans*,12*cis* isomer. The 20:3 conjugated isomer was mainly detected in the adipose tissue of animals fed the 9*cis*,11*trans* diet and to a lesser extent in those from animals fed the CLA mixture.

*Identification of the 16:2 and 18:3 conjugated metabolites of the 10*trans,*12*cis-*18:2*. The identification of the 16:2 and 18:3 metabolites was carried out using the adipose tissue lipids of rats fed the 10*trans*,12*cis*-18:2 isomer. The fractions containing the 16:2 and the 18:3 conjugated isomers were isolated from the total lipids as reported in the Materials and Methods section. These were further converted into the MTAD adducts and into the DMOX derivatives.

The mass spectrum of the MTAD adduct with 16:2 is represented in Figure 3. There is a strong $M^+$ ion (*m/z* 379). The alkyl chain on one side of the ring ($M^+ - R_2$, *m/z* 236) and the other one on the other side of the ring ($M^+ - R_1$, *m/z* 308) permitted location of the position of the ring between C8 and C11 of the C16 chain, and therefore the location of the 8,10-dienoic system in the parent fatty acid. The two intense ions at *m/z* 348 and 276 were accounted for by the loss of a methoxy radical from the molecular ion and loss of methanol from the ion at *m/z* 308.

Similarly, the mass spectrum of the MTAD adduct of the 18:3 conjugated isomer showed a strong $M^+$ ion at *m/z* 405. Two characteristic fragments ($M^+ - R_1$ and $M^+ - R_2$) at *m/z* 236 and 334, respectively, indicated a 10,12-dienoic system (data not shown). Unfortunately, it was impossible to locate the last double bond on fragment $R_2$ using this type of derivatives. Examination of the DMOX derivative permitted us to

**TABLE 2**
**Some Polyunsaturated Fatty Acids (% of total lipids) of Liver Phosphatidylcholine and Phosphatidylethanolamine[a]**

| | Phosphatidylcholine | | | | | Phosphatidylethanolamine | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 9*c*,11*t* | 10*t*,12*c* | CLA mixture | SE | Control | 9*c*,11*t* | 10*t*,12*c* | CLA mixture | SE |
| 20:4n-6 | 27.50 | 24.43 | 26.16 | 26.69 | 1.747 | $24.63^{a}$ | $22.02^{a,b}$ | $19.03^{b}$ | $19.02^{b}$ | 1.032 |
| 22:4n-6 | 0.20 | 0.08 | 0.17 | 0.12 | 0.046 | $0.27^{a}$ | $0.19^{a}$ | $0.47^{b}$ | $0.25^{a}$ | 0.031 |
| 22:5n-6 | $0.36^{a}$ | $0.29^{a}$ | $0.89^{b}$ | $0.53^{a}$ | 0.086 | $0.64^{a}$ | $0.48^{a}$ | $1.73^{b}$ | $0.79^{a}$ | $0.131^{a}$ |
| 22:5n-3 | $0.22^{a}$ | $0.18^{a}$ | $0.3^{b}$ | $0.34^{b}$ | 0.029 | $0.67^{a}$ | $0.56^{a}$ | $1.00^{b}$ | $0.89^{a}$ | $0.068^{a}$ |
| 22:6n-3 | 4.63 | 4.34 | 5.86 | 5.46 | 0.387 | $12.36^{a,b}$ | $11.18^{b}$ | $14.93^{a}$ | $13.07^{a,b}$ | $0.706^{a}$ |

[a]Values are means of three or five determinations. For each phospholipid class, values in rows having different roman superscripts are significantly different ($P < 0.05$).

**TABLE 3**
**Incorporation (wt%) of Conjugated Fatty Acids in Liver Lipid Classes of Rats Fed Different Types of CLA[a]**

| | PL | | | | TAG | | | | CE | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9*c*,11*t* | 10*t*,12*c* | CLA mixture | SD | 9*c*,11*t* | 10*t*,12*c* | CLA mixture | SD | 9*c*,11*t* | 10*t*,12*c* | CLA mixture | SD |
| Conj. 16:2 | — | — | — | — | — | 0.10 | 0.06 | 0.011 | — | — | — | — |
| 9*c*,11*t*-18:2 | 0.40[a] | 0.02[b] | 0.24[c] | 0.015 | 3.54[a] | 0.36[b] | 2.37[c] | 0.061 | 3.69[a] | — | 1.45[b] | 0.175 |
| 10*t*,12*c*-18:2 | — | 0.34[a] | 0.20[b] | 0.014 | — | 1.18[a] | 0.59[b] | 0.086 | — | 1.18[a] | 0.69[b] | 0.033 |
| *c*,*c* conj. 18:2 | 0.03 | — | Trace | 0.007 | 0.23[a] | 0.09[b] | 0.15[c] | 0.010 | — | — | — | — |
| *t*,*t* conj. 18:2 | 0.04 | 0.08 | 0.05 | 0.014 | — | — | — | — | — | — | — | — |
| Conj. 18:3 | — | — | — | — | 0.17[a] | 0.61[b] | 0.41[c] | 0.037 | — | — | — | — |
| Conj. 20:3 | — | — | — | — | 0.23[a] | 0.04[b] | 0.12[c] | 0.009 | — | — | — | — |

[a]Values are means of five determinations. For each lipid class, values in rows having different roman superscripts are significantly different ($P < 0.05$). Conj., conjugated; PL, phospholipids; TAG, triacylglycerol; CE, cholesteryl ester.

locate the double bond in the Δ6 position. Mass spectra of the DMOX derivative of the 18:3 isomer gave an intense molecular ion at *m/z* 331, an intense ion at *m/z* 152, and ions of about equal intensity at *m/z* 166 and *m/z* 167 (data not shown). A similar type of fragmentation has been reported for unsaturated fatty acids having the first double bond in the Δ6 position (42). The other two double bonds can be located by fragments at *m/z* 208 (C9) and 220 (C10) and fragments *m/z* 234 (C11) and 246 (C12).

## DISCUSSION

Most of the studies so far published on CLA have been carried out using commercial mixtures of isomers, where the 9*cis*,11*trans*- and the 10*trans*,12*cis*-18:2 were accompanied by different amounts of the other positional and geometrical isomers. In order to know the impact of the two major isomers on lipid metabolism, the 9*cis*,11*trans*- and the 10*trans*,12*cis*-18:2 were synthesized (32,33) and administered separately to rats.

The fatty acid composition of the liver lipid classes can be modulated by the dietary intake of CLA isomers, as was already reported using a mixture of CLA (31,44). Ingesting the 10*trans*,12*cis*-18:2 resulted in an increase of 18:0 with a commensurate decrease of 18:1n-9 in the three lipid classes, suggesting an inhibition of the Δ9 desaturase as already proposed by Li and Watkins (30) when feeding a CLA mixture. However, this effect is not observed with the 9*cis*,11*trans*-18:2, which indicates that only the 10*trans*,12*cis* isomer may be effective. These *in vivo* results confirm the data of Bretillon *et al.* (45) on the Δ9 desaturation of stearic acid using rat liver microsomes. These data are also consistent with those of Lee *et al*. (46) and Park *et al.* (47).

Ingestion of the 10*trans*,12*cis*-18:2 induced a decrease of

**TABLE 4**
**Fatty Acid Composition (wt%) of Total Lipids from Adipose Tissue of Rats Fed the Control Diet or the Experimental Diets[a]**

| | Control | 9*c*,11*t* | 10*t*,12*c* | CLA mixture | SE |
|---|---|---|---|---|---|
| 16:0 | 20.13[a,b] | 19.50[b] | 24.64[c] | 21.30[b] | 0.434 |
| 16:1n-9 | 0.69[a] | 0.62[a] | 0.84[b] | 0.85[b] | 0.031 |
| 16:1n-7 | 6.02[a] | 5.55[a] | 3.32[b] | 4.29[a,b] | 0.475 |
| 18:0 | 2.28[a] | 2.02[b] | 2.99[c] | 2.36[a] | 0.074 |
| 18:1n-9 | 55.12[a] | 48.85[b] | 48.89[b] | 49.70[b] | 0.646 |
| 18:1n-7 | 3.45 | 3.50 | 3.24 | 3.34 | 0.161 |
| 18:2 isomers[b] | 0.13[a] | 0.24[a] | 0.04[b] | 0.10[b] | 0.014 |
| 18:2n-6 | 8.57 | 9.00 | 8.61 | 9.32 | 0.196 |
| 18:3n-3 | 0.70[a] | 0.72[a] | 0.60[b] | 0.71[a] | 0.027 |
| Conj. 16:2 | ND* | ND | 0.19 | 0.09 | 0.021 |
| 9*c*,11*t*-18:2 | ND | 6.49[a] | 0.40[b] | 3.16[c] | 0.093 |
| 10*t*,12*c*-18:2 | ND | ND | 3.31[a] | 1.70[b] | 0.062 |
| 9*c*,11*c*-18:2 | ND | 0.33[a] | ND | 0.09[b] | 0.017 |
| 10*c*,12*c*-18:2 | ND | ND | 0.04[a] | 0.05[b] | 0.016 |
| Conj. 18:3 | ND | ND | 0.23 | 0.18 | 0.020 |
| *tt* Conj. 18:2 | ND | 0.21[a] | 0.06[b] | 0.10[b] | 0.017 |
| 20:1n-7 + n-9 | 0.27[a] | 0.32[a,b] | 0.51[c] | 0.39[b] | 0.028 |
| 20:3n-9 | 0.06[a] | 0.11[b] | ND | ND | 0.016 |
| 20:4n-6 | 0.20 | 0.24 | 0.11 | 0.18 | 0.037 |
| Conj. 20:3 | ND | 0.33[a] | ND | 0.07[b] | 0.019 |

[a]Values are means of five determinations. ND, not detected under our experimental conditions; SE, standard error. For other abbreviations see Table 1.
[b]Nonconjugated isomers. Values in rows having different roman superscripts are significantly different ($P < 0.05$).

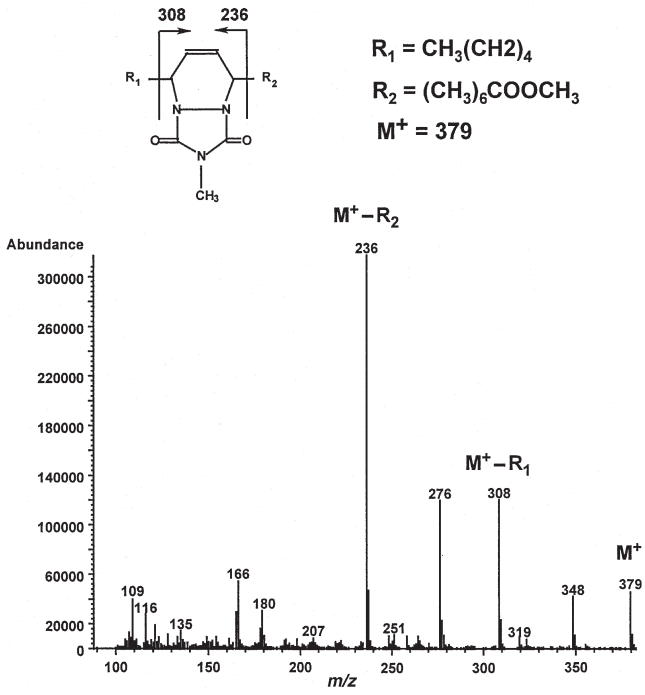


**FIG. 3.** Gas chromatographic–mass spectrometric spectrum of the 4-methyl-1,2,4-triazoline-3,5-dione derivative of 8,10-16:2.

38% in the liver content of TAG compared to the control group, while a small but nonsignificant decrease was observed for the animals fed the 9*cis*,11*trans* isomer. The CLA mixture resulted in a decrease of 24% in TAG compared to the control group (Fig. 1). As the consumption of the 10*trans*,12*cis* isomer in the group fed the CLA mixture was half of that fed the pure isomer, it is obvious that the 10*trans*,12*cis* isomer is responsible for the decrease in the hepatic TAG content. However, this decrease could be due to either an increase in the secretion of TAG by the liver or a decrease in the TAG synthesis or both. The results presented here as well as *in vitro* data (45–47) indicate that the 10*trans*,12*cis* CLA isomer is able to decrease the Δ9 desaturase activity and mRNA expression. As this enzyme activity has been correlated to the TAG secretion in hepatocytes (48), one may speculate that this could be an explanation of our results.

Major changes were also observed when looking at the n-6 and n-3 long-chain PUFA. The 9*cis*,11*trans*-18:2 only decreased the arachidonic acid content in liver PL. The same effect was observed when feeding the 10*trans*,12*cis*-18:2 or the CLA mixture, but this decrease was balanced by an increase in the $C_{22}$ PUFA. This effect has been recently described (30) as a result of feeding a CLA mixture to rats. Again, 10*trans*,12*cis*-18:2 seems to be the isomer mainly responsible for the increase in long-chain PUFA, which could be due to stimulation of the peroxisomal fatty acid metabolism. Such an effect was not reported by Belury and Kempa-Steczko (31), who studied the effect of different levels of CLA added to a corn oil diet at the expense of dextrose. These modifications of the content in PUFA must be further studied to appreciate the impact of such alteration on eicosanoid synthesis.

The selectivity of incorporation of CLA isomers, which was already observed in total neutral lipids (31), has been found in both TAG and CE (Table 4). Interestingly, both the 9*cis*,11*trans* and the 10*trans*,12*cis* isomers are converted in long-chain metabolites, as previously reported (40). However, the present study using pure isomers has shown that the 9*cis*,11*trans* is converted in 18:3 and in 20:3 conjugated fatty acids, the latter being previously identified as the 8,11,13-20:3 (43). Injection of a synthesized 20:3 molecule on both $AgNO_3$-HPLC and GC revealed that this metabolite is the 8*cis*,11*cis*,13*trans* isomer (Berdeaux, O., personal communication). The 10*trans*,12*cis*-18:2 isomer is mainly converted into an 18:3 having double bonds in Δ6,10,12, whereas only smaller quantities of the conjugated 20:3 have been detected. The structure of this 20:3 was previously established as being an 8,12,14 isomer. Further work will have to be carried out to fully identify the 18:3 metabolite of the 9*cis*,11*trans* isomer, which is present only in small amount in liver TAG. Furthermore, the 10*trans*,12*cis* isomer can also be converted into a conjugated 16:2 fatty acid having the ethylenic position in Δ8 and Δ10. Similar peroxisomal metabolic pathways have already been described by Luthria and Sprecher (49) for linoleic acid, which was transformed into a 7,10-16:2 and 5,8-14:2. This 16:2 conjugated isomer was only found in liver lipids from animals fed the 10*trans*,12*cis*-18:2. This may suggest that the 9*cis*,11*trans* and the 10*trans*,12*cis* are metabolized differently through the peroxisomal β oxidation pathway.

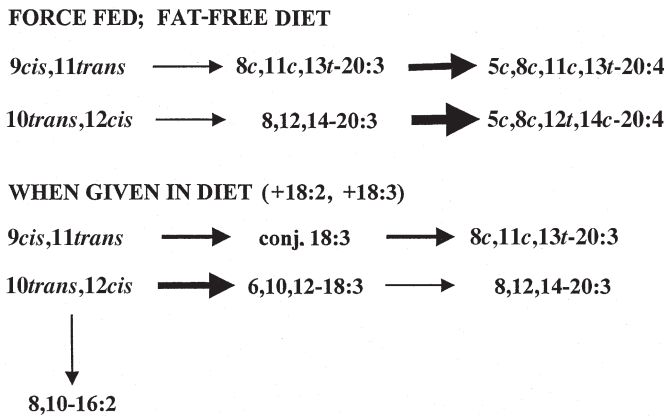


**FIG. 4.** Metabolism of the 9*cis*,11*trans*- and 10*trans*,12*cis*-18:2 isomers by fat-free-fed rats after force-feeding (40) and when given in an equilibrated semisynthetic diet for 6 wk (this study). The thickness of the arrows suggests the importance of the metabolic pathway.

All these results differ from our previous study (40) in which we force-fed CLA to rats consuming a fat-free diet (Fig. 4), where the two 20:4 conjugated isomers were the major metabolites of the 9*cis*,11*trans*- and 10*trans*,12*cis*-18:2 (50). In the present study, the animals received an equilibrated diet. Consequently, the biosynthesis of conjugated 20:4 isomer may be low, as already suggested by Banni *et al.* (41), who detected only the 20:3 conjugated metabolites from CLA in rats. Another possibility is that 20:4 may be formed and rapidly converted. Interestingly, the levels of metabolites from the 10*trans*,12*cis* CLA isomer indicate that its conversion is higher than that of the 9*cis*,11*trans* isomer. Synthesis of the labeled metabolites will permit study of the impact the 18:3 and 20:3 isomers may have on lipid metabolism.

## ACKNOWLEDGMENTS

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# A Single Oral Administration of Conjugated Linoleic Acid Enhanced Energy Metabolism in Mice

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**ABSTRACT:** We investigated the effect of a single oral administration of conjugated linoleic acid (CLA) on energy metabolism in mice. Male Std ddY mice were orally administered CLA (5 mL/kg weight) or linoleic acid (5 mL/kg weight) (both solutions at concentrations of 73.5%) as a control. Oxygen consumption was significantly greater in the CLA-administered mice than in the control mice. Respiratory quotient was slightly lower in the CLA-administered mice than in the control mice. We calculated fat and carbohydrate oxidation from oxygen consumption and respiratory quotient. Fat oxidation in the CLA-administered mice was significantly higher than in the control mice, and there was no difference in carbohydrate oxidation. Serum concentrations of noradrenalin and adrenalin in the CLA administered mice were significantly higher than in the control mice. These results suggested that CLA enhanced sympathetic nervous activity and energy metabolism.

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Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers that are derived from linoleic acid. It is found in foods such as ruminant meats, pasteurized dairy products, and processed cheeses. The major dietary source of CLA for humans is beef or dairy products; beef tallow contains ~0.5% fatty acids as CLA (1). Interest in CLA is increasing because it has been found to have protective properties against cancer (2–8) and atherosclerosis (9,10).

Intake of CLA for several weeks has been reported to reduce body fat content. Adipose tissue of mice given a CLA-supplemented diet (0.5–1.5% CLA) weighed less (11–13). In addition, energy metabolism was higher in mice fed a CLA-supplemented diet than in mice fed a control diet for 6 wk (13).

We assumed that CLA promotes body fat consumption and followed the effect of CLA for 3 h after administration. We gave a diet supplemented with either CLA or linoleic acid (LA) to mice *ad libitum* in a preliminary study. With this method we were not able to evaluate the change in respiratory gas or body temperature after feeding, because the amount of food intake in the CLA group was markedly lower than in the LA group, and oxygen consumption and body temperature were similarly lower. Therefore, the mice were administered CLA *via* a stomach tube in the present study. We now report the effect of a single oral administration of CLA on oxygen consumption, body temperature, and blood components.

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Abbreviations: CLA, conjugated linoleic acid; FFA, free fatty acid; HPLC, high-performance liquid chromatography; LA, linoleic acid; PPAR, peroxisome proliferator-activated receptor.

## MATERIALS AND METHODS

*Animals.* Five-week-old male Std ddY mice (mean body weight 25.5 ± 0.3 g; Japan Shizuoka Laboratory Center, Hamamatsu, Japan) were used. They were housed in standard cages (33 × 23 × 12 cm) under controlled conditions of temperature (22 ± 0.5°C), relative humidity (50%), and lighting (lights on from 1800 to 0600). Mice had free access to water and a stock diet (type MF; Oriental Yeast, Tokyo, Japan) containing (g/kg diet): water, 70; protein, 240; fat, 51; fiber, 32; carbohydrates, 545. The mice were allowed to adapt to the laboratory housing for at least 1 wk before respiratory gas measurement, surgery for telemetry transmitter implant, or blood sampling. The care and treatment of the experimental animals conformed to the guidelines of Kyoto University for the ethical treatment of laboratory animals.

*Materials.* We used unesterified safflower LA as a control and synthesized CLA from unesterified safflower LA. These two samples were synthesized from safflower oil by Linoleic Oils and Fats Co., Ltd. (Tokyo, Japan). The composition of samples was 17.4% palmitic acid, 2.3% stearic acid, 6.5% oleic acid, and 73.5% LA or CLA. The composition of CLA was 34.0% *cis*-9,*trans*-11 and *trans*-9,*cis*-11 CLA; 35.1% *trans*-10,*cis*-12 CLA; 2.5% *cis*-9,*cis*-11 and *cis*-10,*cis*-12 CLA; and 1.9% *trans*-9,*trans*-11 and *trans*-10,*trans*-12 CLA.

*CLA administration.* To avoid circadian variations in physical activity, experiments were carried out from 1100 to 1600, in the dark period when the mice were eating or moving most of the time. One group was administered LA and the other was administered CLA, both in compositions of 73.5% (5 mL/kg body weight) *via* a stomach tube, so the amount of administered LA or CLA was about 200 mg. Body temperature, activity, oxygen consumption, and respiratory quotient were measured when mice were sedentary (baseline values), and measurement was begun after oral administration. The blood samples was taken from mice at 1, 2, and 3 h after oral administration of LA or CLA.

*Respiratory gas analysis.* The instruments (all from Alco System, Tiba, Japan) used for the measurement of oxygen consumption and respiratory quotient in the mice consisted of six acrylic metabolic chambers, gas analyzers (model RL-600), and a switching system (model ANI6-A-S) to sample gas from each metabolic chamber. Mice ($n$ = 11) were separated into two groups of equal body weights; each mouse was placed into a metabolic chamber designed to measure respiratory gas. The details of methods were described in a previous report (14). Briefly, room air was pumped through the chambers and expired

air was dried in a thin cotton column and then directed to a gas analyzer. The amount of fat and carbohydrate oxidized were calculated from the value of oxygen consumption and respiratory quotient, using software for analysis. The data for each chamber were obtained every 7 min and stored on a spreadsheet. The instruments and software were obtained from Alco System (Tiba, Japan).

*Body temperature and activity.* Body temperature and activity were measured by a telemetry system as described elsewhere (15,16). Briefly, a telemetry transmitter was implanted in the peritoneal cavity of each mouse ($n$ = 8), and the mice were subsequently used for experiments for at least 1 wk. The mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, 1 mg/kg). A 1.5- to 2-cm incision was made in the skin along the midline immediately caudal to the abdominal space. Subsequently, the abdominal wall was opened, and the transmitter was implanted in the peritoneal cavity. The instruments for the telemetry system were purchased from Data Science Inc. (St. Paul, MN).

*Serum components.* Mice ($n$ = 96) were divided into two groups so that the mean body weights were equal in both groups. Blood was taken from the heart of mice at 1-h intervals for 3 h after administration of CLA or control. Each mouse was used only once. Serum was obtained by centrifugation and stored at −20°C until measurement. Serum free fatty acids (FFA) were determined by the acyl CoA-synthetase and acyl CoA oxidase enzyme method with a commercial kit (NEFA C-Test; Wako Pure Chemical Industries, Kyoto, Japan). Glucose was assayed by a combination of mutase and glucose oxidase with a commercial kit (Glucose CII Test; Wako). Triglycerides were assayed with a commercial kit (Triglyceride G Test; Wako). Noradrenalin and adrenalin were assayed by a high-performance liquid chromatography (HPLC)-electrochemical detector.

*Statistical analysis.* Data are expressed as means ± SE. The effects of time, treatment, and time × treatment were evaluated by two-way repeated measures analysis of variance (ANOVA); for comparison between two groups at certain time points, Student's $t$ test was used. Statistics were calculated with the Stat View software package (Macintosh Version J 5.0; Abacus Concepts, Berkeley, CA). Probability levels of $<0.05$ were considered to indicate significance.

## RESULTS

Oxygen consumption and respiratory quotient obtained with the gas analyzer, and the amounts of fat and carbohydrates oxidized, which were calculated from these values, are shown

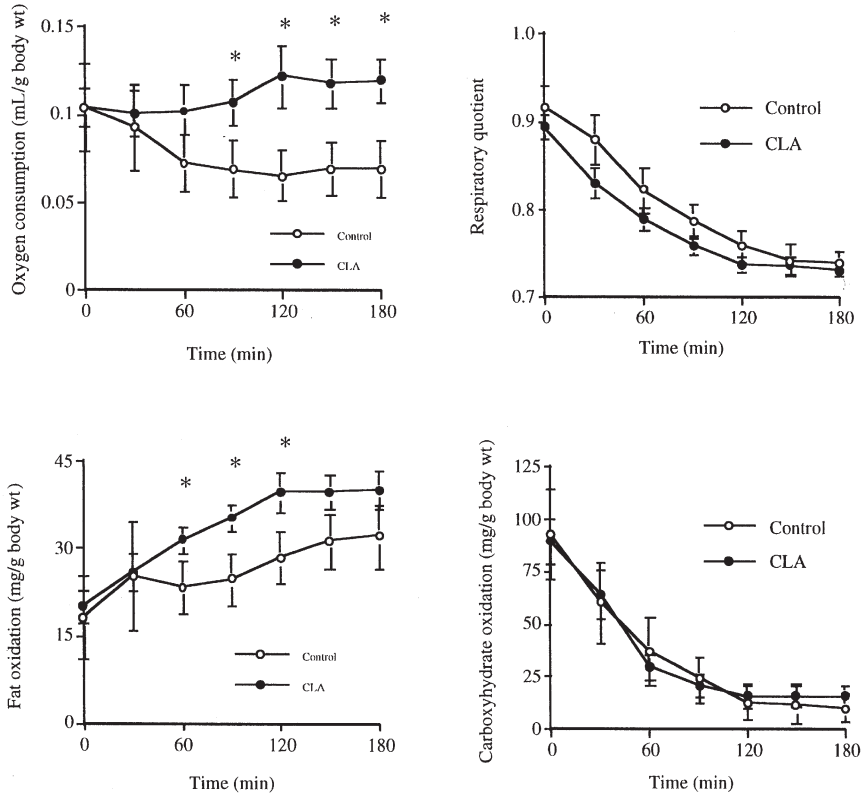


**FIG. 1.** Oxygen consumption (upper left), respiratory quotient (upper right), fat oxidation (lower left) and carboxyhydrate oxidation (lower right) in the mice administered conjugated linoleic acid (CLA) and linoleic acid (control). Values are means ± SEM ($n$ = 5 or 6) Oxygen consumption and fat oxidation were significantly higher in the CLA group than in the control group (time × treatment effect, $P < 0.05$ by two-way repeated measures analysis of variance). *Significantly different from control group ($P < 0.05$ by Student's $t$ test).

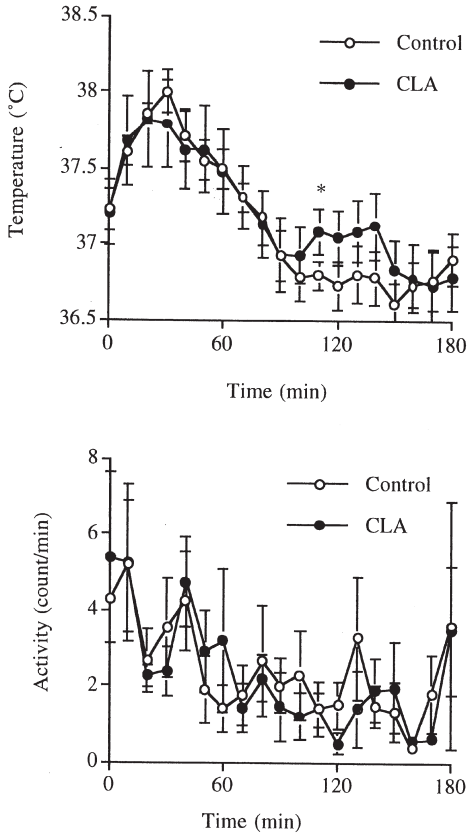


**FIG. 2.** Body temperature (upper panel) and locomotor activity (lower panel) of mice administered CLA or linoleic acid (control). Values are means ± SEM ($n$ = 6–8). *Significantly different from control group ($P$ < 0.05 by Student's $t$ test). For abbreviation see Figure 1.

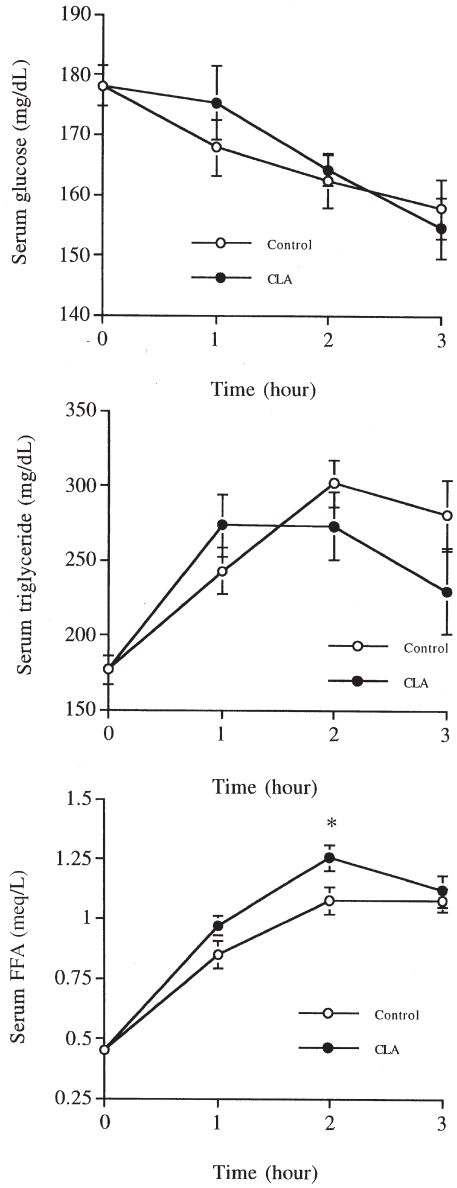


**FIG. 3.** The concentrations of serum glucose (top), serum triglyceride (middle), and serum free fatty acids (FFA) (bottom) in mice administered CLA and linoleic acid (control). Values are means ± SEM ($n$ = 7–15). The concentration of serum FFA was significantly higher in the CLA group than in the control group (time × treatment effect, $P$ < 0.05 by two-way repeated measures analysis of variance). *Significantly different from control group ($P$ < 0.05 by Student's $t$ test). For abbreviation see Figure 1.

in Figure 1. Oxygen consumption was significantly higher from 90–180 min after the administration of CLA as compared with the control group, and the fat energy consumption was significantly higher from 60 to 120 min after administration of CLA. The respiratory quotient in the CLA group was slightly lower than in the control group at 30 and 90 min after administration ($P$ < 0.1), and there was no difference between CLA and control at the baseline value and 3 h after administration ($P$ > 0.9).

Body temperature and activity after administration of CLA were measured using a telemetry system (Fig. 2). Body temperature tended to be higher 2 h after the administration of CLA, and was significantly different from the control group at 110 min.

Figure 3 shows the change of serum FFA, glucose, and triglyceride assayed with commercial kits after the administration of CLA or control. There was no difference between the two groups in the amounts of blood glucose and triglycerides. Serum FFA concentration tended to be increased, and was significantly higher 2 h after CLA administration in mice compared with the control group.

Figure 4 shows the changes in serum noradrenalin and adrenalin concentrations as assayed with an HPLC-electrochemical detector after the administration of CLA or control. The concentration of noradrenalin was significantly higher in the mice administered CLA compared to the control group. The concentration of serum adrenalin tended to increase after the administration of CLA and was significantly different from the control group at 2 h after the administration.

## DISCUSSION

CLA has been reported to suppress body fat accumulation in mice *via* enhancement of body fat metabolism (11,13). How-

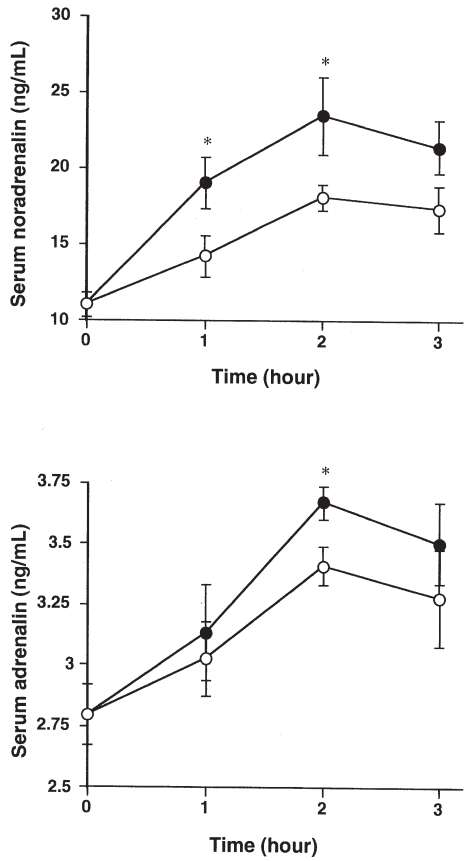


**FIG. 4.** The concentrations of serum noradrenalin (upper panel) and adrenalin (lower panel) of mice administered CLA or linoleic acid (control). Values are means ± SEM ($n$ = 6–8). The concentrations of serum noradrenalin and adrenalin were significantly higher in the CLA group than in the control group (time × treatment effect, $P < 0.05$ by two-way repeated measures analysis of variance). *Significantly different from control group ($P < 0.05$ by Student's $t$ test). For abbreviation see Figure 1.

ever, the mechanisms underlying the stimulation of body fat oxidation have not been elucidated.

The higher oxygen consumption of mice administered CLA *via* a stomach tube was consistent with the elevation of the body temperature. The energy source of this excess oxygen consumption was attributable to fat oxidation, as the results on respiratory quotients in Figure 1 (upper right panel) indicate. There was no difference in carbohydrate oxidation between the CLA and the control groups throughout the experimental period. Analyses and the measurement of sampled gases were carried out in a separate room to avoid exciting the mice.

Throughout the measurement of body temperature, locomotor activity of both groups of mice showed no difference, suggesting that the increment of body temperature was due not to excessive locomotion but to metabolic heat generation. The measurement of the body temperature was carried out in a separate room by using a temperature information telemetry system that was noninvasive and that caused less stress on the experimental animals.

The mechanisms for increased fat oxidation by CLA have been discussed in other reports. Park *et al.* (11) found that carnitine palmitoyltransferase, which catalyzes a rate-limiting process of β-oxidation of fatty acids in mitochondoria, was increased; and lipoprotein lipase activity in the adipose tissue was decreased. West *et al.* (13) reported that energy expenditure was significantly increased after 6 wk of CLA feeding. However, their studies were not intended to explain the cause of the excess metabolic rate associated with CLA feeding. Accumulation of the rapid increases in oxygen consumption and fat oxidation found in the present single administration of CLA would explain the consequent chronic increase in fatty acid β-oxidation and fat utilization reported by Park *et al.* (11) and West *et al.* (13).

In addition, we cannot rule out the possibility that the free CLA directly stimulated body fat oxidation. Belury *et al.* (17) reported that CLA administration activated peroxisome proliferator-activated receptor (PPAR)-α in the liver in mice. The induction of PPAR-α, which plays an important role in fat metabolism (18–20), may be a stimulus for increased fat oxidation by CLA. PPAR regulates acyl-CoA oxidase, cytochrome P4504A1, and liver fatty acid-binding protein. This may activate body fat oxidation. On the other hand, Hida *et al.* (19) reported that fish oil induces PPAR-γ in the organs and induces uncoupling proteins in brown adipose tissue, which causes enhanced energy expenditure by heat generation. Further studies are needed on CLA as a ligand for master regulators like PPAR.

Overall, CLA is expected to be useful in dietary therapy to prevent obesity by increasing energy expenditure. The present study demonstrates that the administration of CLA induces catecholamines, and it enhances not only use of fatty acids as a fuel but also directly stimulates fatty acid utilization *via* heat generation. It should be noted that the CLA used in this study was not pure, and it is possible that the different isomers have different metabolic effects. Further studies using pure reagents are needed to fully understand the metabolic specificity of these different isomers of CLA.

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# Effect of Dietary Restriction on Age-Related Increase of Liver Susceptibility to Peroxidation in Rats

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**ABSTRACT:** Dietary restriction (DR) increases life span and decreases age-related diseases in experimental animals. It has received a great deal of attention in connection with the relationship between aging, nutrition, and oxidative stress because oxidative injury in several organ systems is a prominent feature in aging. We investigated the possibility that DR can protect vulnerable liver lipids against age-related increases of peroxidation. Male Fischer 344 rats fed *ad libitum* (AL) or dietarily restricted (maintained on 60% of AL food intake) were killed by decapitation at 4 (young) or 12 mon (adult) of age. Phosphatidylcholine hydroperoxide (PCOOH) concentration of liver was determined using a chemiluminescent high-performance liquid chromatographic method. Liver PCOOH increased with age in adult rats, but less of an increase of PCOOH was seen in DR rats, which is consistent with results on production of thiobarbituric acid-reactive substances and oxygen-derived free radicals. No significant differences were found in liver superoxide dismutase and catalase activity between AL and DR groups of young and adult rats. Liver triglyceride and cholesterol contents were lower in DR than AL rats at 12 mon. Fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine indicated that the ratio of (20:3n-6 + 20:4n-6)/18:2n-6, an index of linoleic acid (18:2n-6) desaturation, was lower in DR than in AL rats. We concluded that DR suppresses age-related oxidative damage in liver by modulating the amount of lipid as well as fatty acid composition.

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Aging can be characterized as an accumulation of deleterious changes that increase the risk of death. These changes can be attributed to both genetic and environmental factors (1), and they inevitably compromise an organism's ability to meet both internal and external challenges. Oxidative stress is causally related to irreversible damage due to endogenously generated free radicals (2,3). Under normal conditions, cells of aerobic organisms utilize reactive oxygen species (ROS) as physiological messengers. The redox balance is altered by the age-related decline in an organism's ability to counteract oxidative damage (4,5).

Reducing the caloric intake of laboratory animals while maintaining nutrition can increase their lifespans (6–8). Dietary restriction (DR) delays age-associated pathological and physiological changes and extends longevity. DR postpones the accumulation of damaging effects that accompany *ad libitum* (AL) food intake. One major mechanism by which DR retards the aging process is its remarkable ability to reduce oxidative damage (5,9). For example, Kim *et al*. (10) showed that DR decreases the malondialdehyde content of cardiac mitochondria, indicating a decrease in lipid peroxidation. DR feeding regimens enhanced the organism's ability to attenuate levels of harmful reactive free radicals in various organs (11,12). Oxidative stress-mediated injury is causally related to aging since ROS-caused damage accumulates with age (13,14). Although several previous studies reported a relationship between aging and lipid peroxidation, most examined thiobarbituric acid-reactive substance (TBARS) levels to indicate peroxidative status.

In the present study, we determined phospholipid hydroperoxide (PLOOH), a sensitive key indicator for oxidative injury, because phospholipids (PL) are important structural and functional components of the biological system (15,16), and are commonly recognized as a major target of lipid peroxidation. The technique we used to determine PLOOH was a chemiluminescence–high-performance liquid chromatography (CL-HPLC) method. Previous reports from this laboratory described a simple and continuous one-step flow injection system based on cytochrome c-amplified chemiluminescence for the assay of radical scavenging activity (17,18). The present study attempts to quantify age-associated changes of the lipid profile and the degree of attenuation on oxidative damage in DR.

The liver is an important metabolic organ, and is susceptible to a wide variety of disorders, possibly because it is constantly exposed to potentially harmful agents. It is generally recognized that oxidative end-products accumulate with age and therefore free radical-mediated damage to liver cells occurs. Thus, the liver was selected as a model organ for this study in recognition of the significant health benefits of DR.

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Abbreviations: AL, *ad libitum*; CL-HPLC, chemiluminescence-high-performance liquid chromatography; DR, dietary restriction; FI-CL, flow injection-chemiluminescence; FID, flame-ionization detector; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; PE, phosphatidylethanolamine; PL, phospholipid; PLOOH, phospholipid hydroperoxide; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SOD, superoxide dismutase; SPF, specific-pathogen-free; TBARS, thiobarbituric acid-reactive substances; TC, total cholesterol; TG, triglyceride.

## EXPERIMENTAL PROCEDURES

*Animals*. Specific-pathogen-free (SPF) male Fisher 344 rats were housed in plastic cages at the University of Texas Health Science Center with approval of the Institutional Animal Care and Utilization Committee. The SPF status of shipments of rats was verified and maintained as described by Yu *et al*. (19). Dietary restriction (60% of *ad libitum* fed) was begun at 6 wk of age as described by Yu *et al*. (20), and continued throughout life. Rats were weighed biweekly, and were killed by decapitation at 4 or 12 mon of age. The required tissues were then removed.

*Antioxidant activities measurement by the flow injection (FI)-CL system*. To determine changes in antioxidant activity in liver CL intensity was measured with a filter-equipped photon counting-type spectrophotometer (CLD-110; Tohoku Electronic Industry, Miyagi, Japan) connected to a pump (model 303; Gilson Medical Electronics S.A., Villiers-le-Bel, France) and a sample injection valve (model 7125; Rheodyne, Cotati, CA). The mobile phase was 50 mM phosphate buffer (pH 7.4) containing 50% methanol (for solvent-soluble samples), cytochrome c (10 mg/L), and luminol (2 mg/L). The flow rate was maintained at 1.0 mL/min with the pump. For the purpose of measuring the abilities of radical scavengers, a mixture of 0.06% $H_2O_2$ (5 μL) and sample solution (5 μL) was injected. The reduced CL intensity of the mixture compared to the CL intensity of 0.06% $H_2O_2$ (5 μL) enables analysis of radical-scavenging activity (17).

*Phosphatidylcholine hydroperoxides (PCOOH) measurement*. Total lipids were extracted from the liver by the method of Folch *et al*. (21). The CL-HPLC procedure for quantification of PCOOH concentration followed the method of Miyazawa *et al*. (15). Standard PCOOH was prepared by oxidation of PC using a method of Terao *et al*. (22).

*TBARS measurements*. TBARS were quantified as described by Buege and Aust (23) using 1,1,3,3,-tetraethoxypropane as a standard material (Sigma, St. Louis, MO).

*Antioxidant enzyme activities measurements*. Liver catalase activity was determined spectrophotometrically by observing the decomposition of hydrogen peroxide at 240 nm. One catalase unit is defined as the amount of enzyme required to decompose 1.0 μmol $H_2O_2$/min at pH 7.0 at 25°C (24,25). Total superoxide dismutase (SOD) activity was determined by monitoring the inhibition of reduction cytochrome c at 550 nm using xanthine and xanthine oxidase system. One SOD unit was defined as the amount of enzyme that inhibited reduction of cytochrome c by 50% (26).

*Analysis of lipids*. Liver total lipids were extracted and purified by the method of Folch *et al*. (21). PL, total cholesterol (TC), and triglyceride (TG) were measured as described by Gu *et al*. (27). PC and phosphatidylethanolamine (PE) were separated by thin-layer chromatography with chloroform/ethanol/water/triethylamine (30:35:6:35, by vol) as the developing solvent (28). The fatty acid composition of PC and PE were analyzed by gas–liquid chromatography (Varian, Palo Alto, CA) in an Omegawax 320 capillary column (30 m × 0.32 mm i.d., 0.25 μm film, Supelco, Bellefonte, PA) equipped with a flame-ionization detector (FID) after direct transmethylation with 14% $BF_3$ in methanol at 70°C (28). Chromatographic conditions were as follows: column temperature 200°C; carrier gas $N_2$ 30 mL/min; FID temperature 260°C; injector temperature 250°C. Fatty acid methyl esters were identified by comparing their retention times with those of standard methyl esters (Supelco).

*Statistics*. Differences between the means of the individual groups were assessed by one-way analysis of variance with Duncan's multiple range test (SPSS version 7.5, SPSS Institute, Chicago, IL). Differences of $P < 0.05$ were considered to be significant (29).

## RESULTS

DR decreased body weight of each group. DR rats had body weights lower than those of AL rats by 59.7 and 55.7% at 4 and 12 mon, respectively.

To determine the effect of DR on lipid peroxidation, PCOOH (as a representative PLOOH), TBARS production, and active oxygen-derived radical levels were examined in AL or DR rats at 4 and 12 mon. The liver peroxidation indices increased with age in AL rats. PCOOH and TBARS concentrations in livers of AL rats were 3 and 1.4 times higher in the 12-mon group than in the 4-mon group (top and middle panels, Fig. 1). Consistent with these results, the radical activity evaluated by FI-CL assay was significantly higher in the 12-mon group than in the 4-mon group ($P < 0.05$; bottom panel, Fig. 1). However, DR was associated with much less peroxidation in the 12-mon rats. Concentrations of PCOOH or TBARS in adult rat livers were 50 and 76% less, respectively, in the DR rats as compared to those of AL rats (top and middle panels, Fig. 1).

No significant differences were found in catalase and SOD activity between AL and DR groups (Fig. 2). These results demonstrate that lipid peroxidation products increase with age, while DR attenuates the extent of free radical damage.

We further analyzed lipid contents in each group as shown in Figure 3. The TC and TG concentrations of AL rats increased with age of rats (top and middle panels, Fig. 3). The TC and TG concentrations were significantly lower in the DR group than the AL group at 12 mon ($P < 0.05$). No significant difference was found in the PL concentration among all groups (bottom panels, Fig. 3).

The constituent fatty acids in PC and PE are indicated in Table 1. At 4 mon, PC showed an increasing trend of 18:2n-6 (linoleic acid). At 12 mon, a decreasing trend of 20:4n-6 (arachidonic acid) in DR rats was evident when compared to that of AL rats. Consequently, the ratio of (20:3n-6 + 20:4n-6)/18:2n-6, an index of linoleic acid desaturation, was lower in the DR group, and the proportion of 22:5n-6 in the DR group at 12 mon also decreased. A similar, but lesser degree of difference was observed in PE and there was a nonsignificant trend toward decreasing 22:6n-3 in the DR rats.

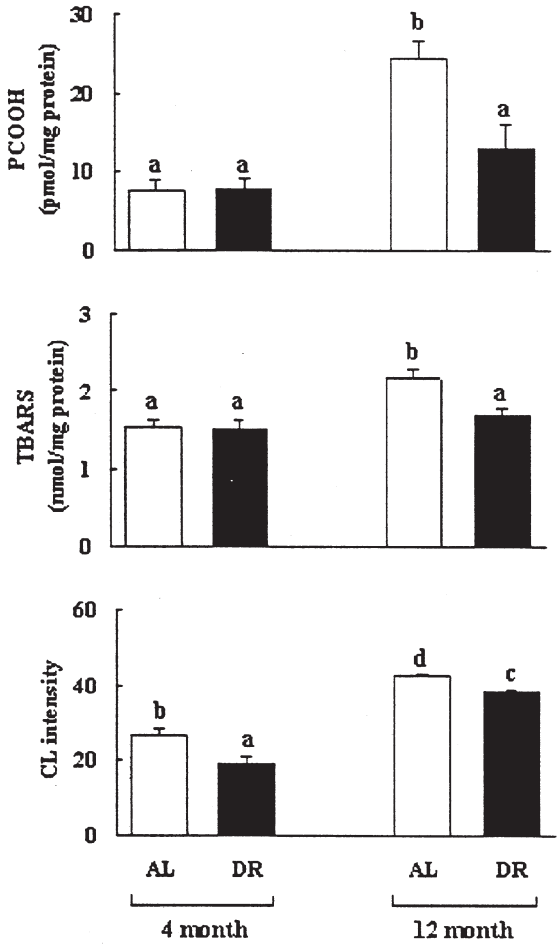


**FIG. 1.** Effects of dietary restriction (DR) on the amount of phosphatidylcholine hydroperoxide (PCOOH; top panel), thiobarbituric acid-reactive substances (TBARS; middle panel), and oxygen-derived radical activity (bottom panel) in the liver of rats between 4 and 12 mon. Each bar represents the mean ± SEM of five rats. Mean values with different superscripts are significantly different ($P < 0.05$). CL, chemiluminescent; AL, *ad libitum*-fed rats.

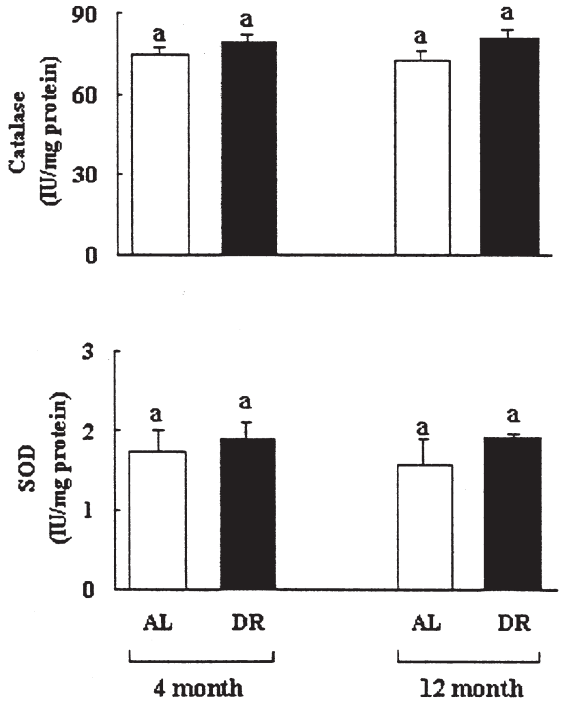


**FIG. 2.** Effects of DR on the cytosolic catalase activity (top panel) and superoxide dismutase (SOD) activity (bottom panel) in the liver of rats between 4 and 12 mon. Each bar represents the mean ± SEM of five rats. Mean values with different superscripts are significantly different ($P < 0.05$). For other abbreviations see Figure 1.

## DISCUSSION

In this study we demonstrated that indices of hepatic oxidative stress increased with age and were less prominent in DR rats. The index of linoleic acid desaturation and unsaturation degree of fatty acids in PC and PE was significantly reduced by DR. A number of investigators have previously examined the effect of DR with age on lipid peroxide concentrations, mostly using TBARS (13,30). They suggested that fatty acid unsaturation is a main factor in determining the sensitivity to lipid peroxidation. To evaluate the effect of DR on oxidative stress, we measured PCOOH as a sensitive marker of oxidative liver injury because of PC's vulnerability to peroxidation (31).

PLOOH was shown to increase significantly with age in AL rats (top panel, Fig. 1). Moreover, polyunsaturated fatty acid (PUFA) contents of 20:4n-6, 22:5n-6, and 22:6n-3 increased in 12-mon AL rats (Table 1) over those present at 4 mon (Table 2), implying an age-related increase of PUFA susceptibility to peroxidation. However, major PUFA amounts were markedly lowered by DR in adult rats, with lower TBARS and PCOOH levels than in AL rats.

The present study found that catalase and SOD activities were not significantly different between DR and AL rats (Fig. 2). Catalase and SOD activities at basal levels were not statistically different between the young and the adult groups. Interestingly, DR led to significant changes in cholesterol and TG levels, indicating that DR primarily modulates the reduction of neutral lipids, not PL (Fig. 3).

To further explore the notion that tissue lipid vulnerability is a crucial factor contributing to cellular oxidative status, constituent fatty acids in PC and PE were analyzed. The trends in fatty acid composition to increase toward peroxidizable PUFA with age are evident (Table 1). Among the most obvious modifications by DR have been the compositional changes related to membrane lipid composition, specifically the age-related membrane fatty acid composition (32). It has been proposed that the modulation of the fatty acid profile by DR results in lower age-related oxidative stress as a possible adaptive strategy (9,11,14). In our study, 18:2n-6 and 18:3n-3 increased in PE of DR rats, whereas the content of PUFA derivatives (20:4n-6, 22:5n-6, and 22:6n-3) is decreased (Table 2).

We conclude that DR protects liver against age-related increases in oxidative stress *in vivo* by modifying lipids and their composition to reduce peroxidizable substrates such as TG and PUFA. Our study strongly supports earlier data

**TABLE 1**
**Effect of Dietary Restriction (DR) Compared with *ad Libitum* (AL) Feeding on the Fatty Acid Composition of Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) in the Liver of Rats Between 4 and 12 mon[a]**

| Fatty acids | PC | | | | PE | | | |
|---|---|---|---|---|---|---|---|---|
| | 4 mon | | 12 mon | | 4 mon | | 12 mon | |
| (weight %) | AL | DR | AL | DR | AL | DR | AL | DR |
| 14:0 | 0.2 ± 0.0[a] | 0.2 ± 0.0[a] | 0.1 ± 0.0[a] | 0.1 ± 0.0[a] | 0.2 ± 0.0[a] | 0.3 ± 0.1[a] | 0.6 ± 0.4[b] | 0.2 ± 0.1[a] |
| 16:0 | 15.4 ± 0.4[b] | 16.3 ± 0.9[b] | 13.2 ± 0.4[a] | 13.2 ± 1.1[a] | 14.7 ± 0.6[a] | 17.6 ± 1.4[b] | 12.3 ± 1.6[a] | 13.2 ± 0.9[a] |
| 16:1 | 0.9 ± 0.0[c] | 1.1 ± 0.1[c] | 0.5 ± 0.0[b] | 0.3 ± 0.0[a] | 0.9 ± 0.1[b] | 0.9 ± 0.2[b] | 0.5 ± 0.2[a] | 0.4 ± 0.1[a] |
| 18:0 | 25.1 ± 0.8[a] | 24.4 ± 1.5[a] | 27.6 ± 5.1[b] | 24.2 ± 2.1[a] | 22.7 ± 0.5[a] | 23.0 ± 1.0[a] | 25.1 ± 1.4[b] | 22.7 ± 0.7[a] |
| 18:1n-9 | 2.7 ± 0.1[b] | 3.6 ± 0.3[c] | 2.2 ± 0.1[a] | 2.6 ± 0.2[b] | 3.9 ± 0.3[b] | 5.1 ± 1.0[c] | 2.3 ± 0.8[a] | 3.9 ± 0.6[b] |
| 18:2n-6 | 11.3 ± 0.9[b] | 15.2 ± 0.7[c] | 8.8 ± 0.3[a] | 11.1 ± 0.9[b] | 8.7 ± 0.9[b] | 9.1 ± 0.9[b] | 5.1 ± 0.7[a] | 14.3 ± 0.8[c] |
| 18:3n-3 | 0.3 ± 0.0[a] | 0.4 ± 0.0[a] | 0.2 ± 0.0[a] | 0.3 ± 0.0[a] | 0.2 ± 0.0[a] | 0.3 ± 0.1[a] | 0.2 ± 0.0[a] | 0.2 ± 0.0[a] |
| 20:3n-6 | 0.7 ± 0.1[a] | 1.2 ± 0.1[b] | 0.5 ± 0.0[a] | 0.4 ± 0.1[a] | 0.5 ± 0.1[b] | 0.6 ± 0.1[b] | 0.3 ± 0.1[a] | 0.5 ± 0.0[b] |
| 20:4n-6 | 31.8 ± 0.6[b] | 28.1 ± 1.0[a] | 34.0 ± 0.3[c] | 30.7 ± 2.7[b] | 29.2 ± 0.9[b] | 29.2 ± 1.6[b] | 25.2 ± 2.8[a] | 27.1 ± 1.1[a,b] |
| 22:4n-6 | 0.9 ± 0.0[a,b] | 0.8 ± 0.1[a] | 1.0 ± 0.1[b] | 1.0 ± 0.1[b] | 2.0 ± 0.1[b] | 1.5 ± 0.1[a] | 2.4 ± 0.1[c] | 2.3 ± 0.1[c] |
| 22:5n-6 | 2.3 ± 0.3[b] | 1.4 ± 0.1[a] | 4.3 ± 0.3[c] | 2.3 ± 0.4[b] | 4.7 ± 0.5[b] | 2.5 ± 0.2[a] | 9.1 ± 0.9[c] | 4.2 ± 0.6[b] |
| 22:5n-3 | 0.5 ± 0.0[a] | 0.7 ± 0.1[b] | 0.4 ± 0.0[a] | 0.6 ± 0.1[a,b] | 1.1 ± 0.1[b] | 1.1 ± 0.1[b] | 0.7 ± 0.1[a] | 1.2 ± 0.1[b] |
| 22:6n-3 | 3.0 ± 0.1[a] | 2.9 ± 0.3[a] | 3.3 ± 0.1[a] | 2.6 ± 0.3[a] | 7.2 ± 0.4[b] | 5.8 ± 0.5[a] | 7.9 ± 1.0[b] | 5.4 ± 0.3[a] |
| 20:3+20:4 | | | | | | | | |
| 18:2 | 3.0 ± 0.3[c] | 1.9 ± 0.1[a] | 3.9 ± 0.1[d] | 2.8 ± 0.1[b] | 3.7 ± 0.5[b] | 3.5 ± 0.5[b] | 5.1 ± 0.8[c] | 2.0 ± 0.2[a] |

[a]Values represent the mean ± SEM of five rats. Values in row with a different roman superscript are significantly different ($P < 0.01$).

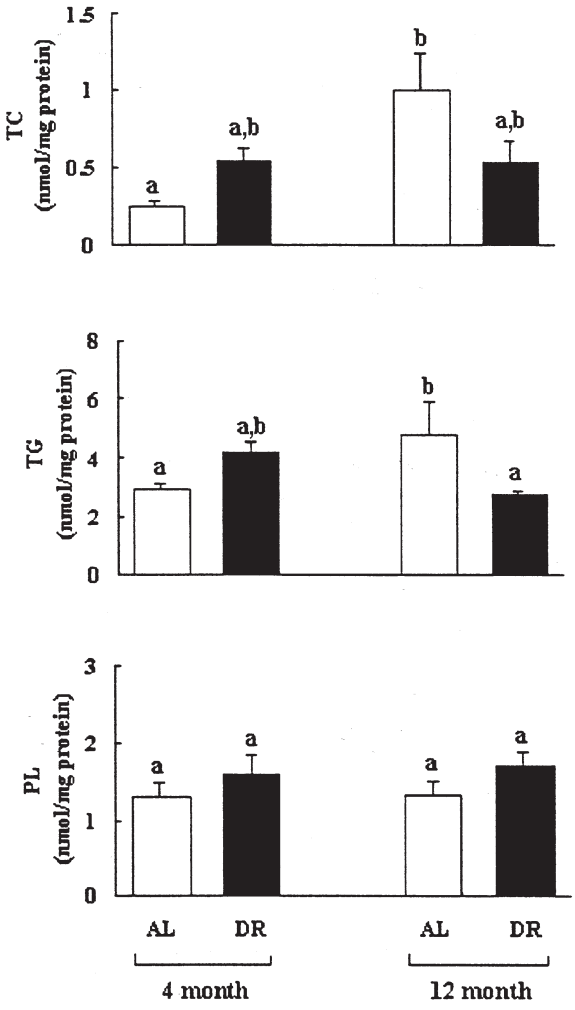


**FIG. 3.** Effects of DR on the concentrations of total cholesterol (TC; top panel), triglyceride (TG; middle panel), and phospholipid (PL; bottom panel) in the liver of rats between 4 and 12 mon. Each bar represents the mean ± SEM of five rats. Mean values with different superscript are significantly different ($P < 0.05$).

(7,11,14,33) that DR is a potent for antioxidative strategy to alleviate liver oxidative injury, thereby slowing down the progression of aging.

## ACKNOWLEDGMENT

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# Overexpression of Acyl-CoA Binding Protein and Its Effects on the Flux of Free Fatty Acids in McA-RH 7777 Cells

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**ABSTRACT:** Overexpression of acyl-CoA binding protein (ACBP) was induced in a rat hepatoma cell line (McA-RH 7777) by stable integration of rat ACBP cDNA. The transfected cells (ACBP-27) had 3.5-fold higher concentrations of ACBP than control cells (14 vs. 4 ng/μg DNA). Both ACBP-27 and control cells were cultured in the presence of various concentrations of radiolabeled palmitic acid; and the effects of ACBP on lipogenesis and β-oxidation were studied. Incubation of the cells with 100 μM palmitic acid resulted in 42% greater incorporation of the fatty acid in ACBP-27 cells as compared to that in the control cells. This increased incorporation of the fatty acid was observed predominantly in the triglyceride fraction. Higher concentrations of palmitic acid (200 to 400 μM) were associated with a significant decrease in the production of $^{14}CO_2$ in the ACBP-27 cell line than in the control cells, while lower concentrations had no effect. Our data suggest a role for ACBP in the partitioning of fatty acids between esterification reactions leading to the formation of neutral lipids and β-oxidation. ACBP may play a regulatory role by influencing this important branch point in intermediary lipid metabolism.

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Acyl-CoA binding protein (ACBP) binds long-chain acyl-CoA esters with very high affinity; it is a ubiquitous protein that probably acts as a housekeeping protein with no pronounced built-in specificity (1). The role of long-chain fatty acyl-CoA esters in the regulation of metabolism and cell signaling has been reviewed by Faergeman and Knudsen (2). It has been reported that the free cytosolic concentration of acyl-CoA esters is very low (nanomolar range) under physiological conditions (2). ACBP is a 10-kDa protein found in various species including mammals (3–7), duck (8), frog (9), insects (10), yeast (11), and plants (12,13); ACBP is expressed in all mammalian tissues tested (14–17). Although the specific biological function of the protein in cells is presently unknown, a number of *in vitro* studies indicate that it is most likely involved in intracellular lipid transport and metabolism of acyl-CoA moieties (18–21). Jolly *et al.* (22) recently identified a novel role of ACBP in microsomal phosphatidic acid biosynthesis that is mediated primarily through the activity of glycerol-3-phosphate acyltransferase. ACBP has been shown to act in the generation of an intracellular acyl-CoA pool-former in yeast (18); it stimulates the synthesis of long-chain acyl-CoA esters by mitochondrial long-chain acyl-CoA synthetase (19) and regulates the activity of outer mitochondrial carnitine palmitoyl transferase (20,21). Previous studies have demonstrated that ACBP can mediate the transport of long-chain acyl-CoA esters immobilized on phospholipid-coated nitrocellulose membranes to mitochondria or microsomes for β-oxidation or glycerolipid synthesis (23), respectively. More recently, the *ACB1* gene encoding ACBP was disrupted in yeast, and the results strongly indicate that yeast ACBP is involved in the transport of newly synthesized acyl-CoA esters from the fatty acid synthetase to the site of glycerolipid synthesis and acyl-CoA desaturation (24). It is also known that the promoter region of the yeast ACBP gene contains two essential elements: one is necessary for expression of the yeast fatty acid synthetase genes (11), and the other element contains sequences corresponding to motifs known as the β-oxidation boxes (25).

ACBP synthesis is regulated by peroxisome proliferators (26–28). Both *in vitro* and *in vivo* studies have shown an increase up to twofold in the expression of rat hepatic ACBP mRNA and protein level after treatment with peroxisome proliferators (26–28). In our previous studies (21), we showed that the levels of ACBP in rat liver decrease with fasting and increase after consumption of a high-fat diet (21). These results indicate that liver ACBP responds to dietary changes and further support a role for this protein in lipid metabolism.

The aim of the present study was to investigate the effects of ACBP on hepatic fatty acid metabolism using a rat hepatoma cell line (McA-RH 7777) with increased expression of ACBP (ACBP-27).

## MATERIALS AND METHODS

*Construction of plasmid pNUT-ACBP and stable integration of ACBP cDNA into McA-RH 7777 cells.* Construction of plasmid pNUT-ACBP was achieved by inserting rat ACBP cDNA into the mammalian expression vector pNUT as previously described (29,30). This was carried out by removal of

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Abbreviations: ACBP, acyl-CoA binding protein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; FABP, fatty acid binding protein; LCAT, lecithin: cholesterol transferase; TLC, thin-layer chromatography.

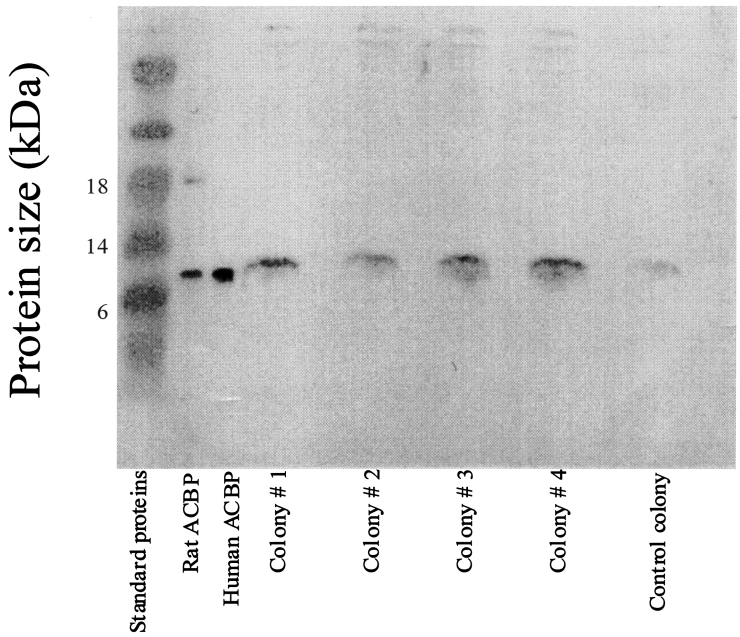


**FIG. 1.** Western blot analysis indicating overexpression of acyl-CoA binding protein (ACBP) (at ~10 kDa size) by ACBP gene-transfected colonies 1–4 as compared to control colony which was transfected with vector only.

the lecithin:cholesterol acyltransferase (LCAT) cDNA from pNUT-LCAT (31) using restriction sites *Xho*I and *Bam*HI.

McA-RH 7777 cells (American Tissue Culture Collection, Rockville, MD) were cotransfected with pNUT-ACBP (or pNUT for construction of the control cell line) using the vector pSV2neo; this vector carries a neomycin resistance gene. Transfection mixtures were made in a 3-mL polypropylene tube as follows: 10 μg/plasmid DNA was suspended in 0.45 mL sterile water, and 0.5 mL HEPES-buffered saline, pH 6.95, was added slowly while mixing with 0.05 mL 2.5 M $CaCl_2$. After 30 min of incubation at room temperature, 1 mL of the above-mentioned transfection mixtures was added to each culture dish containing McA-RH 7777 cells at approximately 50% confluency cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Burlington, Ontario, Canada) containing 10% fetal bovine serum, 10% horse serum, and 1% antibiotic-antimycotic (Geneticin, Sigma, Oakville, Ontario, Canada). The dishes were incubated overnight at 37°C under 5% $CO_2$ concentrations. Subsequently, the medium was refreshed and 0.1 mL Geneticin (50 mg/mL) was added to give a final Geneticin concentration of 500 μg/mL. Dishes were continuously incubated, and the medium was changed every 2–3 d until the majority of the cells sloughed off. After 2 wk of culturing in the presence of Geneticin, virtually none of the parent cells had survived. Macroscopic colonies of surviving cells were harvested and further cultured using multiwell culture plates. These colonies were subsequently transferred to single dishes for further growth. The cells were then collected and screened for the production of ACBP by Western blotting and radioimmunoassay methods (21). ACBP content was expressed relative to DNA content, which was determined according to the method of Labarca and Paigen (32). Briefly, cell homogenates in which deoxyribonucleoprotein structure of chromatin was dissociated were incubated with Hoeschst H 33258 (American Hoechst Corp, currently Hoechst Marion Roussel, Cincinnati, OH). This method has been shown to enhance the fluorescence seen with binding of reagent (Hoeschst H 33258) with DNA (32). Figure 1 shows overexpression of ACBP in ACBP gene-transfected cells as compared to controls. It should be noted that we used only vector (pNUT) transfected cells as control colonies (Fig. 1) vs. ACBP gene-transfected colonies (colonies #1–4 in Fig. 1). One of the ACBP gene-transfected colonies (ACBP-27) was chosen for further study on the basis of its augmented ACBP content. ACBP antibody was generated as previously described (21). Briefly, ACBP was coupled to thyroglobulin and injected into rabbits. The antibody generated against ACBP was purified and used in cross-reactivity studies to determine its specificity; it showed negligible cross reactivity with purified rat liver FABP, bovine serum albumin (BSA), or rat serum albumin (21).

*Determination of the effects of constitutively expressed ACBP on the metabolic flux of free fatty acid.* Cell lines expressing various amounts of ACBP were grown to approximately 80% confluence in 24 multiwell plates and subsequently incubated for 4 h with various concentrations of [U-$^{14}$C]palmitic acid (Amersham Life Science, Arlington Heights, IL) in HEPES-buffered saline containing 2 mg/mL BSA, 36% DMEM, and 0.72% Geneticin. In the labeled medium, there was approximately $2 \times 10^7$ dpm [U-$^{14}$C]palmitic acid/mL of the medium. Therefore, the specific

activities were 4.2, 1.74, 1.13, 0.87, and 0.71 μCi/μM for 100, 200, 300, 400, and 500 μM palmitic acid concentrations, respectively. Two hundred microliters of either labeled or nonlabeled media was added to each well, three wells for each medium. Subsequently, two overlapping glass microfiber filters saturated with 3.5 N NaOH were placed on top of each well for trapping $^{14}CO_2$. The multiwell plates were then wrapped with plastic wrap and covered with a glass plate secured with an approximately 1-kg weight to prevent $CO_2$ leakage. The above unit was incubated at 37°C. After 4 h incubation, the filters were removed from each well, dried, and the trapped radioactivity was determined by scintillation counting and the incorporation of radiolabeled palmitic acid into $^{14}CO_2$ calculated from the specific activity. Subsequently, the medium was removed and each well was rinsed three times with phosphate-buffered saline (1 mL) to remove unincorporated label. Lipids were then extracted by adding a mixture of methanol and water (methanol/water; 1:0.8 vol/vol) to each well. The monolayer was scraped free and transferred to a glass tube, followed by addition of chloroform (2 mL) and glacial acetic acid (0.05 mL) to each tube. After mixing well, the samples were pelletted by centrifugation and dried under nitrogen. An aliquot of the pellet was used for radioactivity determination and another aliquot was resuspended in chloroform and the lipid fractions were determined using thin-layer chromatography (TLC). Lipid fractions were identified by $R_f$ values, and the radioactivity associated with each fraction was calculated by scintillation counting.

*TLC*. A TLC standard mixture was prepared containing oleic acid, diolein, triolein, and phosphatidylcholine. An aliquot (0.075 mL) of this standard mixture was added to each of the dried lipid extracts and mixed thoroughly. The mixture was applied to TLC plates (20 × 20 cm) that were subsequently developed in hexane/diethylether/acetic acid (90:60:1.5 by vol). This solvent system separated the extractable lipids into four fractions, namely, free fatty acids, diglycerides, triglycerides, and phospholipids. Each fraction was visualized with $I_2$ vapor, and areas of the plates corresponding to various fractions were cut and transferred directly into counting vials. Radioactivity was determined by scintillation counting.

*Statistics*. A two-tailed Student's *t*-test for unpaired data was used to assess the differences between the two groups of cells. Differences were considered significant at $P < 0.05$. Data are expressed as means ± standard deviation.

## RESULTS

*Overexpression of ACBP.* Transfected cells with either pNUT-ABCP (ACBP-27) or pNUT (control cells) were incubated in Puregen cell lysis solution for 10 min. ACBP and DNA analyses were performed as previously described (21). ACBP-27 cells had 3.5 times more ACBP than control cells (14 vs. 4 ng/μg DNA).

*Effects of ACBP on the incorporation of palmitic acid into lipids and the substrate dose-response relationship*. A series of studies were carried out to establish optimal conditions for assessing the oxidation of palmitic acid using the McA-RH 7777 cell line. Control cells were grown to confluence in multiwell plates in culture media containing 100 μM [U-$^{14}$C]palmitate. Our preliminary studies indicated a linear relationship between incubation time (from 1 to 5 h) and the incorporation of uptaken radiolabeled palmitate into radiolabeled $CO_2$ (Fig. 2). Regression analysis showed a strong positive relationship ($R^2 = 0.997$) between the incubation time and the mean of three in-

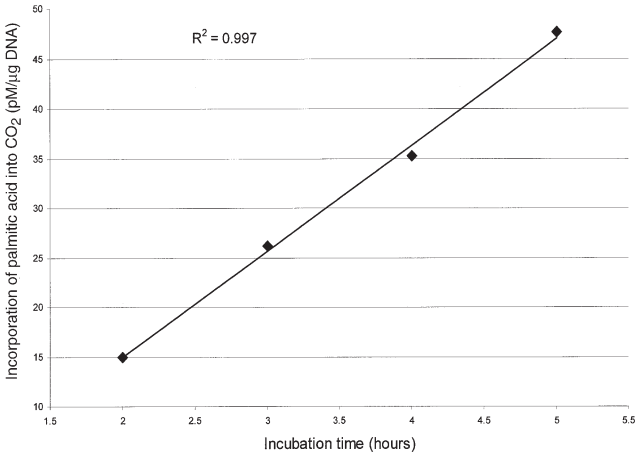


**FIG. 2.** The effects of incubation time on the incorporation of [U-$^{14}$C] palmitic acid into $^{14}CO_2$ in control cells. The cells were grown to confluence in a medium containing 100 μM palmitate (specific activity 3.27 μCi/μM). The incorporation of [U-$^{14}$C]palmitic acid into $^{14}CO_2$ was determined as described in the Materials and Methods section. Results are expressed as means ± standard deviation of triplicate cultures.

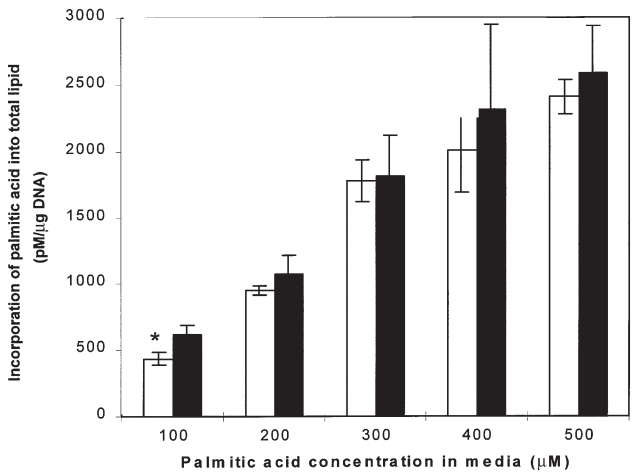


**FIG. 3.** The effects of ACBP on the flux of [U-$^{14}$C]palmitic acid into total lipid and the substrate dose-response relationship. ACBP-27 (solid bars) and control cells (open bars) were incubated for 4 h under varying concentrations of palmitic acid. The lipids were subsequently extracted, and the incorporation of label was determined as outlined in the Materials and Methods section. The data are means ± standard deviation of triplicate cultures. * Indicates significantly different from control levels ($P < 0.02$). For abbreviation see Figure 1.

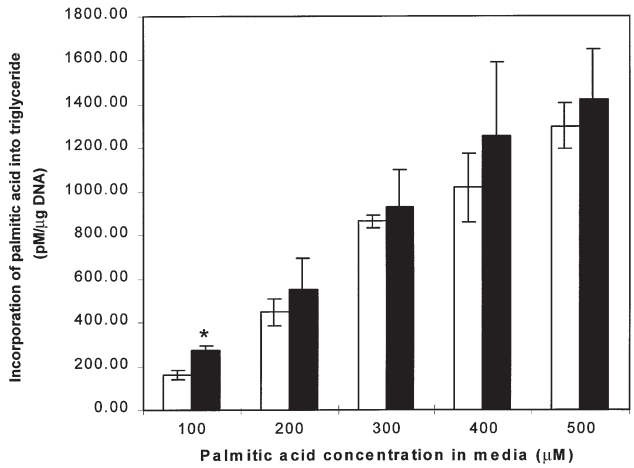


**FIG. 4.** The effects of ACBP on the flux of [U-$^{14}$C]palmitic acid into triglycerides and the substrate dose-response relationship. ACBP-27 (solid bars) and control cells (open bars) were incubated with varying concentrations of palmitic acid for 4 h. Lipids were extracted and separated as described in the Materials and Methods section. The data are means ± standard deviation of triplicate cultures. * Indicates significantly different from controls ($P < 0.01$). For abbreviation see Figure 1.

dependent measurements of generated $CO_2$ (Fig. 2). The 4-h incubation time point was on the linear line and resulted in production of more than 50% of total radiolabeled $CO_2$ measured during the 2–5 h period of incubation (Fig. 2). These criteria were used to choose an incubation time of 4 h.

Cells with increased expression of ACBP (ACBP-27) were grown in the presence of various concentrations of radiolabeled palmitic acid, and the incorporation of label into extractable lipids was monitored after 4 h of incubation. The pattern of incorporation was compared to that obtained in the control cells. Incorporation of palmitic acid into lipids is shown in Figure 3. Although there was a general trend for a greater incorporation of palmitate into extractable lipids in ACBP-27 cells compared to control cells, the difference between the two groups of cells was statistically significant ($P < 0.02$) only at the lowest concentration of fatty acid tested (100 μM). At this concentration, ACBP-27 cells had a 42% greater incorporation of the radiolabel into total extractable lipids than the control cells. The total lipid increase at this concentration was mainly due to an increase in triglycerides; ACBP-27 cells had a 71% greater incorporation of label into triglycerides compared to that in control cells (Fig. 4). Similarly, ACBP-27 cells had a 25% increase (on average) in the incorporation of label into phospholipids when compared to control cells.

*Effects of ACBP on the flux of palmitic acid into β-oxidation and the substrate dose-response relationship.* A similar set of experiments was conducted to examine the effects of the up-regulated ACBP on the flux of [U-$^{14}$C]palmitic acid into β-oxidation as monitored by incorporation of label into $^{14}CO_2$. Incubation with palmitic acid resulted in a significant decrease in the production of $^{14}CO_2$ in ACBP-27 cells as compared to control cells (Fig. 5). This effect appeared to be dose-dependent; ACBP-27 cells produced 51, 47, and 39% lower $^{14}CO_2$ when incubated with 200, 300, or 400 μM of palmitic acid, respectively, as compared to corresponding controls.

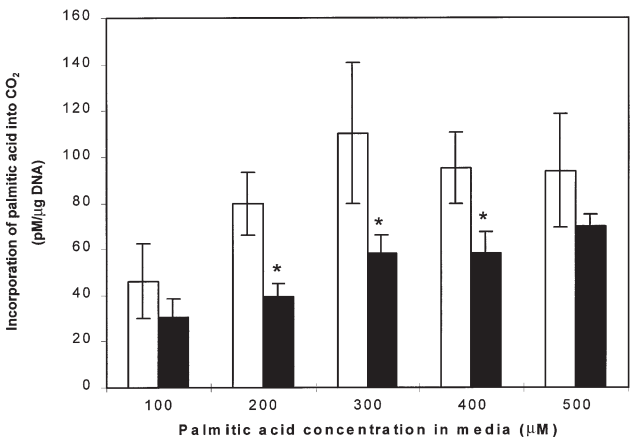


**FIG. 5.** The effects of (ACBP) on the flux of [U-$^{14}$C]palmitic acid into β-oxidation and the substrate dose-response relationship. β-Oxidation of palmitic acid was monitored by trapping of $^{14}CO_2$ during a 4-h incubation time as described in the Materials and Methods section. The data are means ± standard deviation of triplicate cultures. * Indicates significantly different from controls ($P < 0.05$). For abbreviation see Figure 1.

## DISCUSSION

Previous studies have indicated that ACBP stimulates the synthesis of long-chain fatty acyl-CoA (19). It was suggested that ACBP was able to accomplish this by removing newly synthesized long-chain fatty acyl-CoA moieties from the mitochondrial membrane and thereby reducing the negative feedback inhibition by this substrate on the activity of mitochondrial long-chain fatty acyl-CoA synthetase (19). Other studies have suggested that ACBP may facilitate transportation of long-chain acyl-CoA esters to microsomes and mitochondria for glycerolipid synthesis or β-oxidation (23). In previous studies, we showed that tissue levels of ACBP were increased in response to a high-fat diet and were decreased with fasting in the rat (21). Tissue levels of ACBP showed a positive correlation with liver triglycerides in these studies. All of these data suggest a role for ACBP in intermediary regulation of lipid metabolism.

The present studies employed a hepatic cell line in which the expression of ACBP was up-regulated to examine the effects of this protein on the metabolism of palmitic acid *in vitro*. Our data suggest that at a low concentration of palmitic acid, ACBP enhances the incorporation of [U-$^{14}$C]palmitic acid into the synthesis of lipids (particularly triglycerides). This is in accordance with the observations of Rasmussen *et al.* (19), who demonstrated that the saturation of ACBP with long-chain acyl-CoA ester abolished the stimulatory effects of ACBP on the activity of acyl-CoA synthetase. Coincident with the increased label incorporation into synthesis of triglyceride was a decrease in the incorporation of $^{14}CO_2$. As is evident in Figure 5, oxidation of the fatty acid significantly decreased at palmitic acid concentrations of 200, 300, and

400 μM. Since ACBP can influence the flux of fatty acids between esterification into neutral lipids and β-oxidation it may play an important regulatory role in lipid metabolism by influencing this important branch point in intermediary metabolism. Recently, Jolly *et al.* (22), showed that both liver fatty acid-binding protein (L-FABP) and ACBP stimulate microsomal incorporation of the monounsaturated oleoyl-CoA and polyunsaturated arachidonoyl-CoA by 8- to 10-fold, and 2- to 3-fold, respectively. On the other hand, both proteins (L-FABP and ACBP) inhibit microsomal utilization of saturated palmitoyl-CoA by over 60% (22). It has been reported that ACBP protects long-chain fatty acyl-CoA from microsomal acyl-CoA hydrolase activity in the following order: palmitoyl-CoA > oleoyl-CoA > arachidonoyl-CoA (22).

As the relative molar ratio of FABP to ACBP is approximately 8:1 (1,3,33–35), it could be argued that the role of ACBP is likely to be more significant at lower concentrations of long-chain fatty acyl-CoA. FABP may contribute additional function under conditions in which long-chain fatty acyl-CoA levels have exceeded the binding capacity of ACBP. In our studies, ACBP-27 constitutively expressed a 3.5-fold increase in the cellular levels of ACBP when exposed to a low concentration of palmitic acid, which was associated with an enhanced incorporation of radiolabel into total lipids (particularly triglycerides). However, at higher concentrations of palmitic acid the increased incorporation into neutral lipid was not significant. The binding capacity of ACBP may have been exceeded under these conditions.

Although the ACBP gene belongs to the class of housekeeping genes that are ubiquitously expressed, the level of expression for this protein differs markedly among different tissues and cell types (36,37). ACBP is highly expressed in secretory cells such as hepatocytes and steroid-producing cells. This protein is regulated by peroxisome proliferators. The addition of peroxisome proliferators to a rat hepatoma cell line results in an increase in ACBP mRNA (26–28). Several studies indicate that fatty acids and/or acyl-CoA may activate the peroxisome proliferator-activated receptors (38). This in turn would be expected to activate the ACBP gene (38). It may be through this mechanism that intracellular levels of free fatty acids are able to influence key regulatory steps for directing an appropriate metabolic response by the cells. Further studies are required to elucidate the regulatory role of ACBP in intermediary lipid metabolism.

## ACKNOWLEDGMENTS

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# Metabolism of Very Long Chain Polyunsaturated Fatty Acids in Isolated Rat Germ Cells

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**ABSTRACT:** Which cell type is responsible for the high levels of very long chain polyunsaturated fatty acids in testis and whether this fatty acid pattern is a result of a local synthesis are not presently known. In this study, fatty acid conversion from 20:4n-6 to 22:5n-6 and from 20:5n-3 to 22:6n-3 was investigated in isolated rat germ cells incubated with [1-$^{14}$C]-labeled fatty acids. The germ cells elongated the fatty acids from 20- to 22-carbon atoms and from 22- to 24-carbon atoms but had a low Δ6 desaturation activity. Thus, little [$^{14}$C]22:5n-6 and [$^{14}$C]22:6n-3 were synthesized. When Sertoli cells were incubated with [1-$^{14}$C]20:5n-3 for 24 h, an active fatty acid elongation and desaturation were observed. *In vivo* germ cells normally have a higher content of 22:5n-6 or 22:6n-3 than Sertoli cells. An eventual transport of essential fatty acids from Sertoli cells to germ cells was thus studied. Different co-culture systems were used in which germ cells were on one side of a filter and Sertoli cells on the opposite side. When isolated pachytene spermatocytes or round spermatids were added to the opposite side of a semipermeable filter, approximately 1 nmol [$^{14}$C]-22:6n-3 crossed the filter. Little of this was esterified in the germ cells. Similarly, in using [1-$^{14}$C]20:4n-6 in identical experiments, very little [$^{14}$C]22:5n-6 was esterified in germ cells on the opposite side of the filter. Although the very active synthesis of 22:5n-6 and 22:6n-3 observed in Sertoli cells suggests a transport of these compounds to germ cells, this was not experimentally determined.

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n-6 Fatty acids are essential for normal testicular development and fertility (1). The testis and sperm of rats and several other rodents have a high content of docosapentaenoic acid (22:5n-6) (2), whereas in humans (3,4), monkeys (5), and pigs (6) docosahexaenoic acid (22:6n-3) is the dominant fatty acid in testis. A selective n-3 deficiency does not reduce rat fertility (7). However, reduced motility of human spermatozoa has been associated with a declining content of 22:6n-3 in the phospholipids (PL) of the spermatozoa (8).

The germ cells are in close connection with the Sertoli cells. They are located within the seminiferous tubules undergoing mitotic and meiotic divisions, differentiating from diploid spermatogonia (DNA = 2C) to spermatocytes (4C) and finally to spermatozoa (1C), which are released. The content of very long unsaturated polyenes is higher in germ cells than in Sertoli cells both in rat (9) and in monkey (5). Whether the germ cells themselves are able to synthesize the very long unsaturated polyenes from linoleic and α-linolenic acid or whether they must be provided with these fatty acids, e.g., from the Sertoli cells is unknown. Several authors have proposed transport of long polyenes from the Sertoli cells to the germ cells, but such a transport has not yet been determined (10–13).

The aim of the present work was to study the fatty acid metabolism in isolated rat germ cells and eventually the transport of fatty acids from Sertoli cells to germ gells. In the present study centrifugal elutriation followed by application of a Percoll density gradient (14) allowed measurements on highly purified round spermatids (RST) and pachytene spermatocytes (PS). The formation of 22:5n-6 and 22:6n-3 from labeled fatty acid precursors was studied in the light of the revised pathway of the synthesis of these fatty acids (15,16). Sertoli and germ cells were co-cultured to study a possible fatty acid transfer between the different cell types.

## MATERIALS AND METHODS

*Chemicals.* The labeled fatty acids were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO) and the unlabeled fatty acids were from Sigma Chemicals Co. (St. Louis, MO).

*Animals.* Weanling Sprague-Dawley rats were obtained from Møllegaard Laboratory (Ry, Denmark). After separation from their mother, the rats were given a standard rat diet from Grimstone Aldbrough (Hull, UK). The experimental procedures were approved by the local ethical committee.

*Preparation of isolated germ cell suspensions.* Germ cells were isolated from seminiferous tubules of 32-d-old rats. A cell suspension was obtained by consecutive treatment with collagenase, trypsin, and DNase (17,18). The cells were fractionated into PS and RST by centrifugal elutriation followed by separation in density gradients of Percoll (19). By this method purified populations of PS (87–96% purity as evaluated by flow cytometry of the DNA content) and RST (90–95% purity) were obtained (data not shown). The cells were diluted in minimum essential fatty acid medium, and labeled fatty acid substrates were added. More than 90% of the cells were viable, as measured by resistance to uptake of Trypan blue, and more than 80% remained viable after the 24-h incubation period. The cell

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Abbreviations: BSA, bovine serum albumin; FFA, free fatty acids; HBSS, Hanks' balanced salt solution; PL, phospholipid; PS, pachytene spermatocytes; RST, round spermatids; TAG, triacylglycerol.

density in the germ cell suspension was (0.4–1.4) × $10^6$ cells/mL, and the corresponding mean protein concentration was 0.34 mg/mL. Of this preparation, 5.0 mL was used for incubation in NUNC (Roskhilde, Denmark) 50-mL cell culture flasks. The suspensions contained 1.5% (wt/vol) essentially fatty acid-free bovine serum albumin (BSA). Radiolabeled fatty acid (20 nmol) with a specific activity of approximately 55 mCi/mmol was added to each flask.

*Preparation of enriched Sertoli cell suspensions.* The testes were removed, decapsulated, and cut into small pieces in $Ca^{2+}$- and $Mg^{2+}$-free Hanks' balanced salt solution (HBSS). The tissue was then incubated in HBSS containing 0.25% trypsin and 0.2 mg/mL DNase for 15 min at 34ºC in a shaking water bath (120 cycles/min). Subsequently the tubule fragments were allowed to sediment for 5 min, the medium was aspirated, and the pellet was re-suspended in HBSS containing soybean trypsin inhibitor (0.4 mg/mL). The tubule fragments were washed three times in HBSS (with $Ca^{2+}$ and $Mg^{2+}$) by sedimentation at unit gravity for 5 min and then incubated for 30 min in 50 mL HBSS containing 50 mg collagenase for 15 min at 34ºC in a shaking water bath (120 cycles/min). The cells were pelleted by centrifugation at room temperature for 2 min at 15 × *g* and dispersed in HBSS containing 1.25% BSA, followed by centrifugation. The pellet was resuspended in HBSS and filtered through a premoistened 100-µm nylon mesh. The filtrate was centrifuged for 2 min, and the final pellet resuspended in Earle's minimal essential medium supplemented with 2 mM L-glutamine, penicillin ($10^6$ IU/L), streptomycin (25 mg/L), and fungizone (2.5 mg/L). More than 90% of the cells were viable, as measured by resistance to uptake of Trypan blue. After 24 h of incubation, the viability varied from 80 to 95%. The Sertoli cells were isolated essentially as described by Dorrington *et al*. (20).

*Sertoli and germ cells in co-culture.* Costar's (Skipholt, The Netherlands) Transwell cell culture flasks with a growth area of 44 $cm^2$ and a 3.0 µm pore size polycarbonate membrane was used for co-culture experiments. The cell density using the Costar system was (0.8–1.6) × $10^6$ cells/mL in the Sertoli cell suspensions and (0.5–1.4) × $10^6$ cells/mL in the germ cell suspensions. Sertoli cell densities within this range have been used in several other studies (21). Below the membrane 12.5 mL of cell suspension or of medium only was used and 8.5 mL of cell suspension or medium only above the membrane. Some experiments were performed using Falcon cell culture inserts (3.0 mL cell suspension under the membrane and 2.0 mL over) with a membrane pore size of 0.45 µm (Cyclopore®, Whatman, Maidstone, United Kingdom).

*Analytical methods.* Lipids were extracted (22), and aliquots were separated on silica gel 60H thin-layer plates (Merck, West Point, PA) with hexane/diethylether/acetic acid (70:29:1 by vol) and scanned in an imager scanner from Packard (Downers Grove, IL). The PL were isolated using the same thin-layer chromatography system and eluted with 2 × 2 mL $CHCl_3$/MeOH/acetic acid/water 50:39:1:10, by vol. The PL were further separated into phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine on Whatman LK5 thin-layer plates with $CHCl_3$/MeOH/40% aqueous methylamine (120:40:10, by vol) as solvent. The Whatman LK5 thin-layer plates were scanned in a Packard Instant Imager Scanner, and the PL classes were then eluted with 2 × 2-mL $CHCl_3$/MeOH/acetic acid/water 50:39:1:10, by vol.

Aliquots of the total lipids of the cell suspensions, the isolated cells, and the isolated mediums, as well as the PL and the triacylglycerol (TAG) fractions, were transmethylated ($BF_3$-methanol), and the fatty acids were separated with reversed-phase high-performance liquid chromatography (isocratically) on a Supelcosil LC-18 (250 × 4.6 mm) reversed-phase column (Supelco, Bellfonte, PA). The mobile phase was acetonitrile/water (80:20, vol/vol) 2.0 mL/min, or acetonitrile/water (90:10, vol/vol) 0.75 mL/min delivered by a LC-9A Shimadzu apparatus (Kyoto, Japan). Radioactivity was measured in a flow detector A-100 (Radiomatic Instruments & Chemical, Tampa, FL). The fatty acids were identified by comparison with retention times from $^{14}C$-labeled fatty acid standards and with retention times from previous experiments.

The naturally occurring concentration of nonesterified free fatty acids in the sera of the Wistar rats used was approximately 400 µM as measured by capillary gas chromatography with 17:0 as internal standard using a SP-2340 capillary column (30 m × 0.32 mm; Supelco) and a Hewlett-Packard 5890 series II gas chromatograph (Palo Alto, CA) with a flame-ionization detector and helium as carrier gas.

Determination of radioactive acid-soluble products and of radioactive $CO_2$ (as a measure of the rate of oxidation of carbon in the number 1 position) was essentially performed as described by Christiansen (23) and by measuring the labeling of the water phase of the Folch *et al.* extraction (22). The cellular protein was determined (24).

*Statistics*. Mann-Whitney rank test was used, and the *P*-values were Bonferroni-corrected when multiple comparisons were performed.

## RESULTS

*Elongation, desaturation, and retroconversion.* The germ cells were able to perform the elongation reaction from 20- to 22- and from 22- to 24-carbon atom fatty acids (Fig. 1). The fatty acid substrates were elongated to a different degree, and [1-$^{14}C$]20:4n-6 was elongated less than [1-$^{14}C$]20:5n-3. By contrast, [1-$^{14}C$]22:4n-6 and [1-$^{14}C$]22:5n-3 were elongated to an almost equal degree (Fig. 1). The PS were more active in elongating the fatty acids than the RST (Fig. 1 B–D). Little of the labeled fatty acid substrates was Δ6-desaturated, and very small amounts of [$^{14}C$]22:5n-6 and [$^{14}C$]22:6n-3 were synthesized.

*Oxidation and uptake of fatty acid substrate in the lipid fractions.* The fatty acid substrates were esterified to a different degree in PL and TAG (Fig. 2). More [1-$^{14}C$]20:5n-3, [1-$^{14}C$]22:5n-3, and [1-$^{14}C$]22:4n-6 were oxidized or esterified in TAG than [1-$^{14}C$]20:4n-6, [1-$^{14}C$]22:5n-6 and [1-$^{14}C$]-22:6n-3 (Fig. 2). The sum of [1-$^{14}C$]20:5n-3, [1-$^{14}C$]22:5n-3, and [1-$^{14}C$]22:4n-6 that were esterified in TAG was higher than that of [1-$^{14}C$]20:4n-6, [1-$^{14}C$]22:5n-6, and [1-$^{14}C$]22:6n-3,

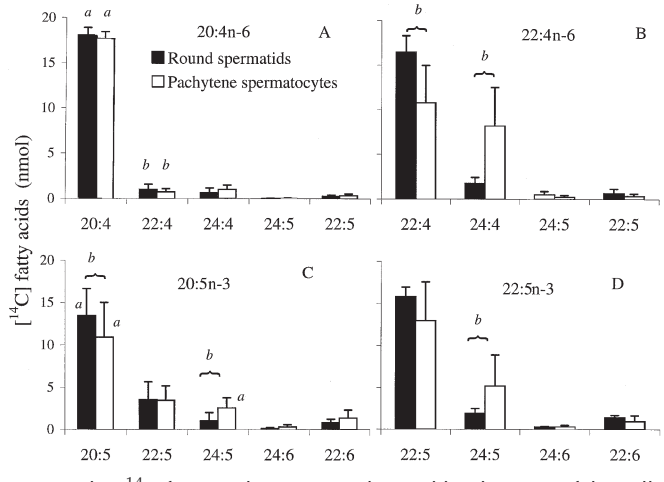


**FIG. 1.** The [$^{14}$C]fatty acid patterns in the total lipid extract of the cells plus media in combination. Germ cells were incubated in NUNC cell culture flasks (Roskhilde, Denmark) with 20 nmol [1-$^{14}$C]-labeled 20:4n-6 (A), 22:4n-6 (B), 20:5n-3 (C), and 22:5n-3 (D) in round spermatids or pachytene spermatocytes as indicated in the legend to panel (A). The [$^{14}$C]-labeled metabolites from the different fatty acid substrates are indicated below each bar. The results are given as the mean of 3–10 (mean 5.4) incubations with 1 SD indicated as T-bars. *a* = $P < 0.05$ when comparing the intermediates formed from [1-$^{14}$C]20:4n-6 with the analog metabolites from [1-$^{14}$C]20:5n-3. *b* = $P < 0.05$ comparing the intermediates formed from round spermatids with the amounts formed from pachytene spermatocytes.

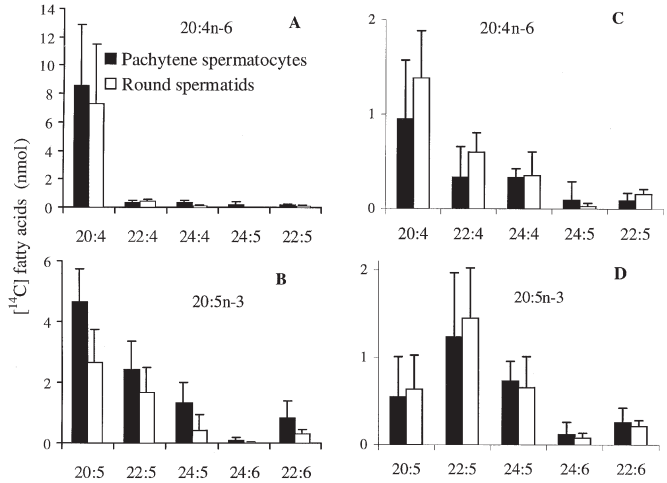


**FIG. 3.** The [$^{14}$C]fatty acid pattern in the phosopholipid (A and B) and triacylglycerol (C and D) fraction after 24 h of incubation with 20 nmol [1-$^{14}$C]20:4n-6 (A,C) or [1-$^{14}$C]20:5n-3 in germ cell suspension (B,D). Key for round spermatids and pachytene spermatocytes are indicated in panel A. NUNC cell culture flasks were used. The [$^{14}$C]-labeled fatty acid metabolites are indicated under the bars. The amounts of the substrates and intermediates recovered are given as a mean of 3–6 (mean 4.5) incubations. T-bars = 1 SD. For manufacturer see Figure 1.

both in PS (rank sum 373 vs. 92, $n_1 = 13$, $n_2 = 17$, $P < 0.0001$) and in RST (rank sum 182 vs. 122, $n_1 = 11$, $n_2 = 13$, $P < 0.01$). The proportion of fatty acid substrates that had been elongated was higher in the TAG fraction than in the PL fraction (Fig. 3). With [1-$^{14}$C]22:5n-6 and [1-$^{14}$C]22:6n-3 as substrates, no products from elongation were detected (data not shown).

Phosphatidylcholine was the major PL class in which the labeled fatty acids were esterified, followed by phosphatidylethanolamine (Fig. 4).

In using PS 1.9–7.1 nmol as nonesterified free fatty acid (FFA) substrates was recovered (depending on the fatty acid substrate used) and in using RST this fraction was 6.3–11.8 nmol. The FFA consisted almost exclusively of the substrate

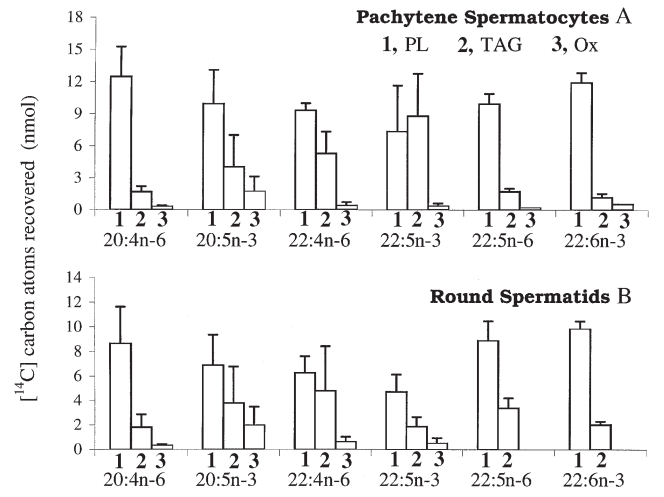


**FIG. 2.** [$^{14}$C]-Incorporation in the phospholipid fraction (PL) and in the triacylglycerol fraction (TAG), and oxidation (Ox) measured by the amount of radioactive acid-soluble products and carbon dioxide formed after 24 h of incubation. Pachytene spermatocytes (A) and round spermatids (B) were incubated with [1-$^{14}$C]fatty acid substrates in NUNC cell culture flasks. The substrates used (20 nmol) are indicated below each cluster of bars. The mean values of 2–10 (mean 4.2) incubations are given. T-bars = 1 SD. For manufacturer see Figure 1.

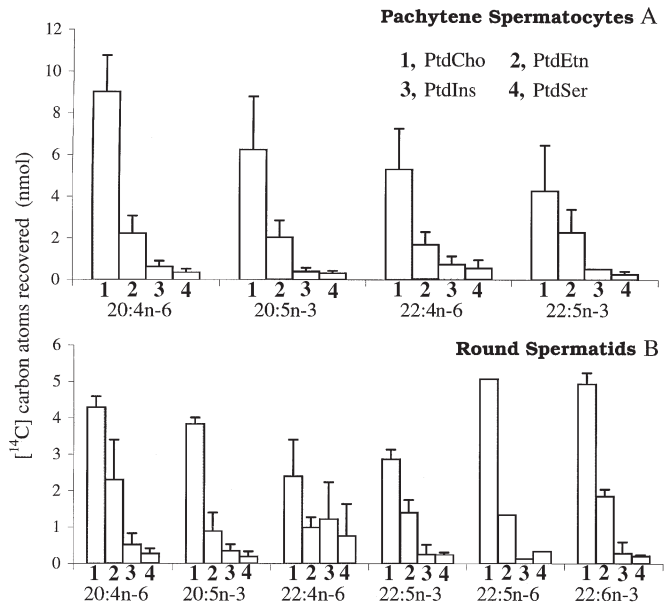


**FIG. 4.** Esterified [$^{14}$C]fatty acids in various phospholipid classes in pachytene spermatocytes (A) and round spermatids (B) after 24 h of incubation in NUNC cell culture flasks. PtdCho = phosphatidylcholine, PtdEtn = phosphatidylethanolamine, PtdIns = phosphatidylinositol, PtdSer = phosphatidylserine. The substrates are indicated below each cluster of bars. The mean amount (nmol) from 2 to 6 (mean value 3.4) incubations are given. T-bars = SD. For manufacturer see Figure 1.

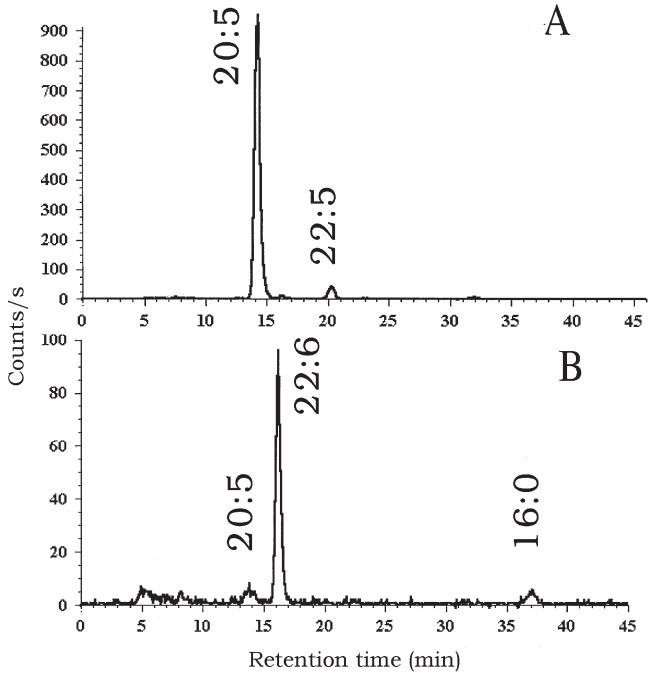


**FIG. 5.** Examples of reversed-phase high-performance liquid chromatograms after incubation with [1-$^{14}$C]20:5n-3. Panel A shows the free fatty acid fraction after incubating round spermatids with [1-$^{14}$C]20:5n-3 for 24 h in absence of Sertoli cells. Panel B shows the labeled fatty acids found in the free fatty acid fraction at the germ cell-side of a 3.0-μm pore membrane when Sertoli cells were incubated with [1-$^{14}$C]20:5n-3 at the opposite side for 24 h. A Costar two-chamber system was used as described in the Material and Methods section.

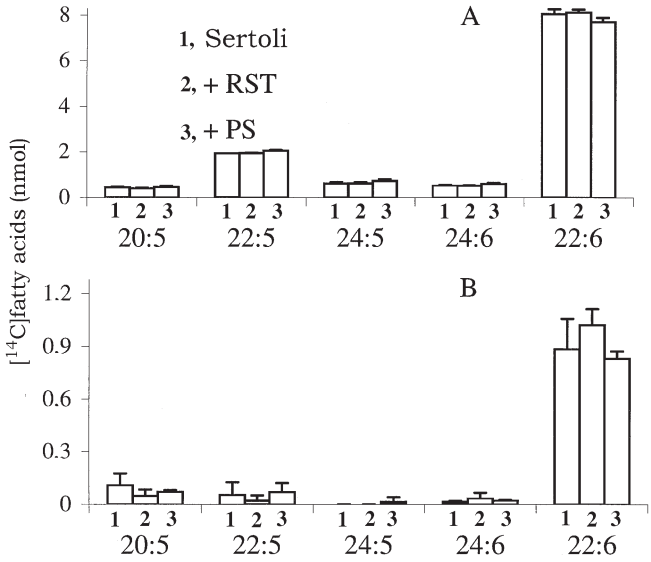


**FIG. 6.** Sertoli and germ cells co-cultured at each side of a 3.0-μm pore membrane using a Costar two-chamber system. The Sertoli cell suspension was incubated with 20 nmol [1-$^{14}$C]20:5n-3 for 24 h at the top of the membrane. Germ cells [pachytene spermatocytes (PS) or round spermatids (RST)] were thereafter incubated at the other side of the membrane and were co-cultured for 48 h. The labeled fatty acids in the lipid extract of cells plus medium at the Sertoli-side of the membrane are shown in panel A and at the germ cell-side of the membrane in panel B. The different [$^{14}$C]-labeled fatty acids are indicated below each bar-cluster. The co-cultures are explained in the legend to panel A. Sertoli = Sertoli cells incubated without germ cells at the other side of the membrane; +RST = Sertoli cells at one side of the membrane and round spermatids at the other; +PS, Sertoli cells with pachytene spermatocytes at the opposite side of the membrane.

used (Fig. 5A) and probably represented the fatty acids that were not taken up by the germ cells. In contrast, [$^{14}$C]22:6n-3 accounted for almost all the FFA when incubating Sertoli cells with [1-$^{14}$C]20:5n-3 (Fig. 5B).

*Studies on a possible transport of fatty acids from Sertoli to germ cells.* A Costar two-chamber incubation system with a polycarbon filter (3.0 μm pores) was used. The Sertoli cell suspension was incubated with [1-$^{14}$C]20:4n-6 or [1-$^{14}$C]-20:5n-3 for 24 h before the germ cells were added to the opposite side of the filter. By doing this preincubation, almost all of the labeled fatty acids were esterified in the Sertoli cells. In the lipid extract from cells plus medium at the Sertoli cell-side of the filter, some [$^{14}$C]22:5n-3, [$^{14}$C]24:5n-3, and [$^{14}$C]24:6n-3 were found, but [$^{14}$C]22:6n-3 dominated after incubation with [1-$^{14}$C]20:5n-3 (Fig. 6A). The conversion of [1-$^{14}$C]20:4n-6 to [1-$^{14}$C]22:5n-6 was less efficient than that of the n-3 analog (data not shown), as described previously. When incubating Sertoli cells with 20 nmol [1-$^{14}$C]22:4n-6, the amounts of labeled 22:4n-6, 24:4n-6, 22:5n-6, and 22:5n-6 found as FFA were 0.4 ± 0.1, 0.1 ± 0.0, 0.1 ± 0.0, and 0.5 ± 0.1 nmol, respectively. When using [1-$^{14}$C]22:5n-3, 1.1 ± 0.0 nmol of [$^{14}$C]22:6n-3 was found in the FFA fraction, which accounted for more than 90% of the labeled fatty acids present there. The labeled fatty acid pattern in the Sertoli cell suspension was not influenced by the presence of germ cells on the contrary side of the filter, and the amounts of [$^{14}$C]-22:6n-3 that crossed the filter from the Sertoli cell side to the germ cell side were independent of the presence of germ cells (Fig. 6). The small amount of [$^{14}$C]22:6n-3 that crossed the filter was mainly found as FFA and was not esterified (data not shown). We conclude that no active transport of labeled fatty acids from Sertoli to germ cells occurred.

## DISCUSSION

Isolated rat germ cells were able to elongate labeled fatty acids from 20- to 22- and from 22- to 24-carbon fatty acids, but little 22:5n-6 and 22:6n-3 were formed from labeled fatty acid precursors. This suggests a low Δ6 desaturation activity in the isolated germ cells, which is very different from the active Δ6 desaturation activity previously observed in rat Sertoli cells (Fig. 6) (26).

Labeled 20:4n-6 and 22:5n-6, two fatty acids which are normally present in significant amounts in rat germ cells, were good substrates for esterification in the PL fraction of the germ cells and little of these acids was oxidized. This was also the case with labeled 22:6n-3. On the contrary, labeled 20:5n-3, 22:4n-6, and 22:5n-3 fatty acids, which are normally present in very small amounts in rat germ cells, were better substrates for esterification in TAG, and partly also for oxida-

tion. The degree of fatty acid esterification in PL may partly contribute to the fatty acid pattern observed in testis of normal rats. The TAG fraction contained a higher degree of elongated fatty acid substrates than the PL. This was similar to previous findings in isolated rat seminiferous tubules (27) and rat Sertoli cells (25), suggesting that the TAG fraction is important in the metabolism of testicular fatty acids.

The limited synthesis of 22:5n-6 and 22:6n-3 observed in the germ cells in the present study was in contrast to the high content of these fatty acids previously observed in germ cells *in vivo* (28). However, the present study shows that Sertoli cells are very active in metabolizing polyunsaturated fatty acids as compared with isolated germ cells. Thus, the Sertoli cells may provide the germ cells with these polyenes. A small amount of [$^{14}$C]22:6n-3, synthetized by Sertoli cells, was released into the incubation medium as FFA and recovered in similar concentrations on both sides of the filter separating germ cells and Sertoli cells. However, [$^{14}$C]22:6n-3 did not accumulate in the germ cells (Fig. 6), and thus a transport of labeled fatty acids from Sertoli cells to germ cells was not observed. The experimental conditions may have an influence on this. The low extracellular concentration of labeled 22:6n-3 and 22:5n-6 (0.1 μM) possibly was too low for [$^{14}$C]22:6n-3 to be efficiently esterified in the germ cells. A cell-to-cell contact may also be obligate for an eventual fatty acid transport to occur, since *in vivo,* germ cells are in close contact with Sertoli cells throughout all steps of the development (during certain stages of their maturation they are actually embedded within the Sertoli cell cytoplasm).

When labeled 18:2n-6 and 20:4n-6 were injected in testis in a previous study, the fatty acids were primarily taken up and metabolized by Sertoli cells, suggesting an important role of the Sertoli cell in the lipid metabolism of testis (29). Further, the results from the present study show that isolated germ cells were inactive in metabolizing labeled polyunsaturated fatty acid, in contrast to Sertoli cells. Together these indicate that Sertoli cells do play a major role in testicular fatty acid metabolism, but it is not demonstrated if there is a transfer of fatty acids from Sertoli cells (or other cells types, e.g., in the vesicula seminalis) to the germ cells. It is possible that the high proportion of 22:5n-6 or 22:6n-3 found in germ cells *in vivo* may partly be due to the loss of most cytoplasm from germ cells along with their maturation (30), possibly altering the fatty acid content, both quantitatively and qualitatively.

In conclusion, the present study showed that isolated rat germ cells were inefficient in synthesizing 22:5n-6 and 22:6n-3 and had a low Δ6 desaturation activity. The rat Δ6 desaturase gene was recently cloned (31), and future studies thus may confirm the major metabolic differences between Sertoli and germ cells observed in the present study on the mRNA level of some key enzymes.

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# Oleate Acutely Stimulates the Secretion of Triacylglycerol by Cultured Rat Hepatocytes by Accelerating the Emptying of the Secretory Compartment

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**ABSTRACT:** The acute effects of addition of oleate on the rate of triacylglycerol (TAG) secretion by cultured rat hepatocytes were studied by monitoring the use of endogenous ($^{14}$C-prelabeled) acyl moieties and exogenous ($^{3}$H-labeled) oleate for the synthesis of secreted TAG simultaneously. Inclusion of exogenous oleate in the medium stimulated the secretion of the endogenous $^{14}$C-labeled acyl moieties by 55–100%. To find out whether the stimulation was due to increased endogenous TAG mobilization or an increased rate of processing of TAG within the endoplasmic reticulum (ER) secretory machinery, use was made of the inhibition of apolipoprotein B (apoB) synthesis (but not degradation) by $Ca^{2+}$ mobilization from the ER. Inhibition of apoB synthesis stopped entry of acyl moieties (from endogenous and exogenous sources) into the secretory pathway. However, even when entry of acyl moieties into the secretory pathway was totally inhibited, exogenous oleate was still able to stimulate (twofold) the secretion [$^{14}$C]TAG, indicating that oleate stimulates the emptying of prelabeled TAG from the secretory compartment at a point distal to apoB synthesis and nascent particle formation. These data indicate that exogenous oleate, besides providing additional acyl moieties for incorporation into secreted TAG, stimulates the secretion of endogenous TAG in a manner (i) that is independent of effects on apoB synthesis and/or degradation and (ii) that involves the enhanced processing of TAG resident within the ER secretory pathway.

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Triacylglycerol (TAG) secretion by hepatocytes is affected by several factors, including the availability of fatty acids and of apolipoprotein B (apoB) for assembly into very low density lipoprotein (VLDL) particles. Through the phosphatidate pathway, exogenous fatty acids are incorporated into diacylglycerols (DAG), which are then partitioned between either the synthesis of cytosolic TAG or, after permeation through the endoplasmic reticulum (ER) membrane, into TAG destined for secretion (1). Within the ER, TAG occur within apoB-containing nascent lipoprotein particles in the rough ER (rER), and as apoB-free TAG droplets within the smooth ER (sER) (2). Addition (fusion) of TAG from this sER pool to the nascent particles (step two of lipidation) at regions of fusion between the rER and sER membrane populations results in the full-sized VLDL particles that are secreted (3,4). Cytosolic TAG undergo a continuous cycle of lipolysis (5) [mostly to DAG (6,7)] and resynthesis. DAG formed through cytosolic TAG lipolysis also contribute to the synthesis of ER lumenal (secreted) TAG (6). Therefore, DAG required for TAG synthesis are contributed both directly through the phosphatidate pathway and from the hydrolysis of cytosolic droplet TAG (1,8).

In freshly prepared rat or rabbit hepatocytes, in which the endogenous cytosolic TAG stores are depleted during the cell isolation procedure, the rate of TAG secretion is almost totally dependent on the addition of oleate (9,10). However, in cultured rat hepatocytes, in which the accumulation of cytosolic TAG is maximized by preculture for up to 24 h with fatty acids, the rate of TAG secretion is less dependent on added fatty acids (11), and the DAG and acyl moieties formed by hydrolysis of cytosolic TAG make a major contribution (about 80%) toward the synthesis of secreted TAG, even in the presence of exogenous oleate (7). Therefore, it appears that in addition to the ability of oleate to stimulate TAG secretion directly, by providing additional acyl moieties, it also stimulates secretion of endogenous acyl groups through an unknown mechanism. The secretion of TAG occurs within VLDL particles, the nascent form of which are produced by rapid but partial lipidation of newly synthesized apoB. Therefore, an experimental approach that distinguishes between the stimulation of the supply of acyl moieties and DAG proximal to apoB synthesis, and of the rate of secretory steps distal to apoB synthesis would be useful in identifying the site of oleate action.

One such approach is provided by the inhibition of TAG secretion when intracellular (ER) $Ca^{2+}$ stores are mobilized (12). The mechanism of this inhibition has not been studied, although it has been suggested that ER lumenal $Ca^{2+}$ is required for the correct folding of apoB (12). However, because ER $Ca^{2+}$ mobilization is known to inhibit protein synthesis (13,14), we considered it possible that TAG secretion may be inhibited as the result of the inhibition of apoB synthesis. If this could be shown to be the case, it would provide the opportunity experimentally to investigate the effect of the addition of exogenous oleate on the rate of TAG secretion by hepatocytes at a step(s) distal to apoB synthesis (and step 1 lipidation). Therefore, in the present study we combined the use of this approach to the

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Abbreviations: Apo, apolipoprotein; DAG, diacylglycerols; TAG, triacylglycerols; EBSS, Earls balanced salts solution; ER, endoplasmic reticulum; rER, rough endoplasmic reticulum; SDS, sodium dodecyl sulfate; sER, smooth endoplasmic reticulum; VLDL, very low density lipoproteins.

previously described dual-labeling technique for the quantification of the utilization of endogenous and exogenous acyl moieties for the synthesis of secreted TAG (7) to study the mechanism of the effect of exogenously added oleate on TAG secretion by cultured rat hepatocytes. The data show that exogenous oleate stimulates the rate of secretion of a pool(s) of TAG resident within the secretory pathway.

## MATERIALS AND METHODS

*Preparation and culture of hepatocytes.* Hepatocytes were isolated from female Wistar rats (200–220 g) by a two-step collagenase perfusion protocol, as described previously (7). They were plated onto 6-cm diameter plastic dishes and allowed to attach in 3 mL of Earls balanced salts solution (EBSS) containing 10 mM HEPES (pH 7.4) and supplemented with new-born calf serum, amino acids, and antibiotics for 4 h, as described previously (7). The medium was then removed, and fresh serum-free EBSS medium was added, supplemented with 0.75 mM oleate complexed to albumin (final concentration 1%), 1 mM pyruvate, 10 mM lactate, 10 nM dexasmethasone, and antibiotics. When [$^{14}$C]-oleate was added the final specific radioactivity of the fatty acid was 0.035 μCi/μmol. After 18 h, the medium was removed and the cells were washed. Fresh medium containing different supplements, as indicated, was added, and the cellular and secreted radiolabeled lipids were quantified for the periods indicated, also as described previously (7).

*Measurement of TAG secretion rates from exogenous and endogenous sources*. The respective contributions of exogenous and endogenous acyl moieties to the secreted TAG were quantified, after extraction and separation of lipids, as described by Lankester *et al*. (7). Cellular TAG were quantified at each experimental time-point so as to obtain the specific activity of endogenous acyl moieties. Secretion rates were expressed in terms of nanomoles of fatty acid equivalent, so that utilization of endogenously generated oleoyl moieties would be directly comparable to that calculated for exogenously added oleate. Preliminary experiments established that all the secreted TAG were associated with VLDL; therefore, routinely no separation of the lipoprotein particles was performed. Incorporation of [$^{3}$H] and [$^{14}$C] labels into cellular and secreted phospholipid was also measured. Routinely, secretion of labeled phospholipid was very low and was negligible compared to that of TAG. Acid-soluble oxidation products, which under these conditions represented >95% of the total oxidation products, were measured after acid precipitation of the fatty acid substrate from solution (7).

*Measurement of apoB synthesis and degradation rates.* Hepatocytes cultured for 18 h as described above were washed and incubated in 3 mL of methionine-free EBSS medium. For the measurement of the rate of synthesis of apoB, the medium was then replaced by 1 mL of methionine-free supplemented medium to which was added 0.75 nmol of [$^{35}$S]methionine (1.14 mCi/μmol); the incorporation of label into cellular protein was allowed to proceed for 20 min, at which time the cells were solubilized by addition of 1.5 mL of a solution containing 100 mM NaCl, 5 mM EDTA, 10 mM $K_2HPO_4$, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecylsulfate (SDS), and protease inhibitors: 1 μg each of antipain, leupeptin, 5 μg *N*-acetyl-Leu-Leu-Nle-CHO and 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride•HCl. The cell extract was sonicated for 30 s and incubated at 40°C for 30 min to complete solubilization. Aliquots (20 μL) of the solubilized cells were acidified with trichloroacetic acid to precipitate total protein. After being washed twice with cold 95% acetone/water, the precipitated protein pellets were used to quantify incorporation of $^{35}$S into total protein.

ApoB was immunoprecipitated from the rest of the solubilized cell material by incubation with sheep anti-rat apoB antibody (4 h at 0°C) followed by incubation (4°C for 18 h) with donkey anti-sheep IgG antiserum. The immune precipitates were dissolved in medium containing 62 mM Tris-HCl (pH 6.8), 8 M urea, 16 mM dithiothreitol, 2% SDS, and 10% mercaptoethanol, aided by sonication. ApoB48 and apoB100 were separated by SDS-polyacrylamide gel electrophoresis as described in Reference 15. Quantification of the radioactivity associated with intact apoB48 and apoB100 was performed by phosphorimage analysis.

*Materials.* Culture media and amino acid, vitamin, and antibiotic solutions were obtained from Gibco (Irvine, Scotland). TAG assay kits were obtained from Sigma (Dorset, United Kingdom). Radiochemicals were obtained from NEN Life Science Products (Hounslow, United Kingdom).

## RESULTS AND DISCUSSION

*Effect of exogenous oleate on the use of endogenous acyl moieties for TAG secretion*. When cells were precultured overnight with [$^{14}$C]oleate, they accumulated [$^{14}$C]TAG and phospholipid intracellularly (7,16). After washing and re-incubation with fresh medium from which oleate was absent, the cells secreted [$^{14}$C]-labeled TAG linearly for at least 3 h (basal rate; Fig. 1A). Addition of oleate (0.75 mM) to the fresh incubation medium at the start of this 3 h period increased the rate of secretion of endogenous ($^{14}$C-labeled) moieties within TAG by 55% (Fig. 1A). In addition, when the exogenously added oleate was $^{3}$H-labeled, it too contributed acyl moieties (about 20% of the total, Fig. 1B) toward TAG secretion (Fig. 1B). Consequently, the overall rate of TAG secretion (from endogenous and exogenous acyl moieties) was increased twofold by the addition of oleate (compare filled symbols in Figs. 1A and 1B), with more than half the stimulation being due to the enhanced secretion of endogenous, prelabeled acyl moieties.

*Secretion of TAG after depletion of ER $Ca^{2+}$*. Depletion of the thapsigargin-sensitive $Ca^{2+}$ store of the ER resulted in inhibition of the utilization of both endogenous and exogenous acyl moieties for TAG secretion (Fig. 2A,B, respectively). However, the pattern of inhibition was different for the two sources. Secretion of [$^{14}$C]oleoyl moieties continued for at least 30 min, after which it was totally inhibited. By contrast,

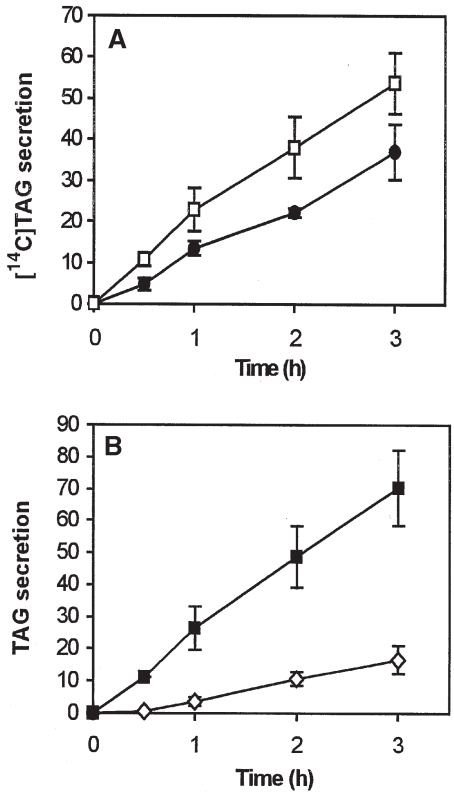


**FIG. 1.** The effect of oleate addition on the secretion of endogenous oleoyl chains within triacylglycerol (TAG). In (A) hepatocytes were cultured for 18 h in the presence of 0.75 mM [$^{14}$C]oleate. They were then washed and incubated for 3 h in fresh medium in the absence (●) or presence (□) of 0.75 mM 9,10[$^{3}$H]oleate. In (B) the rate of appearance of $^{3}$H-labeled acyl chains (◇) within secreted TAG is shown together with total ($^{14}$C- plus $^{3}$H-labeled) TAG secretion (■). Values are expressed as nmol fatty acid per mg of cell protein and are means (± SEM) for three separate preparations, each of which was analyzed in duplicate.

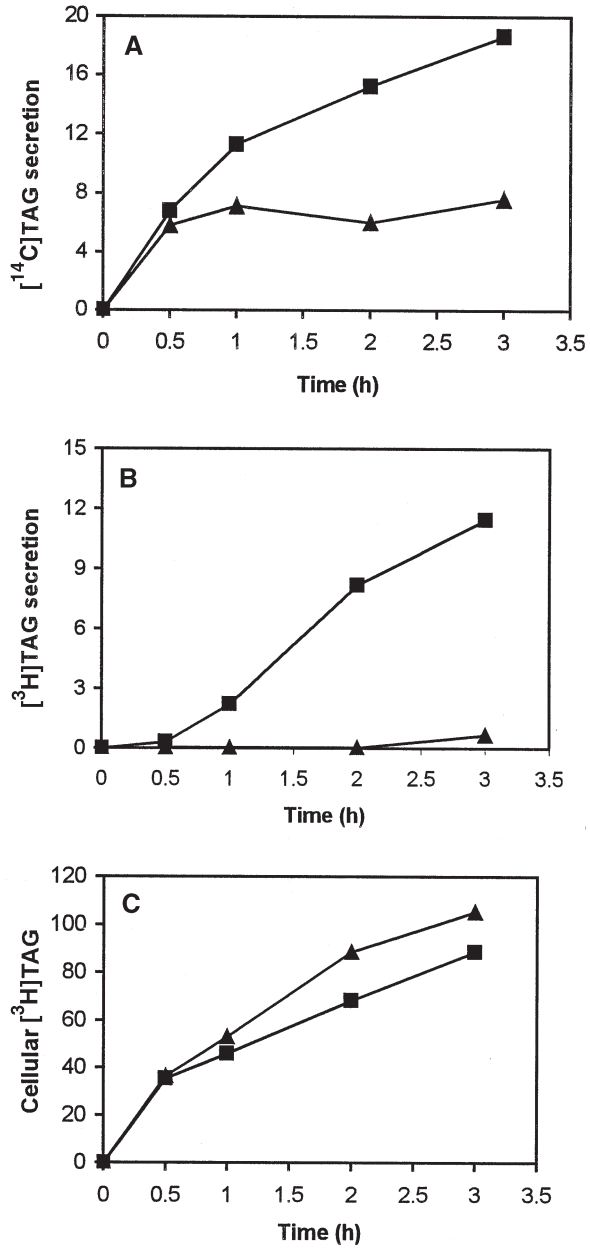


**FIG. 2.** The effects of thapsigargin on the time courses for the secretion and intracellular accumulation of TAG. Cells were prelabeled with [$^{14}$C]oleate for 18 h, washed, and incubated for the indicated times with 0.75 mM [$^{3}$H]oleate in the presence (▲) or absence (■) of thapsigargin. (A) Secretion of $^{14}$C-labeled TAG; (B) secretion of $^{3}$H-labeled TAG; (C) accumulation of $^{3}$H-labeled TAG within the cell. They are from an experiment representative of three similar experiments performed on separate cell preparations. See Figure 1 for abbreviation.

secretion of [$^{3}$H]oleoyl moieties was totally inhibited immediately upon addition of thapsigargin at zero time. Importantly, however, this was achieved without inhibition of TAG synthesis from exogenous [$^{3}$H]oleate (Fig. 2C), which in fact showed a small stimulation, presumably because acyl groups were diverted from TAG secretion to cytosolic TAG accumulation (7). These combined data indicated that depletion of ER $Ca^{2+}$ inhibited the further formation of new lipoprotein particles without inhibiting either (i) the secretion of TAG already within the secretory pathway or (ii) the cellular synthesis of TAG. Thus, the continued secretion of $^{14}$C-labeled TAG for 30 min after addition of thapsigargin is suggested to represent the emptying, from the ER, of secretion-competent [$^{14}$C]TAG pools already present within the secretory compartment prior to the addition of thapsigargin. These intra-ER pools of prelabeled TAG would have included TAG within the nascent apoB-containing particles present within the rER, and non-apoB-associated TAG within the lipid globules of the sER (2–4). The abrupt cessation of [$^{14}$C]TAG secretion after 30 min suggests that, when one or the other of these pools became exhausted, secretion ceased. Since synthesis of TAG was not affected by addition of thapsigargin (Fig. 2C), it would be expected that synthesis of non-apoB-associated TAG within the sER would not have been affected and that this apoB-free pool of TAG would not have become limiting. It is likely therefore that it was the availability of apoB-associated (nascent particle) TAG within the rER that became exhausted after the 30-min time. The time required to empty this pool (approximately 30 min; see Fig. 2A) is of the same order

as that required for assembly of VLDL particles (17). In cells precultured overnight with [$^{14}$C]oleate, the secretion-competent pool of TAG resident within the ER that continued to be secreted after addition of thapsigargin may be calculated to represent approximately 3% of the total cellular TAG.

Thapsigargin inhibits the $Ca^{2+}$-ATPase of the ER (18,19) and therefore produces complete depletion of the relevant pool of $Ca^{2+}$. To verify that inhibition of entry of acyl moieties in newly secreted TAG was proportional to the inhibition of a parameter affected by $Ca^{2+}$ mobilization, we tested conditions that mobilize the ER $Ca^{2+}$ pool suboptimally, as described previously (14). These conditions included additions of hormones and agonists that activate ER $Ca^{2+}$ mobilization, as well as 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ), another inhibitor of the ER $Ca^{2+}$-ATPase (20), in the presence or absence of extracellular $Ca^{2+}$ (Figs. 3,4). The data in Figure 3 show that submaximal depletion of this pool resulted in partial inhibition of the entry of exogenous [$^3$H]oleate into the secretory pathway. The competition between synthesis of cellular (cytosolic) TAG and of secretory TAG for a common intermediate—suggested to be cytosolic DAG (7)—is indicated by the inverse relationship between the graded effects of $Ca^{2+}$ mobilization on the two parameters (Fig. 3A,B). Qualitatively similar inhibition of [$^{14}$C]TAG secretion was also observed (not shown).

*Role of the inhibition of apoB synthesis in the inhibition of TAG secretion by $Ca^{2+}$ mobilization*. The observation that submaximal $Ca^{2+}$ mobilization from the ER resulted in a graded inhibition of the entry of exogenous oleate into the secretory pathway (Fig. 3A) suggested that the inhibition of TAG secretion was due to the inhibition of apoB synthesis, especially since it is well-established that $Ca^{2+}$ mobilization from the ER inhibits the initiation of protein synthesis (21). When the initial rates of incorporation of [$^{35}$S]methionine into apoB48 and apoB100 were measured, inhibition of TAG secretion was found to be proportional to inhibition of apoB synthesis (Figs. 4A–D). This relationship was particularly strong between the rate of incorporation of [$^{35}$S]methionine into apoB48 and the rate of TAG secretion (Fig. 4A,B) as would be expected from the fact that, under the present culture conditions, more than 80% of total apoB secreted was as apoB48 (22). In contrast to [$^3$H]oleate incorporation into secreted TAG, a residual amount of secreted endogenous $^{14}$C-label was insensitive to the inhibition of apoB synthesis (Fig. 4A,C) as would be expected if this residual secretion of [$^{14}$C]TAG were due to the emptying of the $^{14}$C-labeled TAG already present within the ER before the addition of thapsigargin. Importantly, in parallel experiments $Ca^{2+}$ mobilization (in the presence or absence of exogenous oleate) was shown not to affect apoB degradation (data not shown). One may conclude that under conditions of constant apoB degradation, modulation of the rate of apoB synthesis is able to exert strong control over the rate of TAG secretion by becoming rate-limiting for the formation of nascent particles. The observation that both apoB synthesis and [$^3$H]TAG secretion were fully inhibited in the presence of thapsigargin (Fig. 4B,D) explains the instantaneous inhibition of the incorporation of exogenous [$^3$H]oleate into secreted TAG upon addition of the $Ca^{2+}$-ATPase inhibitor at zero-time (Fig. 2B) as expected if, under these conditions, no further nascent particle formation occurred.

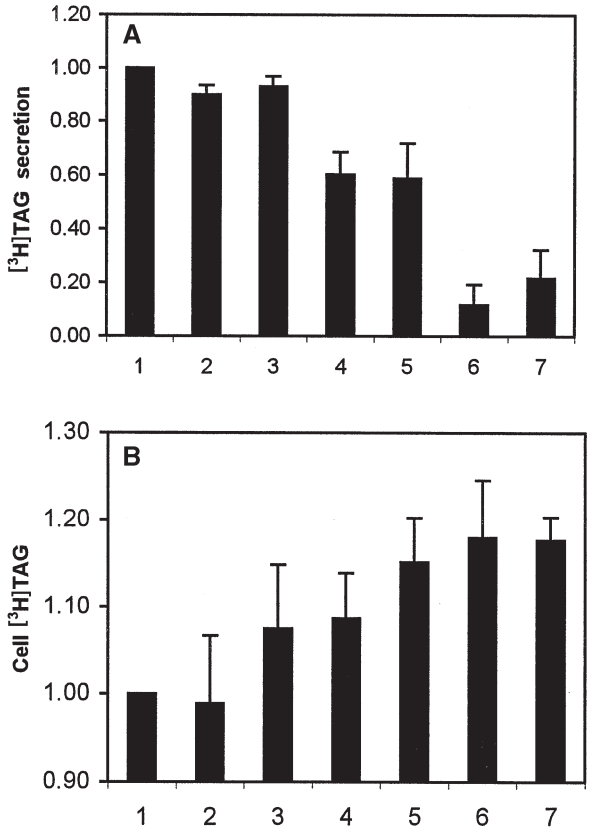


**FIG. 3.** The effect of increasing the degree of $Ca^{2+}$ mobilization in cultured rat hepatocytes on the incorporation of $^3$H-labeled acyl chains into (A) secreted TAG and (B) cellular TAG. Hepatocytes were cultured for 18 h in the presence of 0.75 mM oleate. They were then washed and incubated for 3 h in medium containing 0.75 mM 9,10[$^3$H]oleate. The incorporations of the label into (A) secreted and (B) cellular TAG were measured after 3 h. The acyl chain equivalents (nmol fatty acid/mg cell protein) secreted or accumulated were calculated from the specific activity of exogenous [$^3$H]oleate; see the Materials and Methods section. Values are expressed relative to those of paired control incubations and are means (±SEM) for four separate hepatocyte preparations. Those for control incubations have been set at unity. Conditions were: (1) Control medium, (2) plus 10 nM glucagon, (3) 100 nM vasopressin, (4) 10 nM glucagon plus 100 nM vasopressin, (5) 1 nM phenylephrine plus 10 nM glucagon, (6) 25 μM 2,5-di(*tert*-butyl)-1,4-benzohydroquinone or (7) 100 μM thapsigargin. All dishes had a final concentration of 0.1% dimethylsulfoxide, which was used as solvent for some of the additions. For other abbreviation see Figure 1.

There was a degree of selectivity in the inhibition of [$^{35}$S]-methionine incorporation into apoB48 or apoB100, in that the inhibition of incorporation into total protein was much smaller for any condition tested (approximately 30% of that into the apoB proteins). This may have arisen partly from the fact that $Ca^{2+}$ mobilization inhibits protein synthesis initiation but not peptide chain elongation (23). Therefore, $Ca^{2+}$ mobilization would be expected to have a disproportionate effect on [$^{35}$S]-

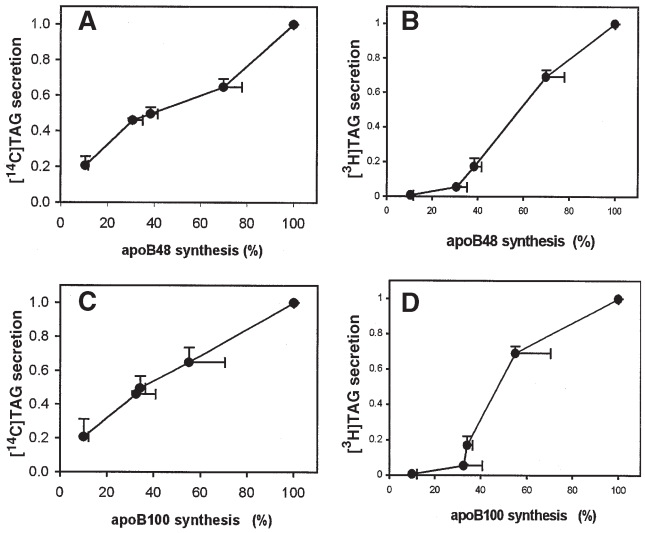


**FIG. 4.** Dependence of TAG secretion on the rate of apoB48 and B100 synthesis in cultured rat hepatocytes subjected to different extents of $Ca^{2+}$ mobilization. In parallel experiments either TAG secretion rates from endogenous ($^{14}C$) and exogenous ($^{3}H$) acyl sources were measured over 3 h or the initial rate of [$^{35}S$]methionine incorporation into apoprotein B48 (apoB48) and apoB100 was quantified under various conditions (control, plus glucagon and phenylephrine, minus calcium chloride, plus EGTA, plus thapsigargin; see legend to Fig. 3). The relationships of the rates of secretion of [$^{14}C$]TAG (A,C) and of [$^{3}H$]TAG (B,D) to the syntheses of apoB48 (A,B) or apoB 100 (C,D) are shown. Values are expressed relative to the rates observed for paired incubations performed in control media, which are set to unity. Data are means (±SEM) for three separate cell preparations. Where the error bars do not show they lie within the symbol.

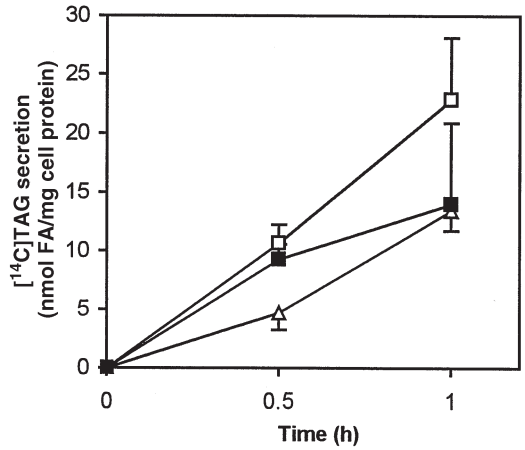


**FIG. 5.** Effect of exogenously added oleate on the rate of TAG secretion from endogenous acyl moieties in the presence or absence of thapsigargin. Cells were prelabeled by culture for 18 h in the presence of 0.75 mM 1[$^{14}C$]oleate. They were then washed and incubated for the times shown either in the absence (△, basal secretion) or the presence of 0.75 mM oleate and either in the absence of thapsigargin (□) or in its presence (100 μM, ■). Data are means (±SEM) for three separate cell preparations. FA, fatty acid; for other abbreviation see Figure 1.

methionine incorporation into larger proteins (e.g., apoB48 and apoB100) with longer chain-elongation times (22).

*Effect of exogenous oleate on the secretion of TAG present within the secretory pathway.* The inability of $Ca^{2+}$ mobilization to inhibit the secretion of [$^{14}C$]TAG already within the secretory pathway while inhibiting the synthesis of apoB enabled us to test the effect of exogenous oleate on the rate of emptying of secretion-competent TAG from the secretory pathway. Figure 5 shows time courses for the secretion of prelabeled, endogenous [$^{14}C$]-labeled moieties within TAG in the presence and absence of exogenous oleate added at zero time. In the absence of thapsigargin, the addition of exogenous oleate markedly stimulated the rate of secretion of [$^{14}C$]TAG (Fig. 1). In the presence of thapsigargin, the initial rate of [$^{14}C$]TAG secretion was equally stimulated (compare 30-min time-points in Fig. 5). Because addition of thapsigargin inhibited entry of acyl groups into the secretory pathway (Fig. 2B) the stimulation of [$^{14}C$]TAG secretion occurred under conditions in which (i) the exogenous oleate itself made no contribution toward overall TAG secretion and (ii) new apoB synthesis was maximally inhibited (Fig. 4). Thus, these data show that the stimulation by exogenous oleate of the rate of secretion of endogenous acyl chains involved a stimulation in the rate of processing, or emptying, of [$^{14}C$]TAG through the secretory pathway. That exogenous oleate did not affect the size of the secretion-competent pool of [$^{14}C$]TAG thus emptied (compare lower two curves in Fig. 5), but only the rate at which it was secreted, suggests that the accelerated emptying of the [$^{14}C$]TAG resident within the ER was not due to recruitment of additional TAG stores within the ER. Instead, accelerated emptying may have been due to indirect activation of the emptying of the ER by an increase in the concentration of an effector metabolite of oleate (e.g., oleoyl-CoA) within the ER lumen rather than to a direct mass-action effect. Previous studies ruled out the possibility that such a metabolite could act by altering the stability of apoB in rat hepatocytes (24), as confirmed in ths study, but other processes, such as second-step lipidation of the nascent particles by the non-apoB-associated TAG within the sER, may be affected by a metabolite generated from exogenous oleate. The identity of such a putative effector is currently under study.

Even when the contribution of cytosolic droplet TAG lipolysis is abolished—by inhibition of the entry of acyl moieties into the secretory pathway that accompanies the inhibition of apoB synthesis—exogenous oleate still stimulates TAG secretion from endogenous sources distal to apoB synthesis and therefore to nascent particle assembly. We conclude that this (these) pool(s) of TAG occur(s) within the secretory pathway itself. That this TAG secretion continued for the same time that it takes to assemble and secrete VLDL particles and that the total amount of TAG secreted was the same, irrespective of its rate of secretion (Fig. 5), suggest that the amount of TAG secreted, after inhibition of apoB synthesis, is determined by the amount of secretion-competent apoB already present within the ER before mobilization of $Ca^{2+}$ occurs.

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# Necrosis and Apoptosis in Lymphoma Cell Lines Exposed to Eicosapentaenoic Acid and Antioxidants

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**ABSTRACT:** The present study is focused on the role of oxidative stress in the induction of either necrosis or apoptosis by eicosapentaenoic acid (EPA) in the lymphoma cell lines Raji and Ramos, respectively. To investigate the different death modes induced by EPA, we assessed the importance of some antioxidants and reactive oxygen species in the two cell lines. We observed that different antioxidants counteracted the necrotic effect of EPA on Raji cells to a different extent, and that vitamin E counteracted EPA-induced accumulation of superoxide anion in this cell line. On the contrary, no effects of antioxidants were observed on development of apoptosis induced by EPA in Ramos cells, and vitamin E did not counteract EPA-induced accumulation of superoxide anions in Ramos cells. Moreover, apoptosis was partly inhibited by transcription inhibitors (actinomycin D) and protein synthesis inhibitors (cycloheximide), suggesting dependency upon new protein synthesis prior to apoptosis. Kinase inhibitors (staurosporin and calphostin C) did not alter the EPA-induced apoptosis. The observed cellular accumulation of superoxide anion following EPA incubation may be important for induction of necrosis in Raji cells. In contrast, none of the other investigated parameters indicated a role of oxidative stress promoted by EPA in the induction of apoptosis in Ramos cells.

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Cross-sectional epidemiological studies suggest that n-3 fatty acids may protect against development of cancer in colon, breasts, and the prostate gland (1,2). Furthermore, reductions in tumor growth and metastasis have been observed in animals fed n-3 fatty acids (3–5). These effects may be due to reduced cell multiplication, as observed in several cancer cell lines derived from breast, colon, prostate, and lung incubated with polyunsaturated fatty acids (PUFA) (6–9). In some of these studies different sensitivity to PUFA has been reported (9–11). This was also observed when we studied the sensitivity of 14 different leukemia/lymphoma cell lines to incubation with PUFA (12). In 10 of these cell lines, reduced cell multiplication was observed with 30–60 μM of arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), or docosahexaenoic acid (22:6n-3), whereas four cell lines were resistant to PUFA. The sensitivity to fatty acids was specific for PUFA as none of the cell lines was sensitive to oleic acid (18:1n-9) or stearic acid (18:0) (13). The reduction in cell number with PUFA could be due to modulation of cell differentiation and proliferation as well as to induction of apoptosis or necrosis. In the promyelocytic HL-60 cell line, differentiation was accompanied by both apoptosis and necrosis after incubation with EPA or AA (13). In Raji cells EPA induced necrosis, whereas apoptosis predominantly was observed in Ramos and U937-1 cells based on data obtained by staining with propidium iodide (PI) and Hoechst 33342 (HO342) and flow cytometry (12,14). Thus, the same bioactive molecule (EPA) induces different biological responses in different cell types. We also have reported that addition of a single dose of vitamin E counteracted necrosis induced by EPA in Raji cells, whereas vitamin E had no effect on EPA-induced apoptosis in Ramos cells (12).

Although growth inhibition of several tumor cell lines has been observed, the molecular mechanisms by which PUFA execute their anticarcinogenic effects still are unknown (10,12,15). Oxidation products of PUFA may contribute to some of the reported effects, whereas deficiencies in cellular antioxidant defense systems may enhance the sensitivity of tumor cells (16). Cells are equipped with a repertoire of antioxidant enzymes, e.g., superoxide dismutase, catalase and glutathione peroxidase, as well as small antioxidant molecules that are mostly derived from fruits and vegetables, including tocopherols, flavonoids, carotenoids, ubiquinol and glutathione (17). Potentially lethal cellular injury can occur if these defense systems are overwhelmed. Accumulation of reactive oxygen species due to incubation with PUFA may cause lipid peroxidation, which may result in a range of products that can be deleterious to cells. Whereas massive amounts of oxidized lipid products are thought to cause gross structural or metabolic damage, moderate amounts may initiate a stress signal cascade inducing cellular death programs leading to apoptosis (18). Lipid peroxidation has been linked to a variety of diseases, including atherosclerosis, ischemia-reperfusion injury, cancer (19), and degenerative disorders like Alzheimer's and Parkinson's diseases and Down's syndrome (20–22) In addition to reactive oxygen species, several other mediators of apoptosis have been identified, e.g., CD95 ligand and receptor, damaged DNA, and ceramide (23–25). The different signals promoting apoptosis may utilize common pathways including caspases (26). During the apoptotic process a series of stereotypic mor-

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Abbreviations: AA, arachidonic acid; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DHE, dihydroethidium; EPA, eicosapentaenoic acid; FCS, fetal calf serum; GSHPx, glutathione peroxidase; HO342, Hoechst 33342, also known as bisbenzimide; NAC, *N*-acetyl-cysteine; PBS, phosphate-buffered saline; PI, propidium iodide; PUFA, polyunsaturated fatty acids; SPBN, *N-tert*-butyl-2-sulfophenylnitrone; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

phological changes occur, like condensation and fragmentation of nuclear chromatin and decrease in cell volume and alteration in the plasma membrane, which cause recognition and phagocytosis of apoptotic cells. In contrast, the necrotic process promotes cell swelling and bursting before release of cell content, which may induce inflammatory response.

The aim of the present study is to elucidate mechanisms by which EPA causes cell death, using Raji and Ramos cells as models for necrosis and apoptosis. We describe the effect of EPA and antioxidants in relation to necrotic and apoptotic cell death. Our experiments demonstrate that EPA-induced necrosis may be counteracted by antioxidants up to 24 h after addition of EPA. On the contrary, we found no effect of antioxidants on EPA-induced apoptosis. Our experiments furthermore indicate that selenium-induced glutathione peroxidase (GSHPx) and protein kinase are not essential for EPA-induced apoptosis.

## MATERIALS AND METHODS

*Materials.* EPA, fatty acid-free bovine serum albumin (BSA), PI, HO342, vitamin E, vitamin C, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, *N*-acetyl-cysteine (NAC), *N-tert*-butyl-2-sulfo-phenylnitrone (SPBN), sodium selenite, dihydroethidium (DHE), and 2,7-dichlorofluorescein diacetate were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphate-buffered saline (PBS), RPMI-1640, fetal calf serum (FCS), L-glutamine, and gentamycin were bought from BioWhittaker (Walkersville, MD). Other chemicals were analytical grade from commercial suppliers. Fujichrome Provia 400 professional color reversal film was supplied from Fuji (Tokyo, Japan). The In Situ Cell Detection kit Fluorescein was purchased from Boehringer Mannheim (Mannheim, Germany).

*Cells*. The leukemic cell lines Raji and Ramos were purchased from BioWhittaker, and cultured in suspension in RPMI-medium supplemented with 10% heat-inactivated FCS, l-glutamine (2 mmol/L), and gentamycin (0.1 mg/mL). Cells were incubated with fatty acids complexed to BSA at a molar ratio of 2.5:1 and then added to regular RPMI medium. The cells were routinely kept in logarithmic growth at 0.2–1.2 $\times 10^6$ cells/mL. For experiments, cells were seeded at a density of $0.3 \times 10^6$ cells/mL. Cell count experiments were performed in duplicate by use of a Coulter Z1 counter (Coulter Electronics Limited, Luton, Bedfordshire, United Kingdom). Cell cultures incubated without fatty acids were used as control in all cell-count experiments and defined as 100%. The effect of certain compounds on cell number was presented as percentage of control. A concentration of 0.1% ethanol, used in the experiments with vitamin E and BHT, affected neither cell viability nor cell multiplication.

*Cell viability*. For microscopic analyses of cell viability (27) 1 mL of the cell cultures was first incubated with 10 µL of PI (0.5 mg/mL) in the dark for 30 min. Thereafter, 10 µL of HO342 (1 mg/mL) was added to the same cultures and incubated for another 30 min in the dark. Staining with PI indicated leaky plasma membranes since this dye cannot cross intact cell membranes. HO342, however, crosses intact as well as distorted plasma membranes and stains the nuclei in all cells; it stains most strongly nuclei with DNA-damage. After staining, the cells were sedimented at $514 \times g$ for 5 min in a swing-out rotor, and the medium was removed. After washing in PBS and resedimentation, the cell pellets were resuspended in 10 µL of pure FCS. Drops of the cell suspensions were placed on a microscope slide and air-dried before at least 200 cells were counted in a Leitz Ortholux II fluorescence microscope (Leica, Wetzlar, Germany). The cells were photographed with Fujichrome Provia 400 professional color reversal film with an MPS 48/52 camera (Leica Mikroskopie und Systeme, Wetzlar, Germany). The film was exposed as 800 ISO, but developed as 400 ISO. DNA fragmentation was quantified by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) using the In Situ Cell Death Detection kit and a FACScan Flowcytometer (Becton Dickinson, San Jose, CA) (28). Five thousand cells from each culture were analyzed.

*Detection of superoxide anion*. Superoxide anion was quantified by DHE in a final concentration of 0.01 µg/mL. After adding the staining solution, the cells were incubated for 15 min at room temperature under an aluminum foil tent before the cells were fixed in 2% paraformaldehyde. Red fluorescence from oxidized ethidium was visualized by fluorescence microscopy (FACScan Flowcytometer) in a total of 10,000 cells per sample. Data were processed using Consort 32 Lysis II software (Becton Dickinson). The percentage of ethidium-positive cells was determined by flow cytometry.

*Determination of GSHPx activity*. GSHPx activity was determined by an assay that couples the reduction of peroxides and the oxidation of glutathione with the reduction of oxidized glutathione by glutathione reductase, using NADPH as a cofactor. Hydroperoxide reduction was followed by a decrease in NADPH absorbance at 340 nM (29).

*Statistics*. Student *t*-test was used to determine the significance level of differences between sample groups. The level of significance was set to $P \leq 0.05$. Results are presented as means ± standard deviation (SD).

## RESULTS

*Antioxidants counteract EPA-induced necrosis in Raji cells, but not EPA-induced apoptosis in Ramos cells.* Raji and Ramos cells were incubated with one of the following antioxidants: 10 µM BHT, 50 µM vitamin E, 20 µM BHA, 100 µM SPBN, 100 µM vitamin C, 1 mM NAC, or 5 µM propyl gallate prior to the addition of 30 µM EPA. Another dose of the respective antioxidants was added to the cells after 12 and 24 h. The cell numbers were counted after 12, 24, and 48 h, and cellular viability was determined after 48 h. EPA in an amount of 30 µM promoted 40% reduction in cell number of Raji cells after 48 h of incubation. Furthermore, EPA induced leaky cell membranes and no sign of nuclear condensation as evaluated by PI and HO342 (12). This indicates necrosis rather than apoptosis (Fig. 1A,B). The reduction in cell num-

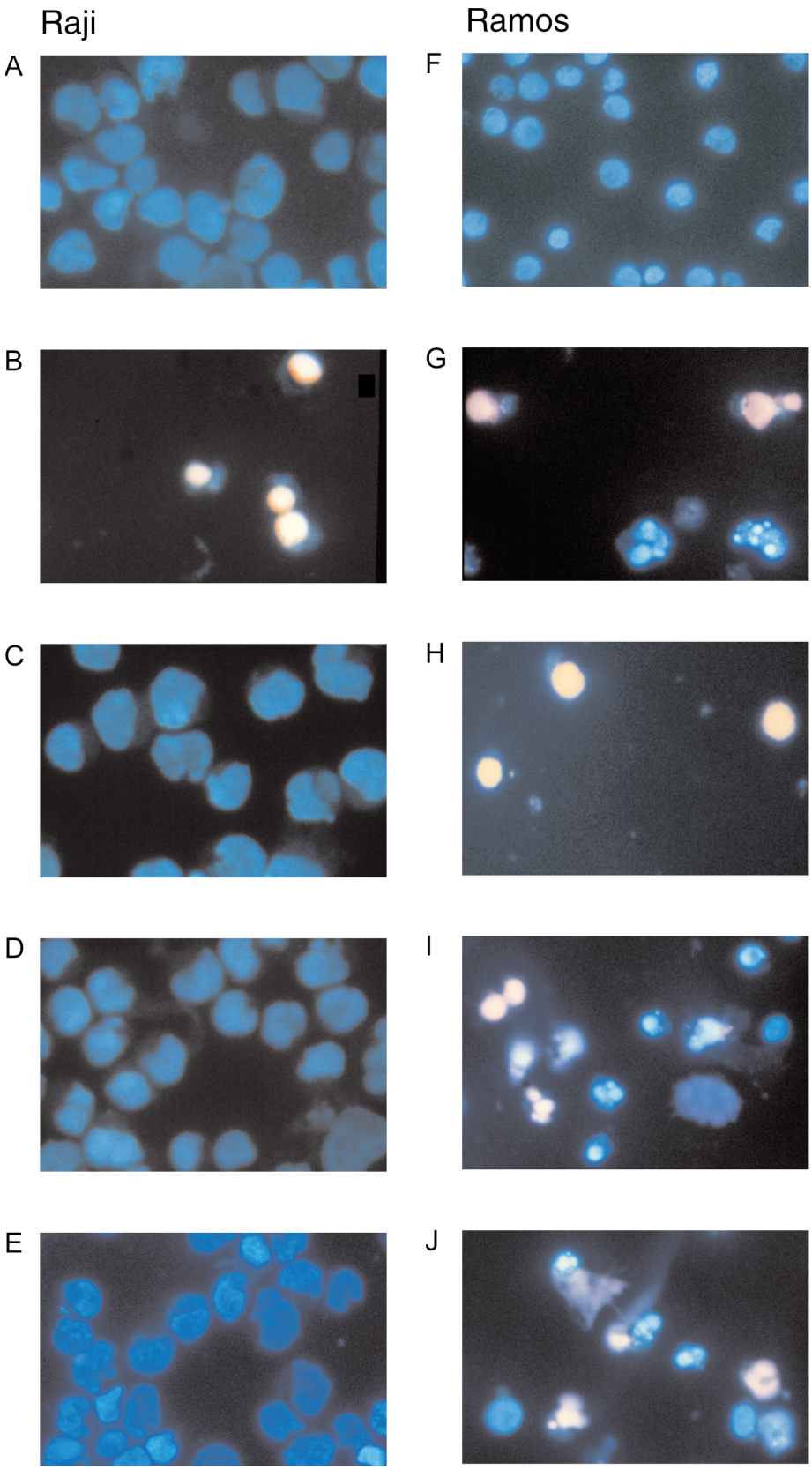


**FIG. 1.** Effect of eicosapentaenoic acid (EPA) with and without antioxidants on Raji (A–E) and Ramos (F–J) cell integrity. Propidium iodide (PI) and Hoechst 33342 (HO342) fluorescence were monitored after 48 h incubation in control cells (A,F), 60 μM EPA (B,G), 100 μM vitamin C + 60 μM EPA (C,H), 50 μM vitamin E + 60 μM EPA (D,I) and 10 μM butylated hydroxytoluene (BHT) + 60 μM EPA (E,J). PI and HO342 associate with DNA and emit red and blue light, respectively, when exposed to ultraviolet (UV) light. Staining with PI indicates leaky plasma membranes since this dye cannot cross intact cell membranes. HO342, however, crosses intact as well as distorted plasma membranes and stains the nuclei in all cells. When HO342 associates with condensed chromatin found in apoptotic cells, the blue color becomes more intense (almost white in the photos). One set of photographs from three separate experiments is shown to provide a qualitative impression about the cellular viability after 48 h.

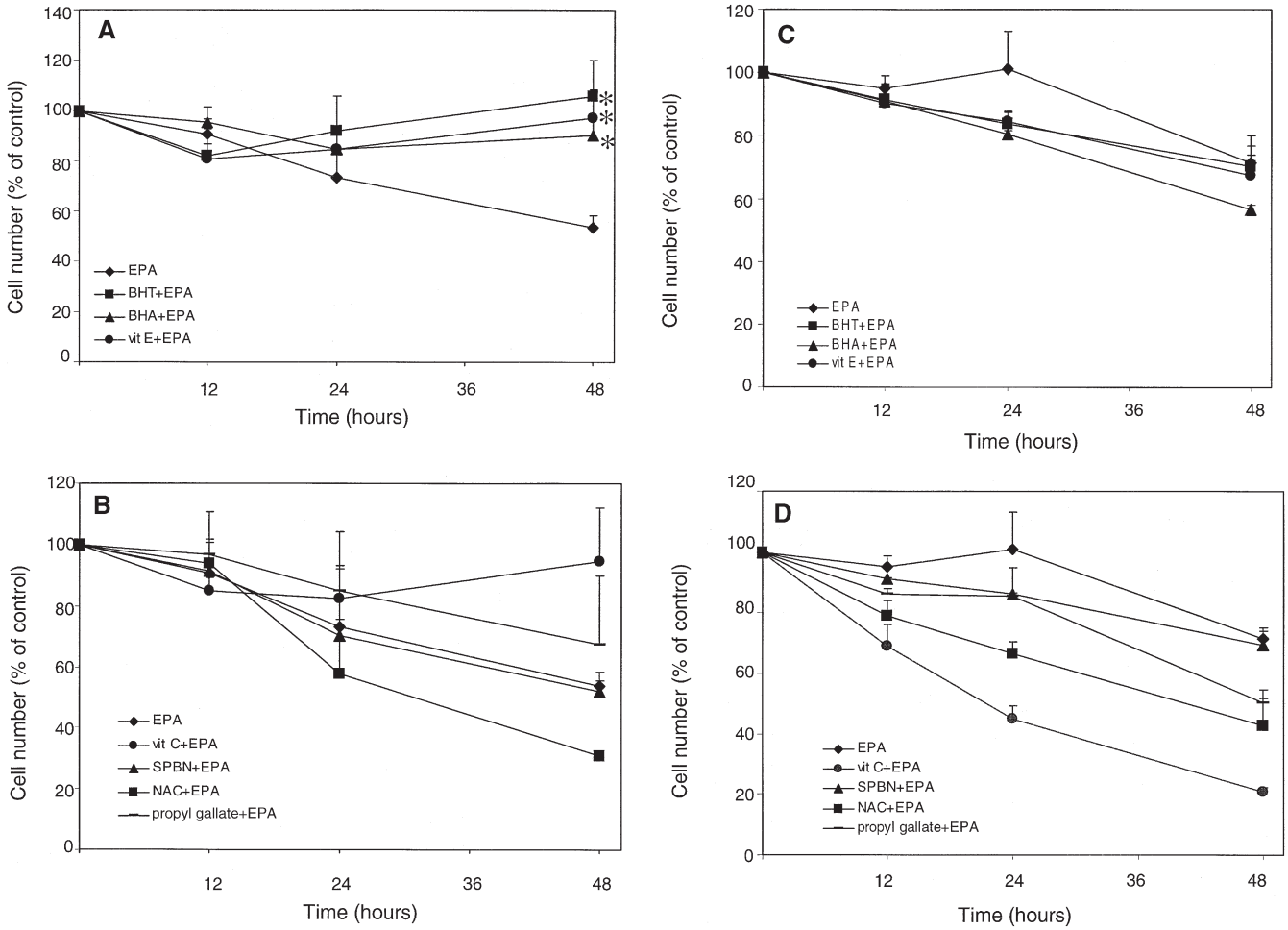


**FIG. 2.** Effect of antioxidants on number of Raji cells (A,B) or Ramos cells (C,D) incubated with EPA. In addition to 30 μM EPA, cells were incubated with water-soluble antioxidants [B,D; 100 μM *N-tert*-butyl-2-sulfophenylnitrone (SPBN), 100 μM vitamin C (vit C), 1 mM *N*-acetylcysteine (NAC) or 5 μM propyl gallate], and lipid-soluble antioxidants [A,C; 10 μM BHT, 50 μM vitamin E (vit E), or 20 μM butylated hydroxyanisole (BHA)]. The antioxidants were added at 0, 12, and 24 h after addition of EPA. Cell numbers were counted at all time points and are presented as percentages of control cells. Data represent means ± SD from triplicates of three separate experiments ($n = 3$). Absolute values for Raji control cells (100%) were 0.38 ± 0.08, 0.57 ± 0.09, and 1.36 ± 0.13 million cells/mL after 12, 24, and 48 h, respectively. For Ramos control cells the absolute values were 0.46 ± 0.11, 0.62 ± 0.17 and 1.44 ± 0.45 million cells/mL. For abbreviations see Figure 1. $^*P \leq 0.05$ compared to EPA-treated cells.

ber and viability of Raji cells was counteracted by BHT, vitamin E, BHA, vitamin C, and to some extent by propyl gallate (Fig. 2A,B). Two of the tested antioxidants did not oppose the EPA-induced reduction in cell number; SPBN had no effect, whereas NAC reduced the cell number more than EPA alone.

The following experiments were limited to the lipid-soluble antioxidants BHT and vitamin E, and the water-soluble vitamin C. The antioxidants were incubated with increasing concentrations of EPA. Incubation of vitamin E or BHT counteracted the effect of 30–60 μM EPA (Fig. 3), whereas vitamin C was less effective than the other antioxidants in counteracting 45 and 60 μM of EPA in Raji cells as evaluated by cell counting. By staining with PI and HO342 to investigate cell integrity, the counteractive effect of vitamin E, vitamin C, and BHT on EPA-incubated Raji cells is clearly demonstrated in Figure 1A–E. It should be noted that there is little difference between the effects of 30 and 60 μM EPA on the number of cells.

In contrast to the marked effects of antioxidants on the EPA-induced necrosis in Raji cells, the antioxidants BHT, vitamin E, and vitamin C had no influence on the apoptotic effect of 60 μM EPA in Ramos cells after 48 h incubation (Fig. 1F–J). Moreover, we found that the lipid-soluble antioxidant BHA did not affect the apoptotic effect of EPA on Ramos cells when counting the cells (Fig. 2C). Incubation of the water-soluble antioxidants SPBN, vitamin C, propyl gallate, and NAC in combination with EPA caused an even greater reduction in cell number compared to Ramos cells incubated with EPA alone (Fig. 2D).

*Effects of vitamin E on EPA-induced necrosis in Raji cells.* We investigated whether the effect of vitamin E differed with preincubation of Raji cells with EPA. Thus, vitamin E (50 μM) was added either at the same time as EPA or after 12 or 24 h incubation. We found that vitamin E added at 12 and 24 h after EPA administration reduced the EPA-induced necrosis to the same extent as when vitamin E was added at the same time as EPA (Fig. 4).

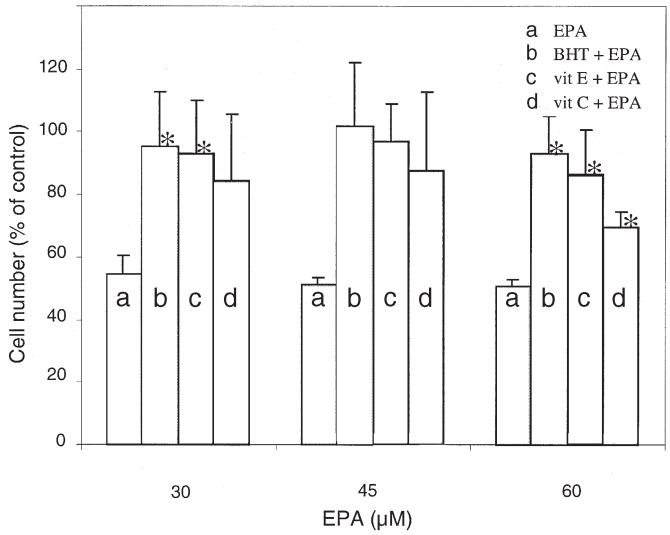


**FIG. 3.** Effect of antioxidants on Raji cells incubated with different concentrations of EPA. BHT (10 μM), vitamin E (50 μM), or vitamin C (100 μM) was added to Raji cells prior to the addition of 30, 45, or 60 μM EPA. The respective antioxidants were added at 0, 12, and 24 h after addition of EPA. Cell numbers were determined after 48 h incubation, and presented as percentages of control cells. Data represent means ± SD from three separate experiments performed in triplicate ($n = 3$). Absolute values for control cells (100%) were 0.45 ± 0.10, 0.56 ± 0.08, and 1.44 ± 0.17 million cells/mL after 12, 24, and 48 h, respectively. For abbreviations see Figure 1. *$P \leq 0.05$ compared to EPA-treated cells.

Furthermore, we wanted to investigate whether preincubation with vitamin E prevented EPA-induced cell death. Therefore, Raji cells were incubated with vitamin E (50 μM) for 1 h, then washed twice and reincubated in the presence of EPA (30 μM) for 48 h. We found that the cell number was 89 ± 21% ($n = 3$) in Raji cells preincubated with vitamin E before addition of EPA, whereas the cell number in EPA-administered cells was 43 ± 14% ($n = 3$) as compared to control cells.

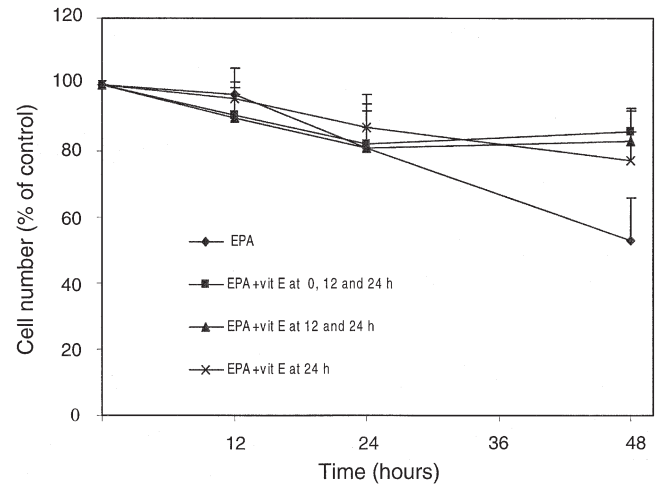


**FIG. 4.** Effect of preincubation of Raji cells with EPA before vitamin E administration. Vitamin E (50 μM) was added either three times (0, 12, and 24 h), two times (12 and 24 h), or one time (24 h) after incubation with EPA (60 μM). Data represent means ± SD from three separate experiments performed in triplicate ($n = 3$). Absolute values for control cells (100%) were 0.34 ± 0.09, 0.78 ± 1.15, and 1.92 ± 0.32 million cells/mL after 12, 24, and 48 h, respectively. For abbreviation see Figure 1.

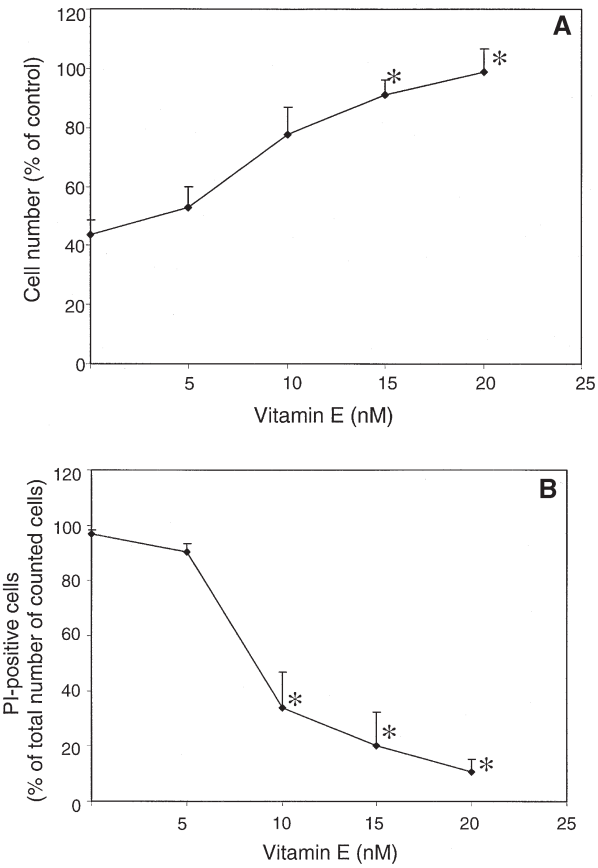


**FIG. 5.** Effect of EPA and different concentrations of vitamin E on number (A) and (B) integrity of Raji cells. Vitamin E was added at 0, 12, and 24 h after incubation with 30 μM EPA. Cell numbers and cell integrity were determined after 48 h incubation, and presented as percentage of control cells (incubated without EPA). Data represent means ± SD from three separate experiments performed in duplicate ($n = 3$). Absolute values for control cells (100%) in part A were 1.39 ± 0.18 million cells/mL. PI, propidium iodide; for other abbreviation see Figure 1. *$P \leq 0.05$ compared to EPA-treated cells.

To determine the concentration of vitamin E necessary to prevent EPA-induced necrosis, Raji cells were incubated with 5–20 nM prior to addition of 30 μM EPA. Vitamin E prevented the reduction in cell numbers in a dose-dependent manner as well as the development of necrosis promoted by EPA (Fig. 5A,B).

*Superoxide anion in necrotic and apoptotic cells incubated with EPA.* The opposing effect of antioxidants on EPA-induced cell death may indicate an elevated level of intracellular reactive oxygen species. This would be consistent with the report that PUFA interfere with cell signal systems by enhancing the level of reactive oxygen species (10). To address this issue, 50 μM vitamin E was added to Raji and Ramos cells prior to addition of 60 μM EPA. DHE, which fluoresces after oxidation by superoxide anion, was added 12, 24, and 48 h after starting the incubation with EPA. In Raji cells incubated with EPA, 95% of the cells were fluorescent after 48 h incubation. The appearance of fluorescence was completely prevented by vitamin E in cells incubated with EPA (Fig. 6A), indicating that vitamin E acts as an expected antioxidant. Incubation of Ramos cells with 60 μM EPA alone caused 40%

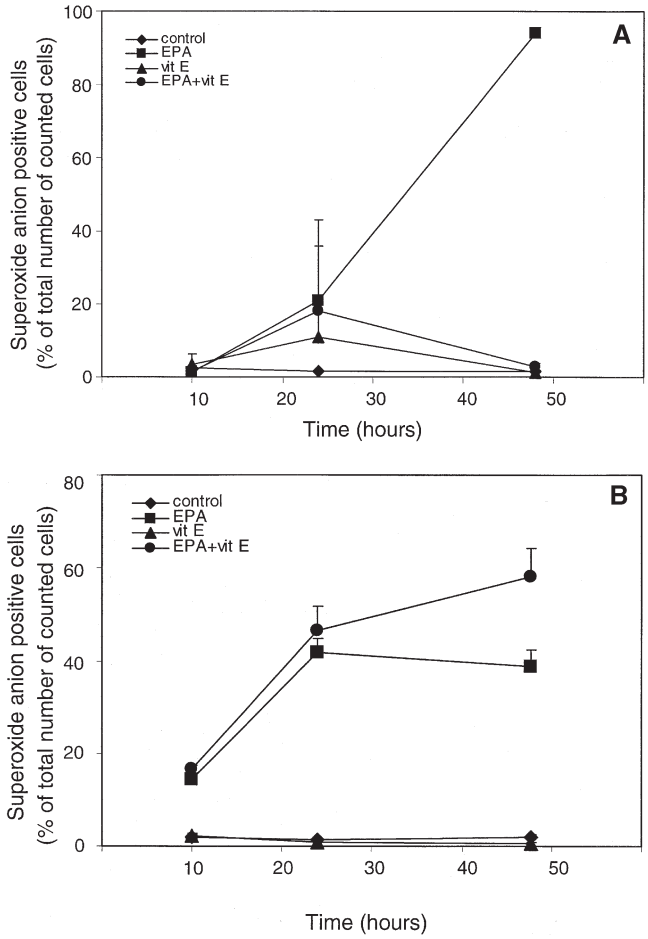


**FIG. 6.** Effect of EPA on superoxide anion level in Raji (A) and Ramos cells (B). Cells were incubated with either control medium, 60 μM EPA, 50 μM vitamin E, or 60 μM EPA + 50 μM vitamin E. Superoxide anion level was measured by flow cytometry after 10, 24, and 48 h. Data represent means ± SD from three separate experiments. For abbreviation see Figure 1.

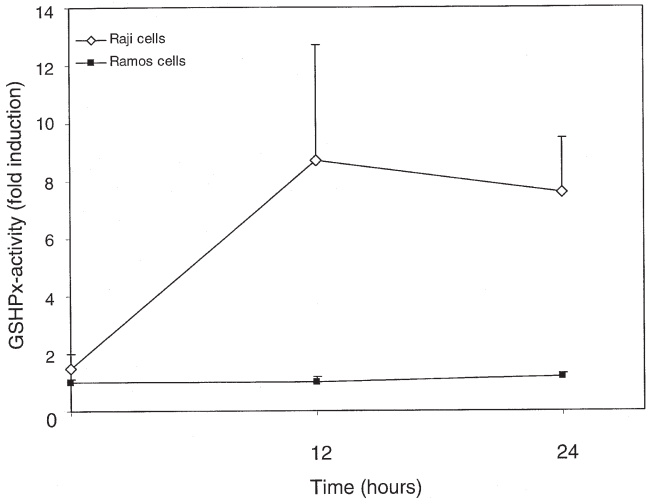


**FIG. 7.** Glutathione peroxidase (GSHPx) activity in cells incubated with 60 μM EPA. Raji and Ramos cells were incubated with 60 μM EPA up to 24 h. GSHPx activity was measured in homogenates of cells, and the data are presented as related to the levels in cells incubated with control medium. Data represent means ± SD from three separate experiments. For other abbreviation see Figure 1.

fluorescent cells, whereas the combination of vitamin E and EPA led to 60% fluorescence (Fig. 6B). These findings indicate that EPA may induce accumulation of superoxide anion in cells undergoing necrosis as well as apoptosis. Addition of vitamin E, however, only prevented development of necrosis and had no significant effect on development of apoptosis.

These experiments showed that we successfully counteracted EPA-induced accumulation of superoxide anion and the onset of necrosis by antioxidants in Raji cells. However, in Ramos cells antioxidants were unable to oppose EPA-induced apoptosis and the accumulation of superoxide anion. Nevertheless, it is possible that local changes in the concentration of reactive oxygen species are influenced by EPA. Therefore, we further evaluated the effects of EPA on glutathione and protein kinase C in Ramos cells.

*Increased GSHPx activity in Raji cells, but not in Ramos cells incubated with EPA*. To further investigate the effects of antioxidant defense mechanisms, we examined the effect of EPA on GSHPx activity. GSHPx uses glutathione as a substrate, and enhanced enzyme activity may protect against cell death caused by PUFA (16). GSHPx is also enhanced by oxidative stress (30,31). Thus, Raji and Ramos cells were treated with 60 μM EPA for 12 and 24 h. This resulted in an eightfold induction of GSHPx activity in Raji cells, whereas no such effect was measured in Ramos cells (Fig. 7). In addition, Raji and Ramos cells were incubated with sodium selenite (pretreatement of 250 nM for 24 h) to induce increased GSHPx activity and then incubated with EPA for another 12 h. This resulted in a 2.5-fold increase in GSHPx activity compared to control cells, whereas there was no protection against EPA-induced cell death (data not shown).

*Inhibitors of transcription and protein synthesis reduced EPA-promoted apoptosis*. Inhibition of either protein or RNA synthesis may prevent or delay apoptosis in different cell types (33,34). We examined whether EPA-induced apoptosis in Ramos cells could be influenced by inhibition of protein synthesis or transcription. Ramos cells were incubated with different concentrations of cycloheximide or actinomycin D, with or without 60 μM EPA. The inhibitors were added 30 min prior to EPA, and after incubation for 24 h cells were HO342-stained to assess apoptosis. Cycloheximide (at 100 nM) and actinomycin D (at 2.4 nM) reduced the apoptotic effect of 60 μM EPA by 21 and 40%, respectively. In addition to microscopic evaluation, we quantified DNA fragmentation by TUNEL-assay using flow cytometry when combining 60 μM EPA and 110 nM cycloheximide. We found that 79% of the cells exhibited fragmented DNA, in accordance with microscopic analyses. That is, with cycloheximide as inhibitor 79 ± 9% of cells showed condensed/fragmented DNA in comparison with EPA-incubated cells (±SD), and with actinomycin D the comparable value was 60 ± 2%.

*Protein kinase C inhibitors did not affect EPA-induced apoptosis*. Protein kinase C activation may reduce as well as promote apoptosis (35). Because PUFA may activate protein

kinase C (36,37) we examined whether inhibition of protein kinases could reduce EPA-induced apoptosis. Calphostin C is an inhibitor of protein kinase C and staurosporin is a potent inhibitor of several types of kinases. Calphostin C (1 nM–1 μM) as well as staurosporin (0.5 nM–0.5 μM) failed to reduce EPA-induced apoptosis in Ramos cells (data not shown).

## DISCUSSION

We observed that EPA caused oxidative stress in the two cell lines Raji and Ramos as evaluated by detection of superoxide anion, but the two cell lines differed in response to antioxidants after incubation with EPA. We demonstrated that several antioxidants effectively reduced the necrotic effect promoted by EPA in Raji cells (Fig. 2A,B). Moreover, vitamin E added up to 24 h after administration of EPA counteracted the effect of EPA in Raji cells. The small amount of vitamin E (20 nM) needed to reduce EPA-induced cell death may be explained by the relative accessibility of vitamin E *in vitro* as compared to the *in vivo* situation. Vitamin E was dissolved in ethanol in our experiments, whereas plasma vitamin E is associated with lipoprotein particles *in vivo* (38). In addition, the amount of cells compared to the number of vitamin E molecules is higher *in vivo* than in our *in vitro* experiments, which also may contribute to the observed effects of low vitamin E concentrations.

Antioxidants counteracted EPA-induced necrosis in Raji cells, suggesting formation of reactive oxygen species by EPA. This is in accordance with other reports suggesting that n-3 fatty acids and their lipid peroxidation products cause cell death in tumor cell lines (10,39). Electron "leakage" from mitochondria during normal oxidase action may give rise to oxidants such as superoxide anion, which *per se* can oxidize biological substrates but cannot trigger lipid peroxidation by abstraction of allylic hydrogens (40). However, superoxide anion may reduce hydrogen peroxide and thereby give rise to hydroxyl radicals, which are extremely strong and indiscriminant oxidants able to abstract allylic hydrogens. Hydroxyl radicals will abstract electrons from molecules located in close vicinity to the place of generation (site-specific reaction) (41). Since it is very difficult to measure hydroxyl radicals, the level of superoxide anion was measured to clarify the difference in response to EPA and antioxidants in Raji and Ramos cells. We found that vitamin E counteracted EPA-induced accumulation of superoxide anion in Raji cells, but not in Ramos cells. In fact, vitamin E further increased the level of superoxide anion in EPA-incubated Ramos cells, indicating that vitamin E in this case may act as a pro-oxidant (42). Several experiments were performed to further investigate whether reactive oxygen species had a role in EPA-induced apoptosis in Ramos cells. Glutathione is the most abundant nonprotein antioxidant in mammalian cells. Depletion of cellular glutathione has been described for oxidative and alkylating agents, tumor necrosis factor-α, and the CD95 ligand axis in different cell types (43). Cells depleted of glutathione either undergo apoptosis or become more sensitive to death-inducing agents (44–46). Glutathione plays a critical role in the cellular defense against oxidative stress by directly reacting with free radicals and reactive oxygen, or as a substrate for the enzyme GSHPx. Furthermore, GSHPx is reported to be enhanced by oxidative stress (30,31). This may explain the increase in GSHPx activity in Raji cells, which is in accordance with our observations that antioxidants counteract the effect of EPA in these cells. However, the GSHPx activity in Ramos cells was not elevated by EPA, which supports our findings that antioxidants had no effect on EPA-induced cell death in Ramos cells. The low activity of cellular selenium-dependent GSHPx may explain the sensitivity to PUFA of some tumor cell lines, and the availability of selenium may be critical for the activity of this enzyme (16). Although incubation with selenium increased the activity of GSHPx, we observed no effect on EPA-induced apoptosis. This indicates that cell death in Raji and Ramos cells is influenced more by other processes than the activity of cellular GSHPx.

Oxidative stress may trigger CD95 ligand expression, contributing to apoptosis (32). However, increased superoxide anion level in Ramos cells incubated with EPA exhibited no alteration in cellular expression of CD95 ligand (Heimli, H., and Drevon, C.A., unpublished data).

Previous studies have shown that inhibitors of macromolecular synthesis may oppose or promote apoptosis in some circumstances (34,47). The inconsistent effects of these agents on apoptosis may reflect the diverse mechanisms preceding apoptosis in different cell types. Our results show that inhibitors of transcription or protein synthesis partly inhibit apoptosis induced by EPA. This implies that Ramos cells exposed to EPA depend on translation/transcription before onset of apoptosis. This is in accordance with the report that apoptosis in lymphoid and colorectal cancer cells depends on new protein synthesis (48).

To further unravel the relations between fatty acids and apoptosis, we inhibited protein kinase C, since EPA or other PUFA may affect the activity of these proteins (36). Furthermore, protein kinase Cδ is specifically cleaved during apoptosis to a catalytically active fragment that is associated with nuclear fragmentation and cell death (49). However, there was no effect on EPA-induced apoptosis by kinase inhibitors, suggesting that activation of kinases is not necessarily involved in apoptotic processes caused by EPA.

In conclusion, antioxidants opposed EPA-induced elevation of superoxide anion and necrosis in Raji cells at different time-points, indicative of a slow structural or metabolic reversible damage caused by oxidized products. However, apoptosis induced by EPA in Ramos cells is probably not initiated by oxidative stress since vitamin E and other antioxidants, had no effect on either apoptosis or lowering EPA-induced elevation of superoxide anion.

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# Effect of 4-Hydroxy-2(*E*)-nonenal on Soybean Lipoxygenase-1

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**ABSTRACT:** The oxidation of linoleic acid by soybean lipoxygenase-1 (LOX-1) was inhibited in a time-dependent manner by 4-hydroxy-2(*E*)-nonenal (HNE). Kinetic analysis indicated the effect was due to slow-binding inhibition conforming to an affinity labeling mechanism-based inhibition. After 25 min of preincubation of LOX-1 with and without HNE, Lineweaver-Burk reciprocal plots indicated mixed noncompetitive/competitive inhibition. Low concentrations of HNE influenced the electron paramagnetic resonance (EPR) signal of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPODE)-generated $Fe^{3+}$-LOX-1 slightly, but higher concentrations completely eliminated the EPR signal indicating an active site hindered from access by 13-HPODE. HNE may compete for the active site of LOX-1 because its precursor, 4-hydroperoxy-(2*E*)-nonenal, is a product of LOX-1 oxidation of (3*Z*)-nonenal. Also, it was an attractive hypothesis to suggest that HNE may disrupt the active site by forming a Michael adduct with one or more of the three histidines that ligate the iron active site of LOX-1.

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In the animal kingdom it is generally acknowledged that 4-hydroxy-2(*E*)-nonenal (HNE) originates from lipid peroxidation of n-6 polyunsaturated fatty acids (1). Recently, specific autoxidative pathways from both 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HPODE) and 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPODE) have been defined (2). Once formed, HNE has been documented to act as a lipid signal (3). Involvement of HNE in mammalian pathologies is diverse, including Alzheimer's disease (4), Parkinson's disease (5), cancer (6), atherosclerosis (7), sporadic amyotrophic lateral sclerosis (8), and alcoholic liver disease (9).

In plants, a metabolic pathway has been found in faba beans and soybeans that involves a sequence of 9-lipoxygenase oxidation of linoleic acid to 9-HPODE, hydroperoxide lyase cleavage of 9-HPODE to give 3(*Z*)-nonenal, and lipoxygenase and hydroperoxide peroxygenase action on 3(*Z*)-nonenal to produce HNE (10–12). However, there is evidence that the last step of oxidation of 3(*Z*)-nonenal readily occurs by autoxidation, even with plant enzymes (13). Since soybean lipoxygenase-1 (LOX-1) oxidized 3(*Z*)-nonenal stereospecifically to 4(*S*)-hydroperoxy-2(*E*)-nonenal, it is obvious that HNE can access the active site of LOX-1 and other lipoxygenase isozymes (12). Three essential histidines ligate the iron active site of LOX-1 (14). It has been shown that HNE forms a Michael adduct with histidine residues (15,16), thereby implying that HNE can react with and cause suicide inhibition of LOX-1 by compromising the active site. In addition, it has been shown that HNE can react with other amino acid residues, such as cysteine (17) and lysine (18), which also can contribute to the inactivation of LOX-1. The literature abounds with other examples of enzyme inactivation or activation by HNE. Those enzymes inactivated include glucose-6-phosphate dehydrogenase (19), microsomal cytochrome P450 (20), $Na^+$-$K^+$-ATPase (21), aldose reductase (22), cytochrome C oxidase (23), α-ketoglutarate dehydrogenase (24), pyruvate dehydrogenase (24), plasma membrane ($Ca^{2+}$ + $Mg^{2+}$)-ATPase (25), glutathione peroxidase (26), and glyceraldehyde-3-phosphate dehydrogenase (27). Interestingly, multicatalytic proteinase, which clears cells of oxidized protein, is inhibited by HNE-cross-linked protein (28), implying a mechanism for accumulation of age-related lipofuscin. Enzymes activated by HNE are those involved in lipid signaling, phospholipase D (29), and phosphoinositide-specific phospholipase C (30).

In this work we demonstrate that HNE inactivates LOX-1 by a mixed noncompetitive/competitive mechanism and that at least part of the competitive mechanism may be due to the decomposition of HNE in buffer.

## MATERIALS AND METHODS

*Materials.* HNE was synthesized by a modification of a previously described method (31). Instead of oxidizing 3,4-epoxynonan-1-ol to the aldehyde by periodinane, the oxidation of Ratcliffe and Rodehorst was used (32), making certain to wash the ethyl ether solution of product HNE with NaOH, HCl, and $NaHCO_3$ solutions as prescribed by the method. The product HNE was somewhat less pure than that obtained with periodinane. Thus, HNE was purified by silicic acid (100 mesh; Mallinckrodt, Phillipsburg, NJ) column chromatography (40 g, column i.d. 2.5 cm) by sequentially eluting with 300 mL each of 7.5, and 10% acetone in hexane. HNE eluted between 200 and 300 mL in 95.5% purity. Linoleic acid was obtained from Nu-Chek-Prep (Elysian, MN). LOX-1 was isolated as previously described (33) and was stored at 3°C as a suspension in 2.3 M $(NH_4)_2SO_4$; protein concentration was 34.5 mg/mL. 13-HPODE was prepared by the method of Gardner (34).

*Time-dependent inactivation of LOX-1 by HNE.* The oxygen electrode assay (Gilson 5/6H oxygraph, Middleton, WI)

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Abbreviations: cmc, critical micelle concentration; EPR, electron paramagnetic resonance; HNE, 4-hydroxy-2(*E*)-nonenal; 9-HPODE, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 13-HPODE, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; LOX-1, soybean lipoxygenase-1.

was completed in a 2.4-mL cell held at 25˚C. The cell was filled with 0.1 M potassium borate buffer (pH 9.86) containing 0.36 μg LOX-1/mL. For zero-time measurement, 10 μL of HNE dissolved in methanol and 10 μL linoleic acid substrate (240 mM) in methanol were injected into the cell in rapid sequence. The final concentration of linoleic acid was 1 mM. Various concentrations of HNE were tested, 10 μL of 4.8, 15, 24, 30, 60, 120, and 240 mM in methanol per 2.4 mL cell, giving final concentrations of 0.02, 0.0625, 0.1, 0.125, 0.25, 0.5, and1 mM, respectively. For the measurements requiring precise timed incubation of HNE with the LOX-1 borate solution, a batch solution was prepared [24 mL 0.1 M potassium borate buffer (pH 9.86) containing 0.36 μg LOX-1/mL was stirred with 100 μL of methanolic HNE solution at the various concentrations described above]. The batch solution was incubated at 25°C for the prescribed time of 5.5, 11, 17, 30, 55, and 90 min, after which 2.4 mL was transferred to the oxygen electrode cell and 10 μL of 240 mM methanolic linoleic acid was injected into the cell to start the oxygen uptake measurement.

The control measurements were the same as described above, except the same volume of methanol replaced the methanolic HNE solution.

*Lineweaver-Burk kinetic analyses of HNE inhibition.* LOX-1 activity assays were measured by oxygen electrode without or with HNE inhibitor after preincubation for exactly 25 min at 25°C by adding either 0.417% methanol (10 μL methanol/2.4 mL LOX-1 solution) or 0.417% methanolic HNE solution (methanolic HNE tested were 30, 60, and 120 mM, giving 0.125, 0.25, and 0.5 mM final concentration, respectively). The LOX-1 solution was 0.97 μg LOX-1/mL in 0.1 M potassium borate (pH 9.86). After the 25-min preincubation, 2.4 mL was transferred to the oxygen electrode cell and 10 μL methanolic linoleic acid was injected to start the reaction. Methanolic linoleic acid was injected at concentrations of 24, 12, 8, 6, 4.8, or 4 mM giving final concentrations of 0.1, 0.05, 0.0334, 0.025, 0.02, or 0.01667 mM, respectively, all of which are below the critical micelle concentration (cmc) of linoleic acid at pH 9.86 (35). As many as 10 replicates were determined for the "without HNE" activities, and "with HNE" activities were replicated four to eight times. Lineweaver-Burk reciprocal data were plotted and interpreted according to Dixon and Webb (36).

*Electron paramagnetic resonance (EPR) analysis of $Fe^{3+}$-LOX-1 in presence of HNE.* A LOX-1 stock solution (109 μM) in potassium borate buffer (0.1 M, pH 9.9) was prepared. To 120 μL aliquots of this enzyme stock, dilute solutions of HNE in ethanol (20 μL) were added such that the final concentrations of HNE were 0, 0.21, and 1.57 mM. These solutions were incubated for 1 h (25°C), at which point the enzyme was oxidized through addition of a 37 μL aliquot of 13-HPODE (2 mM) in methanol. Samples were immediately frozen in liquid nitrogen and EPR spectra acquired at 77 K in a quartz finger Dewar flask. The samples (177 μL) analyzed as follows: LOX-1 (74 μM), 13-HPODE (418 μM), and HNE = 0, 0.17, and 1.24 mM. All spectra were acquired on a Bruker ESP-300E X-band spectrometer. Key parameters were center field 130 mT, sweep width 100 mT, microwave power 63 mW, modulation amplitude 1.2 mT, and sweep time 80 s.

*Analyses of HNE decrease in buffered solutions.* HNE was trapped as the benzoxime and analyzed by flame-ionization detection gas chromatography as the trimethylsilyloxy ether as described (10). Methanolic HNE (15 μL of 60 mM HNE) was added to 3 mL 0.1 M potassium borate buffer (pH 9.86) or 3 mL potassium borate buffer containing 1.04 mg LOX-1. These solutions were incubated at 25°C, and 0.5 mL aliquots were taken at 0, 10, 40, 90, and 120 min to make the *O*-benzoxime derivative of HNE. Aliquots were added to 0.1 mL reagent containing 50 mM *O*-benzylhydroxylamine-HCl in 100 mM Na piperazine-*N,N′*-bis-(2-ethanesulfonate) (pH 6.5) and 0.4 mL methanol. After permitting the reagent to react for 10 min, 27.5 μg methyl nonadecanoate was added as an internal standard, and the derivative was extracted with 1 mL chloroform. The chloroform extract was evaporated with a stream of nitrogen and reacted with trimethylchlorosilane/hexamethyldisilazane/dry pyridine (3:2:2, by vol). After 10 min, the reagent was evaporated with a stream of nitrogen and taken up in 100 μL hexane for gas chromatographic analysis as reported previously (11). Values were relative based on 100% HNE content at zero time.

## RESULTS AND DISCUSSION

An examination of the persistence of HNE in 0.1 M borate buffer (pH 9.86) revealed the loss of HNE (Table 1) with the appearance of seven unidentified compounds having a greater retention time by gas chromatography. The four main compounds had related mass spectra indicating that they were isomeric. The disappearance of HNE was not markedly affected by the presence of LOX-1, which was not too surprising as the concentration of LOX-1 was many orders of magnitude less than HNE. That is, HNE was present at 0.3 mM, and LOX-1 was only at 0.011 μM. Since HNE was present at only 57% of its original concentration after 40 min, the potential effect of HNE on LOX-1 was diminished at longer times. However, during short incubation times, when the rate of inhibition was greatest (Fig. 1), the amount of HNE was only slightly depleted. At 5.5 and 11 min, the amount of HNE was projected to be about 95 and 91%, respectively, at which time the rate of inhibition was the greatest.

**TABLE 1**
**Loss of HNE with Time When Incubated in 0.1 M Potassium Borate Buffer (pH 9.86) at 25°C With or Without LOX-1[a]**

| | Percentage, relative to zero time | |
|---|---|---|
| Time (min) | Without LOX-1 | With LOX-1 |
| 0 | 100 | 100 |
| 10 | 86.4 | 92.8 |
| 40 | 57.3 | 55.7 |
| 90 | 27.3 | 25.1 |
| 120 | 26.3 | 18.8 |

[a]The concentrations of 4-hydroxy-2(*E*)-nonenal (HNE) and soybean lipoxygenase-1 (LOX-1) were 0.3 mM and 0.35 mg/mL, respectively.

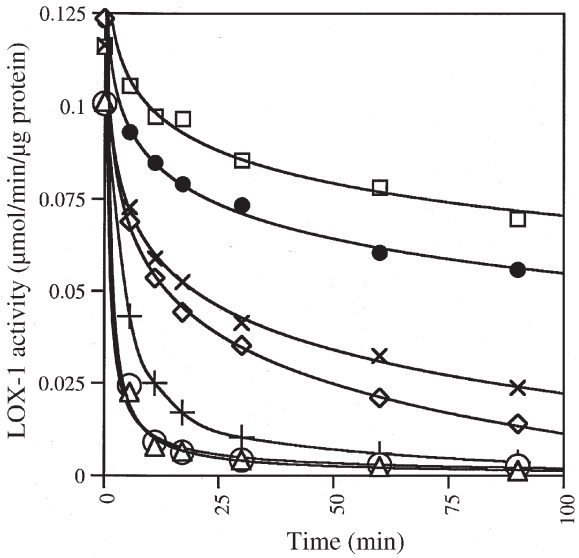


**FIG. 1.** Time-dependent inactivation of soybean lipoxygenase-1 (LOX-1) activity by various concentrations of 4-hydroxy-2(*E*)-nonenal (HNE). □, Control containing methanol only in place of methanolic HNE; ●, 0.02 mM HNE; ×, 0.0625 mM HNE; ◇, 0.125 mM HNE; +, 0.25 mM HNE; ○, 0.5 mM HNE; △, 1 mM HNE. All points represent mean values of replicates. The range in coefficient of variation in percentages was as follows: control, 13 to 18 ($n$ = 19); 0.02 mM HNE, 3 to 31 ($n$ = 4); 0.0625 mM HNE, 5 to 18 ($n$ = 3); 0.125 mM HNE, 9 to 36 ($n$ = 3); 0.25 mM HNE, 10 to 26 ($n$ = 3); 0.5 mM HNE, 0 to 28 ($n$ = 3); and 1 mM HNE, 22 to 81 ($n$ = 3).

It was determined that HNE in concentrations ranging between 20 μM and 1 mM was an effective inhibitor of LOX-1 activity as assayed with 1 mM linoleic acid (Fig. 1). Previous studies with other enzymes showed that the effective concentrations of HNE required for inhibition ranged from a low for cytochrome P450 (0.24 μM to 0.8 mM) (20) and $Na^+$-$K^+$-ATPase (1 μM to 1 mM) (21) to a high for glucose-6-phosphate dehydrogenase (1 to 8 mM) (19). Other enzymes were reported to be effective at HNE concentrations between the foregoing values; thus, the inhibition of LOX-1 by 20 μM to 1 mM was comparable to the effect on many other enzymes. Interestingly, we did not see inhibition when HNE and linoleic acid were added simultaneously; that is, no significant slowing of the rate was seen over the course of the assay of 4 to 5 min. This result indicated that linoleic acid was a more effective competitor for the active site than HNE. However, when HNE was incubated with LOX-1 for a period of time in the absence of linoleic acid, subsequent activity with linoleic acid substrate was markedly reduced. After the data shown in Figure 1 were compensated for the control, the loss of activity was found to be complete after about 17 min of incubation with HNE.

The data presented in Figure 1 are indicative of a slow-binding inhibitor. As outlined by Copeland (37), slow-binding inhibitors are categorized into three types, which can be distinguished by kinetic analysis. The data derived from Figure 1 best fit the "affinity labeling and mechanism-based inhibition" according to Scheme 1, where E, enzyme; $k$, rate constant; and [I], inhibitor.

$$E \underset{k_4}{\overset{k_3[I]}{\rightleftharpoons}} E[I] \xrightarrow{k_5} E\text{-}I$$

**SCHEME 1**

For this type of inhibitor the rate equation is:

$$k_{obs} = \frac{k_5[I]}{K_i + [I]} \quad [1]$$

where $k_{obs}$, apparent (observed) rate constant; $K_i$, apparent concentration of inhibitor required to reach the half-maximal rate of inactivation, and [I], inhibitor concentration.

Since this rate equation is reminiscent of the Michaelis-Menton equation, a reciprocal plot of $k_{obs}$ vs. [I] ideally should give a linear plot. After correcting for the control, exponential extrapolation of the rate of inactivation obtained during the first 11 min (Fig. 1) $k_{obs}$ was derived from $0.693/t_{1/2}$ min. The rate for 1 mM HNE was omitted as being too rapid to obtain reasonable data. The linear reciprocal plot shown in Figure 2 with a nonzero intercept conforms to the inhibitor type shown in Scheme 1. The intercept with the $y$-axis gave the 1/maximal rate of $k_5$, or the maximal rate of inactivation, $k_{inact.}$, that is, $1/k_{inact.} = 5$ or $k_{inact.} = 0.2$ min$^{-1}$. Intersection of the plot with the $x$-axis gave $-1/K_i = -1/3.4$ or $K_i = 0.294$ mM. This type of inhibition is reasonable in view of the fact that HNE is known to form Michael adducts with cysteine (17), lysine (18), and histidine (15,16). It is noted that LOX-1 possesses three essential histidines that ligate the iron active site. It is known from the literature that 4(*S*)-hydroperoxy-2(*E*)-nonenal is a LOX-1 product of 3(*Z*)-nonenal oxidation (12); thus, it seems reasonable that the smaller and structurally related HNE should be capable of accessing the active site.

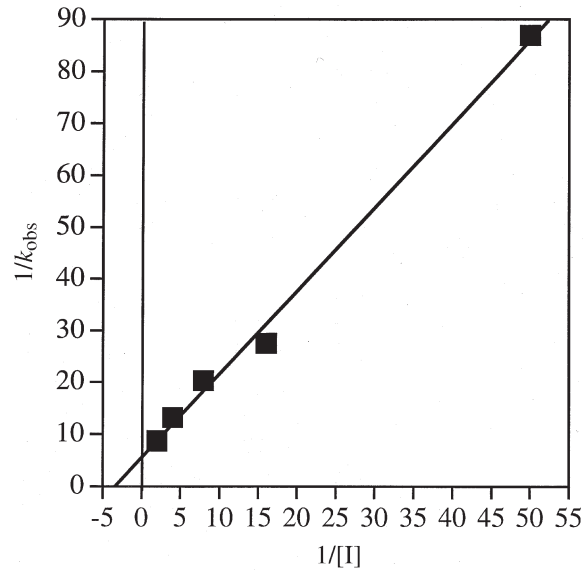


**FIG. 2.** Reciprocal plot of apparent (observed) rate constant, $k_{obs}$ (min$^{-1}$), as a function of inhibitor [I] concentration (mM). Intercept with the $x$-axis is equivalent to $-1/K_i$, and the intercept with the $y$-axis is $1/k_{inact}$ (reciprocal of the maximal rate of inactivation). Derived from the data shown in Figure 1.

For this reason, Lineweaver-Burk kinetic analyses of the inhibitor effect of HNE were completed to confirm the foregoing assessment. In order to determine these data, LOX-1 was preincubated with HNE for 25 min, a time determined to be safely beyond the effective time required for inhibition; however, after 25 min the concentration of HNE was reduced to about 75% of its original value by decomposition in buffer. Also, all activity assays had to be completed below the cmc concentration of linoleic acid, because the true substrate of LOX-1 has been determined to be monomeric linoleic acid, not substrate trapped in micelles (38). This severely restricts the usable range of substrate concentrations, but reasonable data can be collected by the use of replicates. Thus, the $K_M$ of LOX-1 was determined to be 23 μM (Fig. 3), which compares with 15 ± 3 μM reported by Schilstra *et al*. (39). Reciprocal plot analyses in the presence of various concentrations of HNE revealed mixed competitive/noncompetitive inhibition at the two lowest concentrations of HNE tested. Since 3(*Z*)-nonenal served as a substrate of LOX-1 and 4(*S*)-hydroperoxy-2(*E*)-nonenal was a product (12), it was expected that HNE would occupy the active site of LOX-1. The competitive aspect of HNE can be readily explained. The noncompetitive nature of the inhibition was expected due to the slow-binding kinetic analysis outlined above. Surprisingly, at the highest inhibitor concentration tested (0.5 mM), where one would expect the greatest degree of noncompetitive inhibition, the plot showed an almost classical competitive inhibition. In fact, the trend toward competitive inhibition occurred as the inhibitor concentration progressively increased.

This observation of classical competitive inhibition at 0.5 mM HNE does not have a straightforward explanation. Some competitive inhibition could be caused by decomposition products of HNE, which would afford higher concentrations after 25 min preincubation with 0.5 mM HNE. To test this possibility, HNE was preincubated in buffer for 2 to 4 h before testing for its effect on LOX-1 activity. Pretreated HNE was then incubated with LOX-1 for 10 min prior to adding 1 mM linoleic acid. Preincubated HNE, 0.5 and 1 mM, inhibited LOX-1 at 17 and 58%, respectively, compared to controls. Little significant difference in activity could be seen with the time of preincubation between 2 and 4 h. By contrast, a 10 min exposure of LOX-1 to HNE (not preincubated in buffer) at 0.5 and 1 mM inhibited activity 73 and 85%, respectively. As seen in Table 1, the amount of HNE remaining after 2 h preincubation in buffer was less than 25%. It is conceivable that the inhibition observed with preincubation is partly due to residual HNE, but the lack of significant effect over time is suggestive of the contribution from degraded HNE. It is conceivable that the competitive aspect of the Lineweaver-Burk plots observed at higher HNE concentrations was due to degraded HNE from 25 min preincubation of HNE. Nevertheless, some degree of mixed competitive/noncompetitive inhibition should be expected.

Disruption of the active site was supported by EPR results. An EPR signal of LOX-1 was generated by oxidizing native $Fe^{2+}$-LOX-1 to $Fe^{3+}$-LOX-1 by exposing the enzyme to its product, 13-HPODE, followed by immediate liquid $N_2$ freezing (Fig. 4). In the presence of low HNE concentrations (0.21 mM incubation concentration, 0.17 mM after 13-HPODE addition) the EPR signal from oxidized LOX-1 was slightly affected. A 22% decrease in peak-to-peak width of the $g_\perp$ (g = 6) signal was observed: 11.3mT in the absence of HNE and 8.8 mT in the presence of HNE. In view of the half-maximal inhibitor concentration ($K_i$) for HNE of 0.294 mM (Fig. 2), this result was not unreasonable. When the concentration of HNE was increased (1.57 mM incubation concentration, 1.24 mM after 13-HPODE addition), the EPR signal completely disappeared. Since 1.24/1.57 mM HNE is substantially greater than $K_i$, complete inhibition was anticipated; however, the relatively low HNE/LOX-1 incubation ratio of 21 may have depleted the actual HNE concentration by noninhibitory reactions with LOX-1 to some extent. In the present EPR study, the HNE/LOX-1 incubation ratio of 21 compared to literature HNE/protein ratios of 15–143 that were utilized to modify histidine residues (15). From these observations it was concluded that HNE caused the active site to be sufficiently hindered to prevent oxidation of $Fe^{2+}$-LOX-1 to $Fe^{3+}$-LOX-1 by 13-HPODE.

A potential physiological function of HNE is suggested by the results. It is well known that pathogens activate the oxylipin pathway (e.g., Ref. 40). More specifically, Deighton *et al*. (41) found that HNE accumulates at levels as high as 19,000 pmol/g tissue at the site of fungal infection of sweet peppers. In certain experiments HNE was found at even higher levels of 30,000 pmol/g (Deighton, N., unpublished data). Because of HNE reaction with protein and/or glutathione, it is plausible that the amount produced was actually higher. The concentrations found by Deighton translate

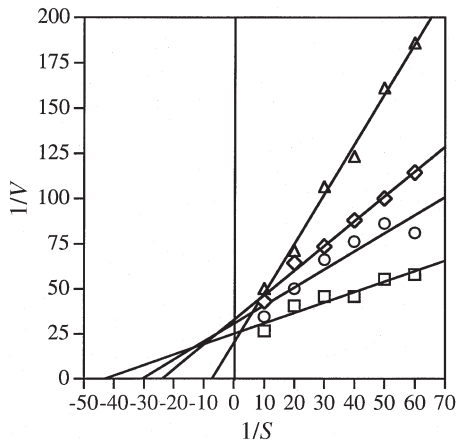


**FIG. 3.** Lineweaver-Burk reciprocal plots of LOX-1 activity with and without HNE inhibitor. Preincubation with or without HNE was 25 min at 25°C before measurement of activity with linoleic acid. Reciprocals are activity, *V*, in μmol/min/μg LOX-1, and linoleic acid substrate, *S*, in mM. □, Activity without HNE; ○, activity with 0.125 mM HNE ◇, activity with 0.25 mM HNE; and △, activity with 0.5 mM HNE. All points are mean values of replicates. The ranges in coefficient of variation in percentages were as follows: "without HNE," 14 to 35 ($n$ = 10); 0.125 mM HNE, 9 to 22 ($n$ = 4); 0.25 mM HNE, 11 to 24 ($n$ = 6); 0.5 mM, 7 to 22 ($n$ = 4). For abbreviations see Figure 1.

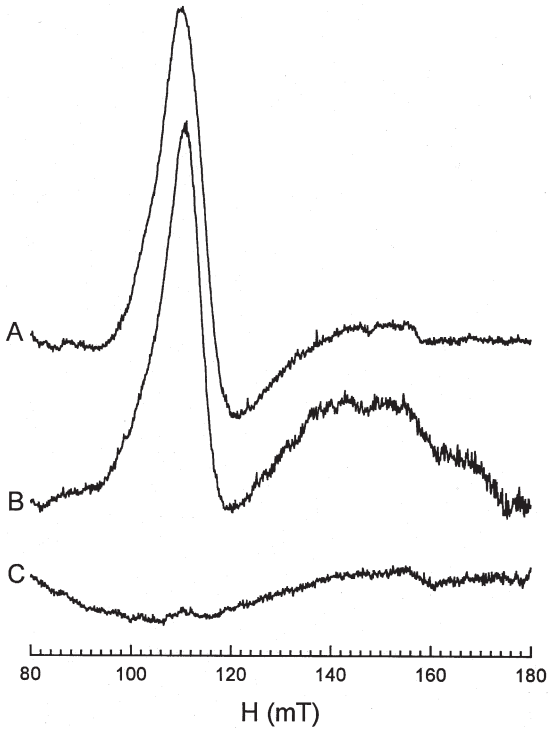


**FIG. 4.** Electron paramagnetic resonance spectra of oxidized LOX-1. The enzyme was oxidized by 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid in the absence (A) and presence of HNE at 0.17 (B) and 1.24 mM (C). For abbreviations see Figure 1.

to 0.019 to 0.03 mM, which brackets the lowest concentration of HNE tested in this study (Fig. 1). Thus, it seems possible that the downstream oxylipin HNE, and possibly related oxylipins, may serve to switch off the lipid signal pathway through inactivation of one of the initiating enzymes. Because linoleic acid effectively competes for the active site, this "feedback suicide" of LOX is suggested to occur only after substrate depletion.

Further research is planned to locate the specific site of LOX-1 inactivation by HNE.

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# Vinyl Sulfide Derivatives of Truncated Oxidosqualene as Selective Inhibitors of Oxidosqualene and Squalene-Hopene Cyclases

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**ABSTRACT:** Various vinyl sulfide and ketene dithioacetal derivatives of truncated 2,3-oxidosqualene were developed. These compounds, having the reactive functions at positions C-2, C-15 and C-19 of the squalene skeleton, were studied as inhibitors of pig liver and *Saccharomyces cerevisiae* oxidosqualene cyclases (OSC) (EC 5.4.99.7) and of *Alicyclobacillus acidocaldarius* squalene hopene cyclase (SHC) (EC 5.4.99.-). They contain one or two sulfur atoms in α-skeletal position to carbons considered to be cationic during enzymatic cyclization of the substrate and should strongly interact with enzyme nucleophiles of the active site. Most of the new compounds are inhibitors of the OSC and of SHC, with various degrees of selectivity. The methylthiovinyl derivative, having the reactive group at position 19, was the most potent and selective inhibitor of the series toward *S. cerevisiae* OSC, with a concentration inhibiting 50% of the activity of 50 nM, while toward the animal enzyme it was 20 times less potent. These results could offer new insight for the design of antifungal drugs.

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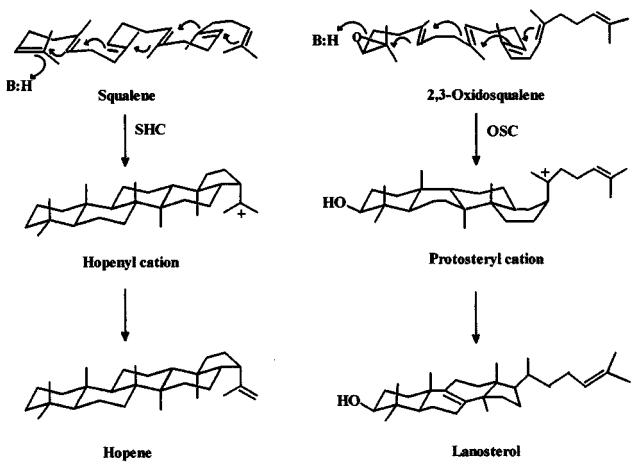


**SCHEME 1**

2,3-Oxidosqualene cyclases (OSC) (EC 5.4.99.7) are membrane-associated enzymes that catalyze the cyclization of (3*S*)-2,3-oxidosqualene (OS) to lanosterol in mammals and yeasts and to cycloartenol in higher plants. OSC have been purified and cloned from different species: *Candida albicans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* among the fungi; higher plants; the rat, the pig, and man (1–14 and references therein).

Squalene-hopene cyclase (SHC) (EC 5.4.99.-) catalyzes the cyclization of squalene to hopene and hopanoids, a class of pentacyclic, sterol-like triterpenes present in many bacterial membranes (2,10,15,16). The two enzymes show many similarities in their substrate cyclization mechanism (Scheme 1). Both enzymes are able to generate carbocationic species by protonation of either a carbon double bond (SHC) or an epoxide (OSC). The formation of the final product in both enzymes is a highly complex and specific process rising from a progression of partially cyclized carbocationic intermediates.

For the formation of lanosterol, as final product of OSC, the pentacyclic C-20 protosteryl cation undergoes rearrangement through a series of 1,2-methyl and hydride shifts, while hopene, the final product of SHC, arises from the loss of the H-29 proton directly from the hopenyl C-22 cation, without skeletal rearrangement. Possibly this is a consequence of the fact that SHC behaves as a more primitive enzyme (16,17). SHC from *Alicyclobacillus acidocaldarius* has recently been overexpressed in *Escherichia coli*, purified, and crystallized (10,18,19). It shows a 17–27% identity with the cloned OSC and a molecular weight of 71,500 Da, and it contains several repeats of a highly conserved motif rich in aromatic amino acids, the QW motif, also present in OSC (20). SHC is so far the only suitable model for studying the mechanism of triterpene cyclization from a structural standpoint. Indeed, all information available about the oxidosqualene cyclization mechanism is inferred from mutagenesis experiments and inhibition studies, since no OSC have been crystallized (21–23).

For many years we have been studying OSC inhibitors. Initially we obtained effective inhibitors of OSC by mimicking the carbocationic intermediates formed during cyclization

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Abbreviations: CIMS, chemical ion mass spectrometry; EIMS, electron ionization mass spectrometry; HRMS, high-resolution mass spectrometry, $IC_{50}$, concentration of inhibitor that reduces the enzymatic conversion by 50%; IR, infrared; 29-MOS, 29-methylidene-2,3-oxidosqualene; NMR, nuclear magnetic resonance; OS, 2,3-oxidosqualene; OSC, 2,3-oxidosqualene-lanosterol cyclase; SHC, squalene-hopene cyclase; THF, tetrahydrofuran.

of OS, designing squalene-derived structures in which the positively charged carbocation was replaced by a nitrogen (1, 24–26). Another strategy that has been adopted is to intercept the enzymatic active-site nucleophiles with a stable allylic cation, resulting in an irreversible covalent modification of OSC. Following this strategy, Prestwich (21,27–29), Corey (23), and our (30,31) groups synthesized various series of 2,3-oxidosqualenoid dienes and vinyl epoxides, some of which were found to be selective and time-dependent inhibitors of yeast or animal OSC. Finally, various series of sulfur-containing oxidosqualene derivatives, in which sulfur has replaced carbons C5, C6, C8, C9, C10, C11, C13, C14, C15, C16, C18, C19, or C20 (squalene numbering), have been synthesized (22,32–36). Some of these compounds are potent inhibitors of OSC, particularly of the *C. albicans* enzyme.

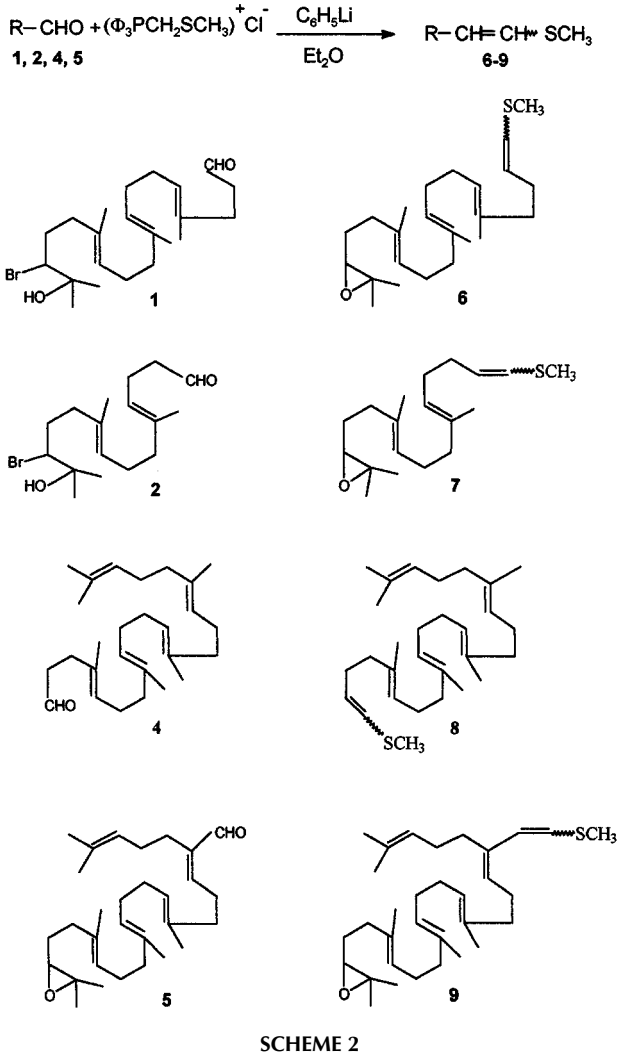
We thus believed that the synthesis of a new class of truncated vinyl sulfide derivatives of OS and of squalene, having the reactive functions at crucial positions 2, 15 and 19, together with other related compounds, might afford new derivatives with better selectivity toward the animal, yeast, or bacterial enzyme. These derivatives should give new insight into the function and the reactivity of the nucleophiles of the active site of the various OSC (and of SHC) that stabilize the C-2, C-13 and C-20 cationic intermediates.

## MATERIALS AND METHODS

*Chemicals.* The $^1$H nuclear magnetic resonance (NMR) spectra were recorded on either a JEOL EX 400 (JEOL, Inc., Peabody, MA) or a Bruker AC 200 instrument (Karlsruhe, Germany) in $CDCl_3$ solution at room temperature, with $SiMe_4$ as internal standard. Mass spectra were obtained on a Finnigan MAT TSQ 700 spectrometer (San Jose, CA). Infrared (IR) spectra were recorded on a PE 781 (PerkinElmer, Wellesley, MA) spectrophotometer. Microanalyses were performed on an elemental analyzer 1106 (Carlo Erba Strumentazione, Milano, Italy). The reactions were monitored by thin-layer chromatography (TLC) on $F_{254}$ silica gel precoated sheets; after development, the sheets were exposed to iodine vapor. Flash-column chromatography was performed on 230–400 mesh silica gel. Tetrahydrofuran (THF) and diethyl ether were dried over sodium benzophenone ketyl. All solvents were distilled prior to flash chromatography.

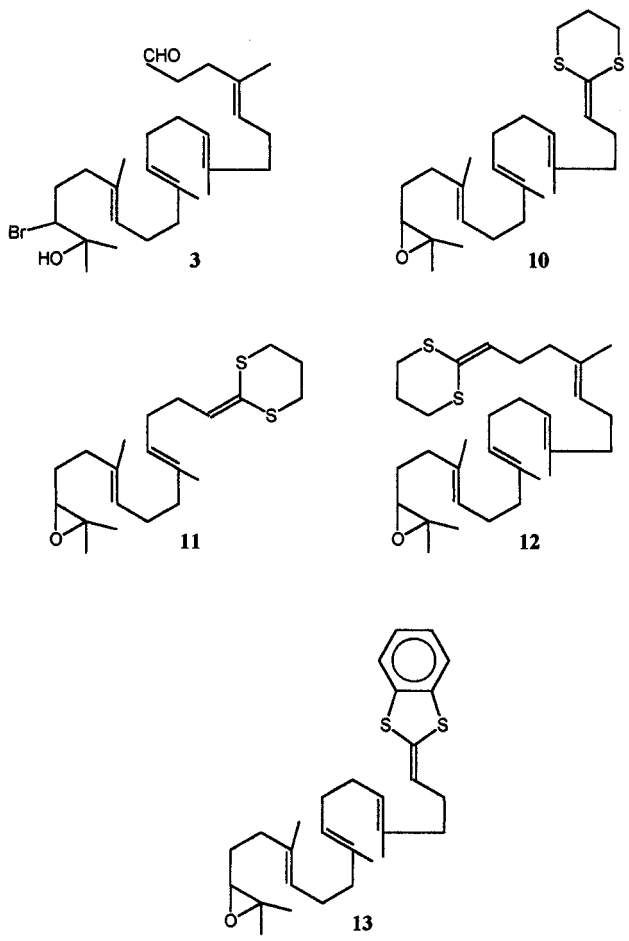
Squalene, lanosterol, and polyoxyethylene 9 lauryl ether were obtained from Sigma Chemical Co. (St. Louis, MO). [$^{14}$C]Squalene and [$^{14}$C]-(3*S*)-2,3-oxidosqualene were obtained through biological synthesis by incubating a pig liver $S_{10}$ fraction with [2-$^{14}$C]mevalonolactone (NEN, Boston, MA).

$C_{22}$ squalene aldehyde monobromohydrin **1** (Scheme 2) was obtained as previously reported (37,38). $C_{17}$ and $C_{27}$ squalene aldehyde monobromohydrins **2** (Scheme 2) and **3** (Scheme 3) were obtained using the same method as reported for **1**, starting from the corresponding $C_{17}$ and $C_{27}$ aldehydes. $C_{27}$ squalene aldehyde **4** (Scheme 2) was obtained as previously described (24). $C_{30}$ squalene aldehyde epoxide **5** (Scheme 2) was obtained as previously described (30).




**SCHEME 2**

*(5*E*,9*E*,13*E*)-17,18-Epoxy-5,10,14,18-tetramethyl-1-methylthio-1,5,9,13-nonadecatetraene (**6**, Scheme 2).* In a two-necked flask, anhydrous diethyl ether (5 mL) and phenyllithium (1.6 M solution in hexane, 2.40 equiv, 360 μL, 0.58 mmol) were added and stirred at room temperature under dry nitrogen. (Methylthiomethyl)triphenylphosphonium chloride (1.20 equiv, 104 mg, 0.29 mmol), dissolved in anhydrous diethyl ether (3 mL), was then added, and the red solution turned to pale yellow. After stirring for 10 min at room temperature, $C_{22}$ squalene aldehyde monobromohydrin **1** (1.0 equiv, 100 mg, 0.24 mmol), in anhydrous diethyl ether (2 mL), was added and left for 30 min under stirring. The mixture was then poured into cold diethyl ether/saturated aqueous NaCl (1:1, 50 mL) and extracted with diethyl ether (3 × 30 mL). The combined extracts were washed with saturated brine (1 × 30 mL), dried with anhydrous sodium sulfate, and evaporated *in vacuo*. The resulting oil was purified by flash chromatography with petroleum ether/diethyl ether, 97:3, to give 32 mg (35% yield from **1**) of compound **6**, as a mixture (about 1:1) of 1*E* and 1*Z* isomers, as a colorless oil. $^1$H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6 H, epoxidic $CH_3$), 1.56–1.68




SCHEME 3

(*m*, 11 H, allylic $CH_3$ and $CH_2$-epoxide), 1.98–2.18 (*m*, 14 H, allylic $CH_2$), 2.22 and 2.26 (2 *s*, 3 H, *E* and *Z* $SCH_3$), 2.70 (*t*, 1 H, epoxidic CH, *J* = 6.2 Hz), 4.98–5.18 (*m*, 3 H, vinylic CH), 5.35–5.56 (*m*, 1 H, *E* and *Z* C*H*=CHS$CH_3$), 5.84–6.01 (*m*, 1 H, *E* and *Z* CH=C*H*S$CH_3$); infrared (IR) ($CCl_4$) 2970, 2930, 2860, 1550, 1450, 1380 $cm^{-1}$; electron ionization mass spectrometry (EIMS) *m/z* 376 (1), 361 (0.4), 329 (0.4), 315 (0.6), 291 (0.5), 249 (0.5), 241 (0.8), 223 (18), 135 (15), 107 (35), 87 (100); chemical ion mass spectrometry (CIMS) (isobutane) *m/z* 377 (100), 359 (42); high-resolution mass spectrometry (HRMS) *m/z* 376.2796 (calc. for $C_{24}H_{40}OS$ 376.2800). Anal. ($C_{24}H_{40}OS$) C, H, O, S.

*(5*E*,9*E*)-13,14-Epoxy-6,10,14-trimethyl-1-methylthio-1,5,9-pentadecatriene (7, Scheme 2).* Compound **7** was obtained starting from aldehyde **2** by using the same method as described for **6**, as a mixture (about 1:1) of 1*E* and 1*Z* isomers, in 33% yield. $^1H$ NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6 H, epoxidic $CH_3$), 1.55–1.68 (*m*, 8 H, allylic $CH_3$ and $CH_2$-epoxide), 2.00–2.16 (*m*, 10 H, allylic $CH_2$), 2.23 and 2.27 (2 *s*, 3 H, *E* and *Z* $SCH_3$), 2.70 (*t*, 1 H, epoxidic CH, *J* = 6.2 Hz), 5.02–5.20 (*m*, 2 H, vinylic CH), 5.41–5.58 (*m*, 1 H, *E* and *Z* C*H*=CHS$CH_3$), 5.85–6.02 (*m*, 1 H, *E* and *Z* CH=C*H*S$CH_3$); IR ($CCl_4$) 2970, 2930, 2855, 1550, 1450, 1380 $cm^{-1}$; EIMS *m/z* 308 (2), 293 (1.8), 275 (1), 260 (2), 243 (1.2), 223 (2), 203 (1.8), 181 (1.8), 175 (3.5), 135 (15), 107 (18), 87 (100); CIMS (isobutane) *m/z* 309 (100), 291 (48), 261 (38); HRMS *m/z* 308.2179 (calc. for $C_{19}H_{32}OS$ 308.2174). Anal. ($C_{19}H_{32}OS$) C, H, O, S.

*(5*E*,9*E*,13*E*,17*E*)-5,9,14,18,22-Pentamethyl-1-methylthio-1,5,9,13,17,21-tricosahexaene (8, Scheme 2).* Compound **8** was obtained starting from aldehyde **4** using the same method as described for **6**, except for the amount of phenyllithium (1.40 equiv). In this case the crude oil was purified by flash chromatography with petroleum ether/diethyl ether, 99:1 vol/vol, to give compound **8**, as a mixture (about 1:1) of 1*E* and 1*Z* isomers, in 47% yield. $^1H$ NMR ($CDCl_3$): δ 1.57–1.68 (*m*, 18 H, allylic $CH_3$), 2.00–2.16 (*m*, 20 H, allylic $CH_2$), 2.23 and 2.27 (2 *s*, 3 H, *E* and *Z* $SCH_3$), 5.00–5.18 (*m*, 5 H, vinylic CH), 5.42–5.56 (*m*, 1 H, *E* and *Z* C*H*=CHS$CH_3$), 5.86–6.02 (*m,* 1 H, *E* and Z CH=C*H*S$CH_3$); IR (liquid film): 2970, 2920, 2850, 1440, 1380 $cm^{-1}$; EIMS: *m/z* 428 (4), 413 (1.2), 381 (1), 359 (2), 291 (6.5), 223 (5.6), 201 (3.8), 175 (12), 154 (40), 139 (25), 107 (35), 87 (100); CIMS (isobutane) *m/z* 429 (100); HRMS *m/z* 428.3473 (calc. for $C_{29}H_{48}S$ 428.3477). Anal. ($C_{29}H_{48}S$) C, H, S.

*(6*Z*,10*E*,14*E*,18*E*)-22,23-Epoxy-2,10,15,19,23-pentamethyl-6-(2-methylthiovinyl)-2,6,10,14,18-tetracosapentaene (9, Scheme 2).* Compound **9** was obtained starting from *Z*-aldehyde **5** using the same method as described for **6**, except for the amount of phenyllithium (1.40 equiv). It was obtained as a mixture (about 1:1) of 1*E* and 1*Z* isomers, in 27% yield. $^1H$ NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6 H, epoxidic $CH_3$), 1.58–1.66 (*m*, 17 H, allylic $CH_3$ and $CH_2$-epoxide), 2.00–2.18 (*m*, 18 H, allylic $CH_2$), 2.29 and 2.31 (2 *s*, 3 H, *E* and *Z* $SCH_3$), 2.70 (*t*, 1 H, epoxidic CH, *J* = 6.2 Hz), 5.02–5.22 (*m*, 5 H, vinylic CH), 5.85–6.29 (*m*, 2 H, *E* and *Z* C*H*=C*H*S$CH_3$); IR ($CCl_4$): 2970, 2930, 2860, 1550, 1450, 1380 $cm^{-1}$; EIMS *m/z* 484 (1), 437 (1), 367 (0.6), 331 (3.4), 270 (1.8), 227 (3.8), 215 (5), 201 (8), 195 (4), 179 (35), 147 (73), 119 (33), 105 (45), 89 (75), 69 (100); CIMS (isobutane) *m/z* 485 (100), 437 (90); HRMS *m/z* 484.3740 (calc. for $C_{32}H_{52}OS$ 484.3739). Anal. ($C_{32}H_{52}OS$) C, H, O, S.

*2-[(4*E*,8*E*,12*E*)-16,17-Epoxy-4,9,13,17-tetramethyl-4,8,12-octadecatrienylidene]-1,3-dithiane (10, Scheme 3).* In a two-necked flask anhydrous THF (5 mL) and *n*-butyllithium (1.6 M solution in hexane, 2.40 equiv, 610 µL, 0.98 mmol) were added and stirred at –20°C under dry nitrogen. (1,3-Dithian-2-yl)triphenylphosphonium chloride (1.20 equiv, 205 mg, 0.49 mmol) was then added, while the solution turned to yellowy orange, and left for 30 min at room temperature under stirring. $C_{22}$ Squalene aldehyde monobromohydrin **1** (1.0 equiv, 170 mg, 0.41 mmol), in anhydrous THF (2 mL), was added and left for 2 h under stirring. The mixture was then poured into cold diethyl ether/saturated aqueous NaCl (1:1, 50 mL) and extracted with diethyl ether (3 × 30 mL). The combined extracts were washed with saturated brine (1 × 30 mL), dried with anhydrous sodium sulfate, and evaporated *in vacuo*. The resulting oil was purified by reversed-phase flash chromatography with acetonitrile/water, 75:25, then

80:20, 85:15, 90:10, 95:5 to give 62 mg (35% yield from **1**) of compound **10**, as a colorless oil. $^1$H NMR ($CDCl_3$): δ 1.25 and 1.30 (2 *s*, 6 H, epoxidic $CH_3$), 1.54–1.66 (*m*, 11 H, allylic $CH_3$ and $CH_2$-epoxide), 1.98–2.35 (*m*, 16 H, allylic $CH_2$ and $SCH_2CH_2CH_2S$), 2.70 (*t*, 1 H, epoxidic CH, *J* = 6.2 Hz), 2.85 (*t*, 4 H, $SCH_2CH_2CH_2S$, *J* = 5.9 Hz), 5.00–5.18 (*m*, 3 H, vinylic CH), 5.93 (*t*, 1 H, fulvenic CH, *J* = 7.1 Hz); IR ($CCl_4$): 2965, 2930, 2860, 1725, 1680, 1450, 1380 cm$^{-1}$; EIMS *m/z* 434 (1), 281 (1.5), 223 (0.5), 213 (2.5), 199 (3), 163 (2), 149 (42), 145 (100); CIMS (isobutane) *m/z* 435 (32), 391 (100); HRMS *m/z* 434.2678 (calc. for $C_{26}H_{42}OS_2$ 434.2677). Anal. ($C_{26}H_{42}OS_2$) C, H, O, S.

*2-[(4*E*,8*E*)-12,13-Epoxy-5,9,13-trimethyl-4,8-tetradecadienylidene]-1,3-dithiane (**11**, Scheme 3).* Compound **11** was obtained starting from aldehyde **2**, using the same method as described for **10**, in 30% yield. $^1$H NMR ($CDCl_3$): δ 1.25 and 1.30 (2 *s*, 6 H, epoxidic $CH_3$), 1.54–1.66 (*m*, 8 H, allylic $CH_3$ and $CH_2$-epoxide), 1.95–2.32 (*m*, 12 H, allylic $CH_2$ and $SCH_2CH_2CH_2S$), 2.70 (*t*, 1 H, epoxidic CH, *J* = 6.2 Hz), 2.85 (*t*, 4 H, $SCH_2CH_2CH_2S$, *J* = 5.9 Hz), 5.02–5.15 (*m*, 2 H, vinylic CH), 5.95 (*t*, 1 H, fulvenic CH, *J* = 7.2 Hz); IR (liquid film): 2970, 2930, 2860, 1725, 1680, 1450, 1390 cm$^{-1}$; EIMS *m/z* 366 (4), 294 (1.2), 258 (1), 211 (1), 145 (100); CIMS (isobutane) *m/z* 367 (100), 351 (8); HRMS *m/z* 366.2054 (calc. for $C_{21}H_{34}OS_2$ 366.2051). Anal. ($C_{21}H_{34}OS_2$) C, H, O, S.

*2-[(4*E*,8*E*,12*E*,16*E*)-20,21-Epoxy-4,8,13,17,21-pentamethyl-4,8,12,16-docosatetraenylidene]-1,3-dithiane (**12**, Scheme 3).* Compound **12** was obtained starting from aldehyde **3** using the same method as described for **10**, in 32% yield. $^1$H NMR ($CDCl_3$): δ 1.26 and 1.30 (2 *s*, 6 H, epoxidic $CH_3$), 1.58–1.68 (*m*, 14 H, allylic $CH_3$ and $CH_2$-epoxide), 2.00–2.35 (*m*, 20 H, allylic $CH_2$ and $SCH_2CH_2CH_2S$), 2.70 (*t*, 1 H, epoxidic CH, *J* = 6.2 Hz), 2.85 (*t*, 4 H, $SCH_2CH_2CH_2S$, *J* = 5.9 Hz), 5.00–5.20 (*m*, 4 H, vinylic CH), 5.95 (*t*, 1 H, fulvenic CH, *J* = 7.2 Hz); IR (liquid film): 2965, 2930, 2860, 1725, 1680, 1450, 1380 cm$^{-1}$; EIMS *m/z* 502 (0.2), 349 (0.2), 281 (0.5), 268 (0.2), 239 (0.3), 145 (100); CIMS (isobutane) *m/z* 503 (100), 485 (18); HRMS *m/z* 502.3305 (calc. for $C_{31}H_{50}OS_2$ 502.3303). Anal. ($C_{31}H_{50}OS_2$) C, H, O, S.

*2-[(4*E*,8*E*,12*E*)-16,17-Epoxy-4,9,13,17-tetramethyl-4,8,12-octadecatrienylidene]-1,3-benzodithiole (**13**, Scheme 3).* In a two-necked flask, 2-dimethoxyphosphinoyl-1,3-benzodithiole (39) (1.05 equiv, 79 mg, 0.30 mmol) was dissolved in anhydrous THF (3 mL) and stirred at room temperature under dry nitrogen. The reaction mixture was cooled at –80°C and *n*-butyllithium (1.6 M solution in hexane, 2.50 equiv, 455 µL, 0.73 mmol) was added, while the solution turned to yellowish orange. After stirring for 10 min at room temperature, $C_{22}$ squalene aldehyde monobromohydrin **1** (1.0 equiv, 120 mg, 0.29 mmol) in anhydrous THF (2 mL) was added and left for 10 min at –80°C and for a further 2 h at room temperature, under stirring. The mixture was then poured into cold diethyl ether/saturated aqueous NaCl (1:1, 50 mL) and extracted with diethyl ether (3 × 30 mL). The combined extracts were washed with saturated brine (1 × 30 mL), dried with anhydrous sodium sulfate and evaporated *in vacuo*. The resulting oil was purified by flash chromatography on a column that had been eluted with petroleum ether/isopropylamine, 99:1 until the eluate was basic, and then it was eluted with petroleum ether/isopropylamine, 99.9:0.1 to give 38 mg (28% yield from **1**) of compound **13**, as a colorless oil. $^1$H NMR ($CDCl_3$) δ 1.26 and 1.30 (2 *s*, 6 H, epoxidic $CH_3$), 1.54–1.64 (*m*, 11 H, allylic $CH_3$ and $CH_2$-epoxide), 2.02–2.18 (*m*, 14 H, allylic $CH_2$), 2.70 (*t*, 1 H, epoxidic CH, *J* = 6.2 Hz), 5.02–5.24 (*m*, 4 H, vinylic CH and fulvenic CH), 7.26–7.55 (*m*, 4 H, aromatic CH); IR (liquid film) 2970, 2930, 2860, 1450, 1380 cm$^{-1}$; EIMS *m/z* 468 (0.3), 315 (1.8), 251 (2.2), 203 (1), 179 (100); CIMS (isobutane) *m/z* 469 (70), 347 (90), 345 (100), 329 (90); HRMS *m/z* 468.2522 (calc. for $C_{29}H_{40}OS_2$ 468.2520). Anal. ($C_{29}H_{40}OS_2$) C, H, O, S.

*Enzymatic assays*. Solubilized and partially purified pig liver and yeast OSC were obtained as previously described (37,40). The enzymatic activity was determined by incubating 2,000 cpm of [$^{14}$C]-(3*S*)-2,3-oxidosqualene and evaluating the amount of lanosterol formed, as previously described (40). [$^{14}$C]-(3*S*)-2,3-Oxidosqualene and [$^{14}$C]squalene were obtained by biological synthesis by incubating 1 µCi of [$^{14}$C]mevalonolactone with an $S_{10}$ supernatant of a pig liver homogenate (25 mg of proteins) in the presence of the OSC inhibitor U-18666A (1), following the method of Poják (41). Recombinant squalene hopene cyclase was provided by Prof. Karl Poralla (Universität Tübingen, Germany).

For enzymatic activity determination of SHC, 10 µM squalene and 2,000 cpm of [$^{14}$C]squalene were dissolved in ethanol in the presence of polyoxyethylene 9 lauryl ether (final concentration 0.05%) in test tubes. The solvent was evaporated under nitrogen; and the enzyme (3 µg) in 1 mL of citrate buffer, 0.1 M, pH 6.0, containing 0.1% polyoxyethylene 9 lauryl ether, was added to the test tubes and incubated for 30 min at 55°C. The reaction was stopped by adding 1 mL of methanolic KOH and heating at 80°C for 30 min in a water bath. After extracting with 2 mL of petroleum ether, the solvent was evaporated. The extracts were redissolved in a small amount of $CH_2Cl_2$ and spotted on TLC plates developed with petroleum ether. The conversion of squalene to labeled hopene was analyzed by radio-TLC scanner (Packard System 2000 Imaging Scanner; Hewlett-Packard, Palo Alto, CA), and the percentage of transformation was calculated by integration. Alternatively, bands corresponding to squalene and hopene were scraped off and counted with a liquid scintillator (Beckman LS500 TD; Beckman Instruments, Fullerton, CA). In these conditions, the amount of diplopterol formed was negligible (less than 1%) and it was ignored.

$IC_{50}$ values (the concentration of inhibitor that reduced the enzymatic conversion by 50%) were determined by adding the inhibitors, as ethanolic solution, to the mixture of nonradiolabeled and labeled substrates and by incubating with SHC, as described.

Time-dependent inactivation of OSC was determined at 35°C as previously described (31). Time-dependent inactivation of SHC was determined at 55°C by adding the inhibitors to the enzyme solution in the absence of substrate. Aliquots

were withdrawn at suitable intervals, and diluted 50-fold by transfer to test tubes containing nonradiolabeled and labeled substrate squalene (10 μM) and polyoxyethylene 9 lauryl ether (0.1%) in 0.1 M citrate buffer, pH 6. Residual activity was determined by comparison to an enzyme solution preincubated in the above conditions. Second-order inactivation constants were determined from *t*/2 values obtained in the time-dependent inactivation experiments.

## RESULTS AND DISCUSSION

As mentioned in the introduction, the Oehlschlager group has developed various nontruncated sulfide analogs of OS by following different strategies (32–35). One approach involved the development of OS analogs containing a sulfur atom at positions normally occupied by carbons of OS considered to be cationic during enzymatic cyclization. In another approach, sulfur substituted a carbon of a double bond of OS adjacent to a position considered to be cationic during OS cyclization. These derivatives are supposed to bind the enzyme and to be cyclized, generating sulfonium intermediates. These two classes of thia analogs therefore lack one double bond in the squalene skeleton. Another approach was the development of analogs of OS usually containing a sulfur atom in α-skeletal position to carbons considered to be cationic during OS cyclization. These compounds are supposed to cyclize enzymatically and the carbocation formed is stabilized by the adjacent sulfur, strongly interacting with enzyme residues normally stabilizing the natural carbocation.

Our previous findings showed that compounds having a truncated squalenoid structure and a correctly located reactive group adjacent to a double bond involved in the cyclization are potent and selective inhibitors of the eukaryotic OSC (17,30,31). Several of these squalenoid derivatives, with a 2,3-oxide function, are able to inhibit bacterial SHC. Squalene is the natural substrate of the bacterial enzyme, but the 2,3-oxidosqualene is also a substrate of SHC *in vitro* (42,43). We have now developed truncated vinyl sulfide derivatives of OS and other new derivatives and compared their activity on the animal, yeast and bacterial enzyme.

In Table 1, the $IC_{50}$ inhibition values obtained testing OSC activity with a solubilized and partially purified pig liver OSC and a microsomal suspension of *S. cerevisiae* are compared with those obtained with a purified recombinant SHC of *A. acidocaldarius*. Most of the compounds tested, as expected, are effective inhibitors of both OSC and SHC. The most promising result was obtained with compound **6** (Scheme 2), which shows an $IC_{50}$ of 50 nM for yeast OSC and for SHC: this is among the best activities described to date for *S. cerevisiae* OSC inhibition and one of the best for bacterial SHC. On *S. cerevisiae* OSC, compound **6** is 240-fold more effective than compound **9**, the homologous compound bearing a lateral chain, and 30-fold more effective than the shorter analog **7**. The $IC_{50}$ of compound **6** toward pig OSC is 1 μM, which is one-fifth and one-half of the activity shown by compounds **7** and **9**, respectively. Therefore, compound **6** is not only very active but also specific for yeast OSC, as it is 20 times less active toward the animal enzyme. This result strongly supports the existence of peculiar differences between animal and fungal OSC in the modality of assisting the cyclization process by the active site, although a great homology exists between yeast and animal cyclases both in the amino acid sequence and in the mechanism of cyclization.

**TABLE 1**
**Inhibition Values ($IC_{50}$) of Pig Liver and *Saccharomyces cerevisiae* Oxidosqualene Cyclase (OSC) and Squalene-Hopene Cyclase (SHC) by Vinyl Sulfide Derivatives of Truncated Oxidosqualene**

| | $IC_{50}$ (μM)[a] | | |
|---|---|---|---|
| Compound[b] | OSC (pig liver) | OSC (*S. cerevisiae*) | SHC (*A. acidocaldarius*) |
| **6** | 1 | 0.05 | 0.05 |
| **7** | 5 | 1.5 | 0.5 |
| **8** | 100 | 50 | 3.1 |
| **9** | 2.2 | 12 | 9 |
| **10** | 0.35 | 0.17 | 10 |
| **11** | 6 | 2.5 | 0.6 |
| **12** | >100 | 35 | 25 |
| **13** | 12 | 3.5 | 0.9 |

[a]$IC_{50}$, inhibitor concentration reducing enzymatic conversion by 50%; *A. acidocaldarius, Alicyclobacillus acidocaldarius.*
[b]For compound structures, see Scheme 2 and 3.

The differences seem mainly to concern the formation or stabilization of the C-20 carbocation, since the shorter inhibitors, designed to affect the formation or the stabilization of the C-13 carbocation, do not show important differences of activity between pig and yeast OSC. A similar pattern of inhibition was observed with the 29-methylidene-2,3-oxidosqualene (29-MOS) and hexanor-29-MOS derivatives (21,27–30): (18*Z*)-29-MOS was a very potent irreversible inhibitor of pig and rat OSC, but failed to inactivate yeast OSC, while the (18*E*)-hexanor-29-MOS, lacking the side chain, was a potent and time-dependent inhibitor of yeast OSC.

With the exception of compound **6** toward yeast OSC and SHC, the activity of the other methylthio derivatives **7** and **9** is in the micromolar range for both OSC and SHC. Another interesting finding about this new series of compounds concerns compound **10** (Scheme 3): this dithiane derivative is a very effective inhibitor of both pig and yeast OSC ($IC_{50}$ = 0.35 and 0.17 μM, respectively) and less effective as an SHC inhibitor ($IC_{50}$ = 10 μM). The shorter derivative **11** and the benzodithiole derivative **13** are less active toward OSC and more active toward SHC, and derivative **12**, bearing a terminal dithiane, is a poor inhibitor of both OSC and SHC. The activity of **11** toward SHC is similar to that of other truncated derivatives, such as compound **7** or the (18*E*)-hexanor-29-MOS, previously studied by us (17). SHC seems to be more affected than OSC by possible interactions with the process of formation or stabilization of the C-13 carbocation, since insertion in the substrate analogs of functionalities in the vicinity of this position is very effective for inhibition. Compound **8** is a very poor inhibitor of OSC and a fairly good inhibitor of SHC, thus confirming our previous finding that the substrate analogs need a 2,3-epoxide ring to behave as OSC

inhibitors. Obviously, the epoxide is not necessary for SHC, as the activity toward **8** on SHC is comparable to the other derivatives of this series.

Theoretical studies (44,45) have shown the excellent properties of sulfur in stabilizing the electron-deficient α-carbon, due to its good π- and σ-donor properties. These new compounds, once cyclized by the enzyme, should therefore form a more stable carbonium ion, because of the adjacent sulfur. This intermediate should react, or at least strongly interact, with enzyme residues normally stabilizing the natural carbocations of the cyclizing substrate. Stabilization by sulfur is obviously greater in the α-carbon of ketene dithioacetals, such as compounds **10** and **11**, that, if cyclized by the OS enzyme, should afford a highly delocalized thiocarbenium ion interacting with the adjacent nucleophiles of the enzyme active site. Finally, the benzo-1,4-dithiafulvene derivative **13**, if cyclized by the enzyme, should afford a stable heteroaromatic 1,3-benzodithiolium ion, which might react with the enzyme nucleophiles or directly afford the cyclized fulvene through elimination of the adjacent proton (39).

In order to get further insight into the mechanism of inhibition by the more active compound **6,** it would be interesting to study the time-dependency of inhibition on *S. cerevisiae* OSC, but the low specific activity found in yeast microsomes does not allow the dilution necessary to test the residual activity after preincubation. We are currently planning to look for a more active and suitable source of yeast enzyme. Recently it has been shown that most OSC activity in yeast is present in the cellular compartment of lipid particles (46). Work is in progress to prepare a lipid particle fraction and to test whether this source of enzyme activity is more suitable to study time-dependency of the more interesting inhibitors on yeast.

So far, time-dependency has only been studied with pig liver OSC and with SHC. SHC was not inhibited in a time-dependent manner up to a concentration of **6** that was 10-fold higher than the $IC_{50}$.

Pig liver OSC was inhibited in a time-dependent manner only at inhibitor concentrations five times higher than the $IC_{50}$: after 5 min of preincubation in the presence of the inhibitor, the residual activity was 60% of the controls, and after 20 min it was reduced to 35% of the controls, as shown in Figure 1. The second-order inactivation constant, $k_{inact}/K_I$, calculated from the $t_{1/2}$ values, was 0.011 $mM^{-1}$ $min^{-1}$. This time-dependent inhibition of pig liver OSC showed a pattern similar to that found with a β-hydroxysulfide derivative of truncated OS that we had previously studied (31). Also in this case, the inhibition might be due to the formation of partially cyclized carbocationic intermediates able to bind the enzyme irreversibly or to form a tightly bound ionic complex of the sulfonium ion with the nucleophilic residues of the active site.

The formation of a 6.6.5 fused tricyclic cation that is responsible for OSC inactivation by (18 *E*)-29-MOS was suggested by Abe *et al.* (47). After incubating SHC with labeled (18*Z*)-29-MOS, a partially cyclized metabolite was isolated (36).

We were not able to show the presence of metabolites of compound **6** by high-performance liquid chromatographic analysis of the incubation mixture. Compound **6** is a very efficient inhibitor, and therefore only traces of metabolites could be formed. It is possible that a large amount of the enzyme is necessary to produce detectable amounts of metabolites of a very effective inhibitor.

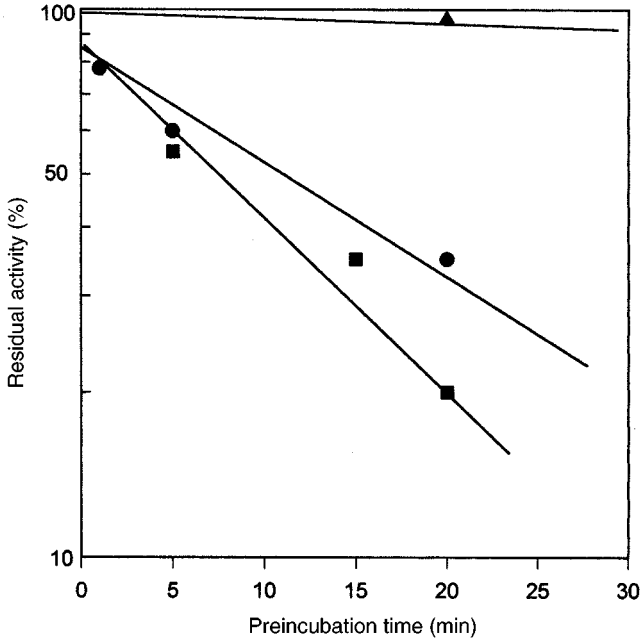



**FIG. 1.** Time-dependent inhibition of pig oxidosqualene cyclase by compound **6** at concentrations of 0.2 (–▲–), 5 (–●–), and 20 μM (–■–).

The high activity and specificity of the methyl sulfide derivative **6** toward yeast OSC, if confirmed with pathogenic fungi, could offer interesting prospects for designing new antifungal drugs.

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# Composition of the Silk Lipids of the Spider *Nephila clavipes*

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**ABSTRACT:** A detailed analysis of the lipids of spider silk is given for the first time. Extracts of the silk from the golden orb weaver, *Nephila clavipes*, were studied by gas chromatography, mass spectrometry, and chemical derivatizations. The major group of the lipids consisted of methyl-branched 1-methoxyalkanes (methyl ethers) with up to four methyl groups in the chain (chain length between $C_{28}$ and $C_{34}$), which are unique to spiders. The position of the methyl branches was determined by conversion into cyanides, which allowed easy location of methyl branches. The second-largest group included alkanes with a wide structural variety; 2-methyl-branched, even-numbered hydrocarbons predominated. A general numerical method for the estimation of retention indices of alkanes and their derivatives is presented. Further components of the web included alkanols and alkanediols, fatty acids, and glyceryl ethers. Some comments on the biosynthesis of these compounds are also given.

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The chemical composition and structure of spider silk is currently under intensive study because of its unique properties like tensile strength and elasticity (1–4). Most of this work has been done on silk of the golden orb weaver, *Nephila clavipes* (Arachnida: Araneae). Although the proteins of the fiber have been investigated in detail, other components of the webbing have not received much attention. The presence of lipids on the silk of some spiders has been reported by us (5,6). The purpose of the present study was to analyze the silk lipids of *N. clavipes* for the first time.

## MATERIALS AND METHODS

*Silk*. *Nephila clavipes* was fed *Drosophila melanogaster* and *Calliphora erythrocephala* flies. The large sexual dimorphism between male and the larger female spiders facilitated the collection of female silk only. Silk was obtained from freshly built webs (<2 d old) produced by female specimens living freely in a rearing room under subtropical conditions. The spiders were not fed during web production in order to reduce the possibility of contamination of the silk with lipids of insect prey. Batches of silk (wet weight between 100 mg and 2 g) were immersed in 1 to 4 mL pentane or $CH_2Cl_2$ for several hours; the silk was removed and then the excess solvent was evaporated at room temperature. Dichloromethane exhibited greater efficiency in the extraction step especially with freeze-dried material, which tended to form hard clumps, but $CH_2Cl_2$ also extracted greater quantities of polar material such as ethanolamine. The silk extracts were then analyzed. For quantitative determinations silk batches were freeze-dried (Christ Alpha 1-2), weighed, extracted, freeze-dried again, and finally weighed to estimate the amount of lipids of the silk.

*Derivatization*. Transformation of methyl ethers into cyanides was performed with pentane or dichloromethane extracts of silk (7). The methyl ethers in the extracts were cleaved with $Me_3SiI$ and the resulting iodides were substituted with cyanide. Although substitution could have been performed with NaCN in dimethylsulfoxide at 50°C, cleaner extracts were obtained using tetraethylammonium cyanide at room temperature in $CH_2Cl_2$, because the solution of the iodides could be used directly without change of solvent. After extraction of excess reagent with water and evaporation of $CH_2Cl_2$, the residue was taken up in pentane. This procedure resulted in a clean, salt-free solution ready for analysis by gas chromatography–mass spectrometry (GC–MS) without formation of artifacts.

Methyl esters were obtained by oxidation of extracts with $RuO_4$ (5). Silylations were performed by adding 30 μL *N*-trimethylsilyltrifluoroacetamide (MSTFA) to an equal amount of extract. After 30 min at 50°C, excess reagent and solvent were evaporated with a gentle stream of nitrogen and the residue was taken up in pentane. Methylations of fatty acids were performed similarly, but with trimethylsulfonium hydroxide solution (Aldrich, Milwaukee, WI) instead of MSTFA. Under these conditions, wax esters are cleaved and alcohols are converted to 1-methoxyalkanes.

*Analysis*. Mass spectra (70 eV) were obtained with a VG 70/250 S mass spectrometer (Manchester, United Kingdom) coupled to a Hewlett-Packard HP 5890 A gas chromatograph (Palo Alto, CA) with on-column injection or a Hewlett-Packard MSD 5973 with splitless injection, both in electron impact (EI) mode with helium as the carrier gas. The gas chromatograph was a Carlo-Erba Fractovap 2101 (Milan, Italy) with on-column injection or a CE Instruments GC 8000 (Milan, Italy) with split/splitless-injection. Separations were performed on different 25-m BPX-5 (SGE; Melbourne, Australia) capillary columns with hydrogen as the carrier gas. The retention indices *I* were determined according to van den Dool and Kratz (8). Well-established procedures for identification of alkanes exist, which have been recently summarized in a general identification scheme based on mass spectra and retention indices (9). These were followed and modified, as

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Abbreviations: EI, electron impact; GC, gas chromatography; *I*, retention index; MS, mass spectrometry; MSTFA, *N*-trimethylsilyltrifluoroacetamide.

discussed in the text, when needed. Several identified compounds were compared with synthetic samples [1-alkanols, 1,3-diols, some 1-methoxyalkanes (5), and 2,6-dimethyloctacosane and 2,22-dimethyloctacosane].

## RESULTS

Two extractions with pentane yielded a lipid content of 2.14 and 2.27% for two samples (173.0 and 224.2 mg dry weight, respectively). An additional extraction with $CH_2Cl_2$ yielded another 2.6 and 4.01% of material, which also contained some nonlipidic compounds. From these experiments, the lipid content of the dry fiber was estimated to range between 3 and 5%. The GC–MS analyses of the $CH_2Cl_2$ or pentane extracts revealed the presence of three major classes of lipid: hydrocarbons, alcohols, and unique methyl-branched alkyl methyl ethers.

*Alkyl methyl ethers*. Long-chain ($C_{28}$ to $C_{34}$) alkyl methyl ethers (1-methoxyalkanes) made up between 50 and 80% of the silk lipids. The ethers were identified by characteristic ions at $m/z = 45$ ($CH_3OCH_2^+$) and $M^+ - 32$ ($M^+ - CH_3OH$) in their mass spectra, as well as their inertness toward MSTFA. Their gas chromatographic *I* and intensive ions arising from cleavage next to methyl groups pointed to multiple methyl branches in these ethers. The gas chromatograms of the crude and derivatized extracts showed one advantage of cyanide derivatization (Fig. 1). Owing to the later elution of the cyanides, other compounds present in the extract were better resolved, thereby allowing the identification of minor hydrocarbons that coeluted with some methyl ethers in the silk extracts.

The ethers from *N. clavipes* contained up to four methyl branches. As an example, the identification of 1-methoxy-16,20,24,28-tetramethylhentriacontane (compound **28** in Table 1) will be described (mass spectrum, Fig. 2A). The number of methyl groups along the chain was calculated by comparison of its retention index *I* (8) with calculated values $I_c$ for different structures according to Equation 1 (5,6), where *N* is the number of carbons in the chain times 100, FG is a functional group increment, Me is an increment for each methyl group according to its position relative to the end of the chain (see Table 2), and *S* is a steric increment for each 1,5-position of two methyl groups, which was determined to

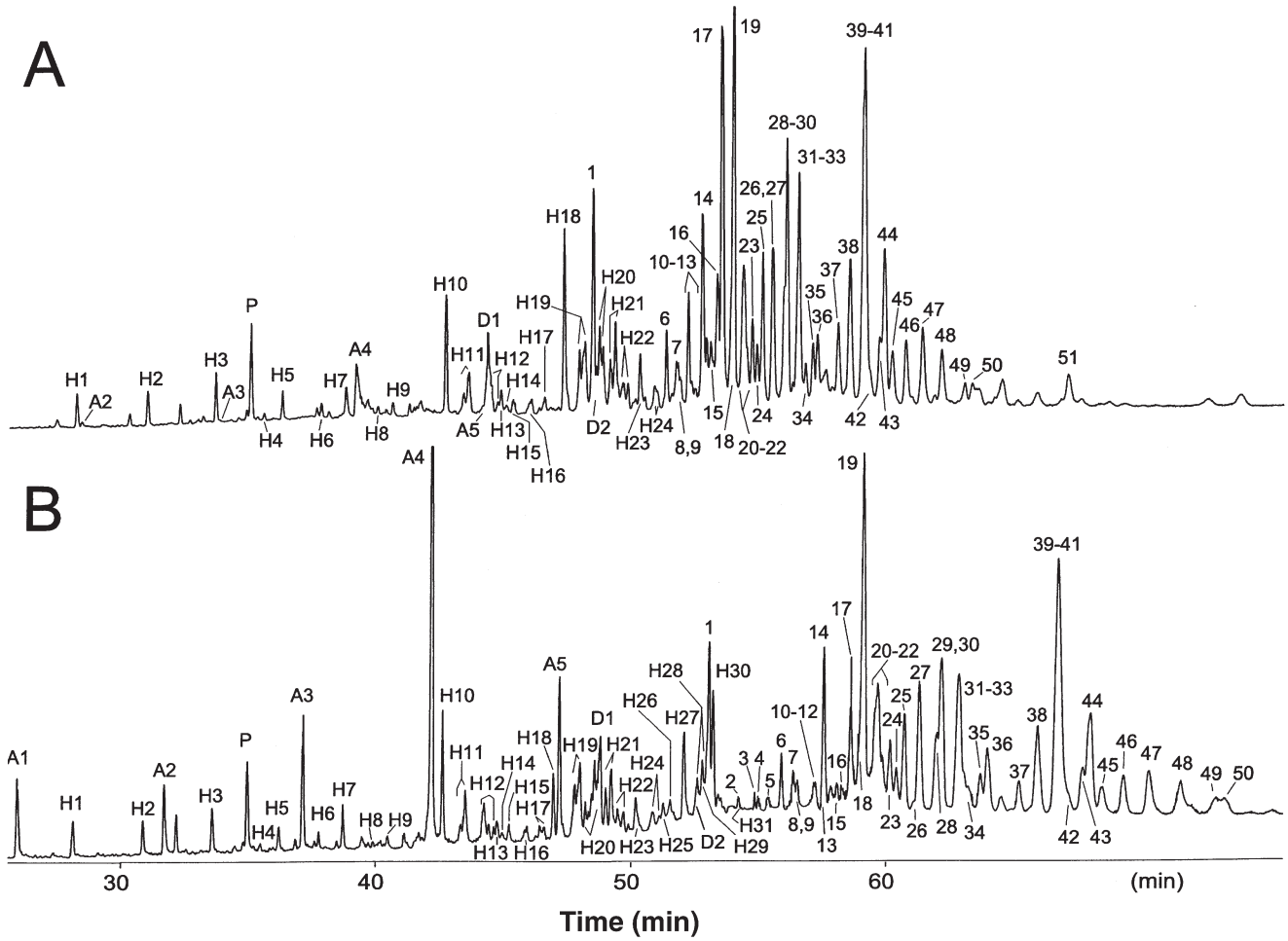


**FIG. 1**. Gas chromatograms of silk lipid extract from *Nephila clavipes* on a 25-m BPX-5 column (SGE, Melbourne, Australia). (A) Crude silk extract; (B) silk extract after transformation into cyanides. Prefixes of peak numbers denote chemical classes: H, hydrocarbon; A, alcohol; P, phthalate; D, diol.

**TABLE 1**
**Presence of 1-Methoxyalkanes in Web Extracts of *Nephila clavipes***

| | $C_{28}$ | $C_{29}$ | $C_{30}$ | $C_{31}$ | $C_{32}$ | $C_{33}$ | $C_{34}$ |
|---|---|---|---|---|---|---|---|
| | | | **8** | **18** | **29** | | |
| 20-Methyl- | | | 3487 | 3589 | 3687 | | |
| | | | + | + | + | | |
| | | | **9** | **19** | **30** | **38** | |
| 22-Methyl- | | | 3491 | 3589 | 3687 | 3785 | |
| | | | + | +++ | ++ | ++ | |
| | **1** | | | | | | |
| 26-Methyl- | 3329 | | | | | | |
| | ++ | | | | | | |
| | | | **14** | | | | |
| 28-Methyl- | | | 3531 | | | | |
| | | | ++ | | | | |
| | | | **17** | | | | |
| 2,28-Dimethyl- | | | 3571 | | | | |
| | | | ++ | | | | |
| | | | **10** | **20** | **31** | **39** | |
| 16,20-Dimethyl- | | | 3506 | 3605 | 3706 | 3803 | |
| | | | + | ++ | + | + | |
| | | **3** | **11** | **21** | **32** | **40** | **48** |
| 18,22-Dimethyl- | | 3425 | 3509 | 3608 | 3707 | 3804 | 3902 |
| | | + | + | ++ | ++ | +++ | + |
| | | **4** | **12** | **22** | **33** | **41** | |
| 20,24-Dimethyl- | | 3431 | 3516 | 3611 | 3712 | 3807 | |
| | | + | + | + | + | + | |
| | | **5** | | | | | |
| 20,26-Dimethyl- | | 3447 | | | | | |
| | | + | | | | | |
| | | | **16** | | | | |
| 20,28-Dimethyl- | | | 3557 | | | | |
| | | | + | | | | |
| | | | **13** | **23** | **34** | **42** | |
| 22,26-Dimethyl- | | | 3529 | 3624 | 3715 | 3814 | |
| | | | + | ++ | + | + | |
| | | | | **24** | **35** | **43** | **49** |
| 16,20,24-Trimethyl- | | | | 3632 | 3737 | 3824 | 3923 |
| | | | | + | + | ++ | + |
| | **2** | **6** | **15** | **25** | **36** | **44** | **50** |
| 18,22,26-Trimethyl- | 3389 | 3466 | 3549 | 3641 | 3744 | 3830 | 3926 |
| | + | + | + | ++ | ++ | ++ | + |
| | | | | **27** | | **45** | |
| 20,24,28-Trimethyl- | | | | 3660 | | 3839 | |
| | | | | ++ | | + | |
| | | **7** | | **26** | | | |
| 14,18,22,26-Tetramethyl- | | 3482 | | 3652 | | | |
| | | + | | + | | | |
| | | | | **28** | **37** | **46** | **51** |
| 16,20,24,28-Tetramethyl- | | | | 3681 | 3766 | 3855 | 3945 |
| | | | | ++ | + | ++ | + |
| | | | | | | **47** | |
| 18,22,26,30-Tetramethyl- | | | | | | 3876 | |
| | | | | | | ++ | |

[a]Bold numbers refer to compounds in Figure 1. Small numbers are retention indices of cyanide derivatives. +++: major component (more than 8 % of total extract); ++: minor component (1–8 % of total extract); +: trace component (less than 1% of total extract).

be 5.[1] Normally, the calculated and measured values did not fall more than 10 units apart.

$$I_c = N + \mathrm{FG} + \Sigma\,\mathrm{Me}_i - \Sigma\,S \qquad [1]$$

A value of $I$ = 3465 was found for compound **28** (FG is 232 for methyl ethers), pointing to four or more methyl groups in

[1]The values were determined with $C_{29}$ and $C_{30}$ components. A slight decrease in increments [see Carlson *et al.* 9] has to be taken into account when applying the method to longer chains. A 1,7-arrangement of methyl groups often shows a similar factor $S$ especially in multimethyl-branched compounds. Note that this algorithm allows only rough estimation of the structure (9), especially the discrimination of several constitutional isomers with different chain length. More precise $I_c$ values can be obtained with molecular mechanics calculations (10), at the expense of considerably more effort.

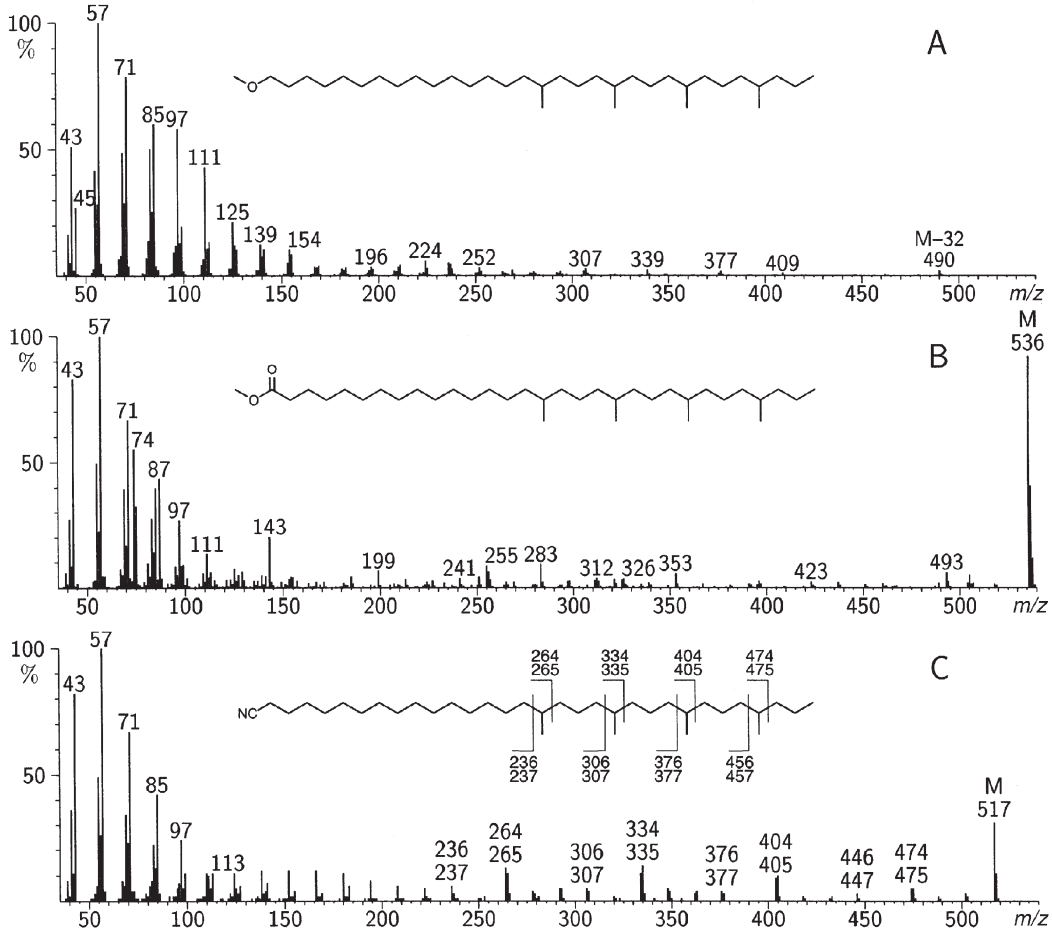


**FIG. 2.** Mass spectra of *Nephila clavipes* silk lipids and their derivatives. (A) 1-Methoxy-16,20,24,28-tetramethylhentriacontane (compound **28** in Table 1); (B) methyl 16,20,24,28-tetramethylhentriacontanoate; (C) 16,20,24,28-tetramethylhentriacontyl cyanide.

the molecule. The EI mass spectrum of the corresponding cyanide allowed easy location of the methyl groups, because chain cleavage predominantly occurs at branching points and preferably furnishes nitrogen-containing cations (7) (Fig. 2C). The original position of the methoxy group is thus evident. Because other fragment ions become more prominent below *m/z* = 115, location of methyl groups between C-2 and C-5 is ambiguous. To overcome this problem, the mass spectra of the corresponding methyl esters allow the localization especially at these positions (11), whereas multiple branchings within the chain are difficult to ascertain in the esters (Fig. 2B), especially when they occur as mixtures (12). The mass spectrum of the corresponding methyl ester of compound **28** exhibits no ions characteristic for methyl groups between C-2 and C-5, leading to the structural assignment of compound **28**. The value $I_c$ = 3461 for compound **28** correlates well with the observed one of 3465, while the respective cyanide (FG 458 for the cyanide group) differs slightly more: $I$ = 3681, $I_c$ = 3688. For comparison, mass spectra derived from 1-methoxy-18,22,26-trimethylhentriacontane (compound **25** in Table 1) are given in Figure 3. A total of 51 methoxyalkanes of *Nephila clavipes* silk were identified by this method and are tabulated in Table 1.

*Alkanes*. Alkanes constituted the second-largest group of compounds in the silk extracts; most of them were methyl-branched. The major components, 2-methyloctacosane and 2-methyltriacontane, were accompanied by a large number of dimethyl, trimethyl, and tetramethyl even- and odd-numbered alkanes (alkanes with an even or odd number of carbon atoms in the chain) as well as some *n*-alkanes (Fig. 1). The identification of even-numbered alkanes with several methyl branches is more difficult than those of the odd-numbered ones (9). As

**TABLE 2**
**Increments $Me_i$ for Calculation of Retention Indices**

| Position | $Me_i$ | Position | $Me_i$ |
|---|---|---|---|
| 2 or n-1 | 60 | 7 or n-6 | 36 |
| 3 or n-2 | 73 | 8 or n-7 | 33 |
| 4 or n-3 | 56 | 9 or n-8 | 31 |
| 5 or n-4 | 46 | 10 to n-9 | 28 |
| 6 or n-5 | 40 | | |

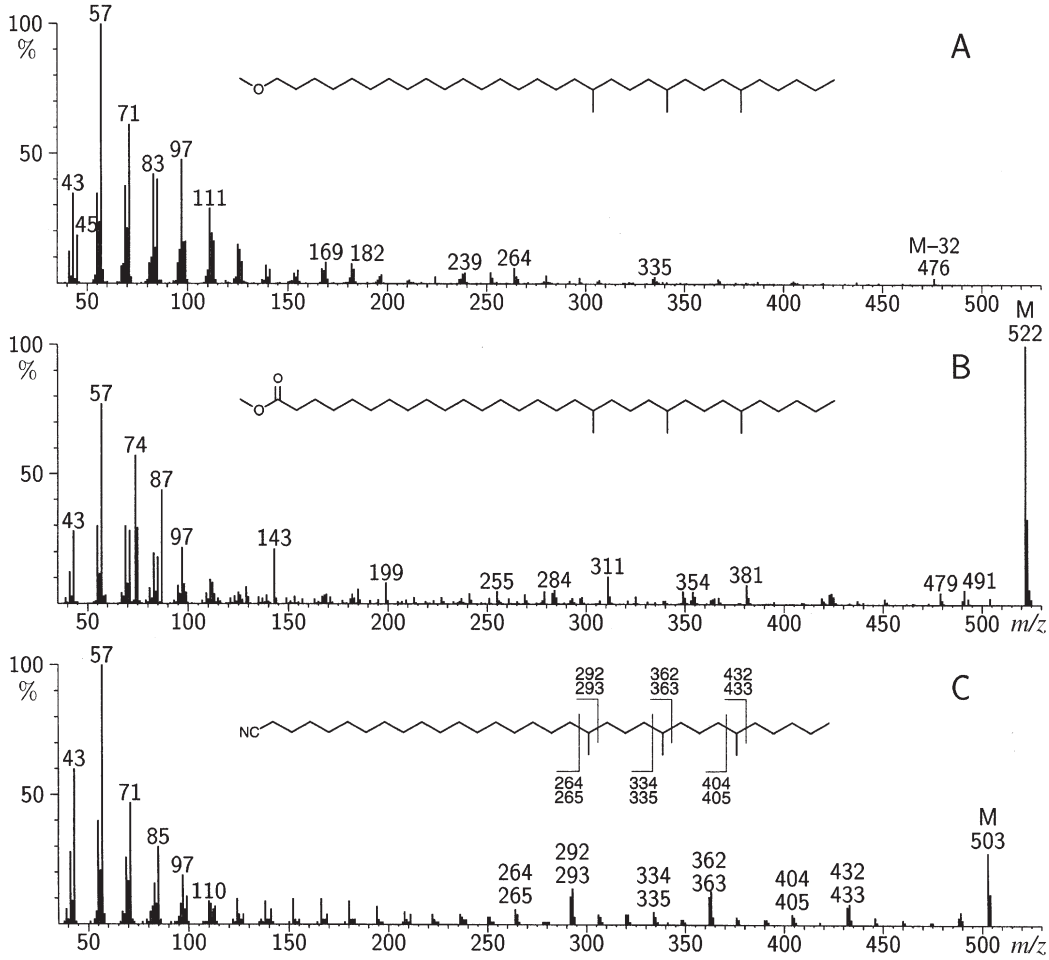


**FIG. 3.** Mass spectra of *Nephila clavipes* silk lipids and their derivatives: (A) 1-Methoxy-18,22,26-trimethylhentriacontane (compound **25** in Table 1); (B) methyl 18,22,26-trimethylhentriacontanoate; (C), 18,22,26-trimethylhentriacontyl cyanide.

pointed out by Pomonis *et al.* (13), but not always considered by later workers, the identification of 2-methylalkanes with an even-numbered carbon chain and additional methyl branches is often ambiguous. In the mass spectra, characteristic ions *a* arise from α-cleavage next to the methyl branch (Fig. 4).

Fragments containing no additional methyl branch also form an intense ion $a - 1$, resulting in characteristic double ions (14,15). Unfortunately, a methyl group at C-2 does not suppress the formation of the $a - 1$ ion (13), as do methyl groups in other positions. Therefore, for every set of ions, two possible structures exist, provided both represent biosynthetically reasonable structures. All multiply branched cuticular alkanes from arthropods identified so far possess at least three methylene units and an uneven number of them between the branches (14,16–18). An even number of spacer units can arise only in 2,X-branched odd-numbered alkanes, which are formed by an isobutyryl starter (derived from leucine) and incorporation of a propionate unit somewhere in the chain. The only compound of this type known so far is 2,5-dimethylheptadecane, a sex pheromone component of the moth *Lambdina fiscellaria* (19). Other reports of compounds not fulfilling the above rules are either not unequivocally supported by the presented data or are misinterpretations (14). Nevertheless, hydrocarbons possessing only a one-methylene spacer are reported from insects (18,20), but these are considerably smaller molecules than typical cuticular alkanes. In odd-numbered hydrocarbons usually only one structure fulfills the rules listed above, but in even-numbered alkanes often both structures are reasonable. Nevertheless, it should be noted that hydrocarbons not following the described biosynthetic requirements have been identified in other organisms, e.g. cyanobacteria (21).

The silk of *N. clavipes* contained a complex, difficult-to-elucidate pattern of hydrocarbons with many positional isomers. For example, the mass spectra from 2,6-dimethyloctacosane ($I$ = 2898, Fig. 4A) and 2,22-dimethyloctacosane ($I$ = 2899, Fig. 4B), which were available as synthetic reference compounds (Schulz, S., unpublished data), showed only small differences which could not be predicted. The presence of 2,6-dimethyloctacosane in the extracts was confirmed by its

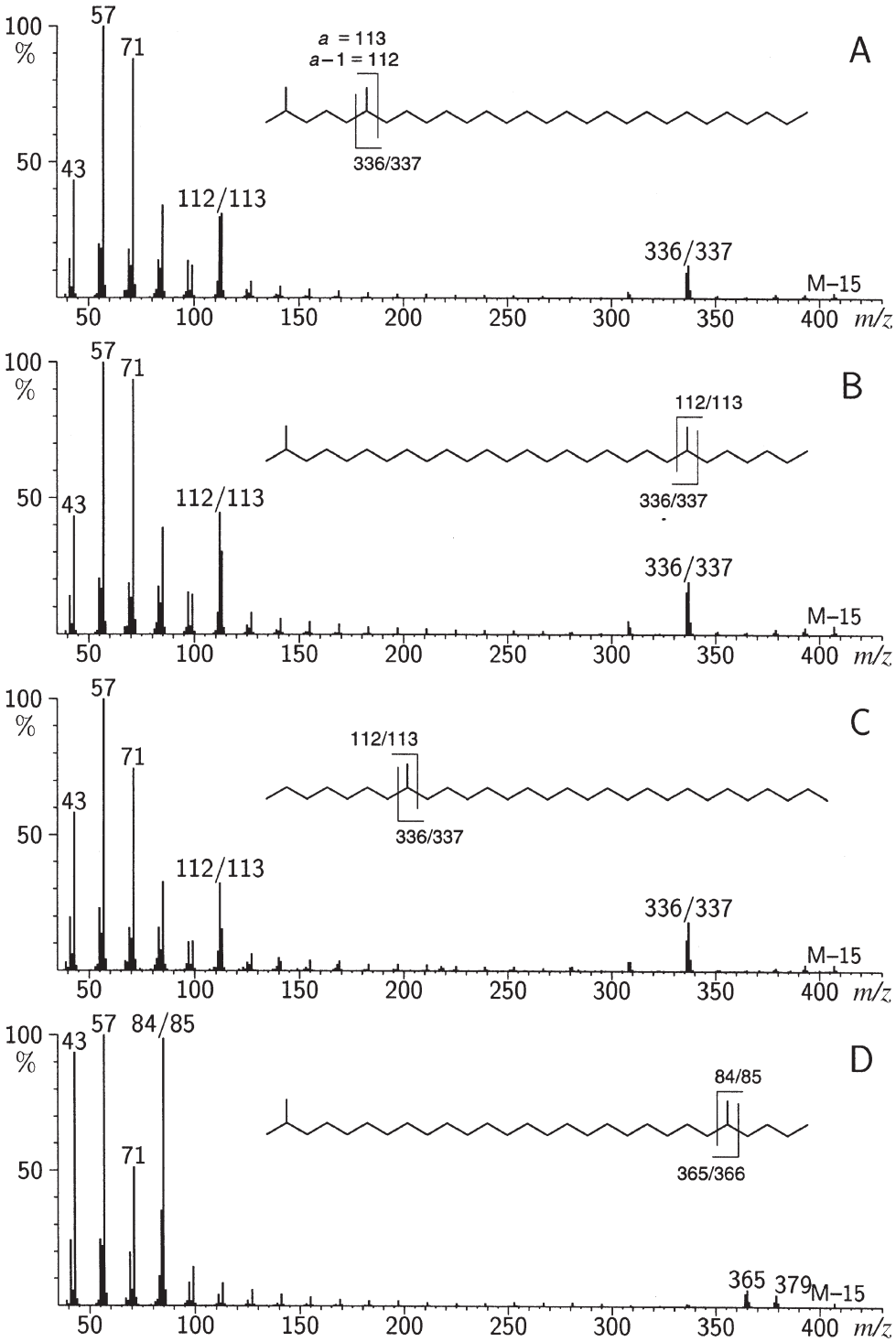


**FIG. 4.** Mass spectra of branched alkanes. (A), 2,6-Dimethyloctacosane (synthetic); (B), 2,22-dimethyloctacosane (synthetic); (C) 7-methylnonacosane (*Nephila clavipes*); (D) 2,24-dimethyloctacosane (*Nephila clavipes*).

mass spectrum, although the presence of the 2,22 isomer remains possible. The third compound with a similar spectrum, 7-methylnonacosane (Fig. 4C), also occurred in the extract but was readily identified because of its greater retention index ($I$ = 2936). We also found spectra with corresponding $I$ values consistent with the isomeric pairs 2,8-/2,20- ($I$ = 2293), 2,10-/2,18- ($I$ = 2291), 2,12-/2,16- ($I$ = 2289), and 2,14-dimethyloctacosane ($I$ = 2289). It cannot be stated with certainty whether only one or both compounds of these pairs were present in the silk. Additionally, 2,24-dimethyloctacosane ($I$ = 2903) was identified, for which no biosynthetically reasonable other structure exists (Fig. 4D). Obviously, a relatively random distribution of methyl branches on even-numbered carbon atoms occurred along the chain.

Identifications became more difficult with additional methyl groups in the chain, because complex overlayings of several isomers with identical or slightly different *I* were found. Although only two isomers (2,6,10- and/or 2,18,22-) dominated in the trimethyloctacosanes eluting at an *I* of 2921, three isomers are possible in the corresponding tetramethyloctacosanes at *I* = 2950, despite the fact that the 2-methyl group and the other branches are certainly located at opposite ends due to biosynthetic considerations. Otherwise, only one methylene unit would be found between two methyl branches (Fig. 5).

Both 2,16,20,24- and 2,14,20,24-tetramethyloctacosane are consistent with the observed mass spectrum. Both were probably present, because the characteristic $a - 1$ fragment occurred at *m/z* = 224 and at 252. These structures do not explain the intense ion at *m/z* = 295, which could be due to the presence of 2,14,18,24-tetramethyloctacosane, but the required corresponding ion at *m/z* = 183 was of relatively low abundance. Similar difficulties arose with the identification of other multibranched hydrocarbons. The major hydrocarbons identified in each peak are shown in Table 3 and were often accompanied by several unstated isomers. A more detailed description of the hydrocarbons of the silk and the cuticle of *N. clavipes* will be published elsewhere.

*Alcohols*. Straight-chain, even-numbered 1-alkanols ($C_{18}$ to $C_{26}$) were also present in the silk extracts. They sometimes exhibited considerable tailing during GC on apolar phases, depending on the inertness of the column. Therefore, silylation prior to analysis is the method of choice to improve peak shape. During the transformation of the ethers into cyanides the alcohols were also smoothly transformed, as can be seen for 1-tetracosanol (**A4** in Fig. 1). Because peak shape was improved, an additional silylation was not necessary. In addition to the 1-alkanols, the diols 1,3-docosanediol (compound **D1** in Fig. 1), 1,3-tetracosanediol (**D2**), and 1,3-hexacosanediol were present in the extracts. They were identified from their mass spectra by an intense ion at *m/z* = 75, representing $HO(CH_2)_2CHOH^+$, which was accompanied by characteristic ions at M – 1, M – 18, and M – 36 (Fig. 6A). These diols formed dicyanides under the conditions reported above. The mass spectrum of the dicyanide of the major 1,3-tetracosanediol showed an intense ion at *m/z* = 348 (M – 40, M – $CH_2CN$), indicative for the terminal cyano group, but the location of the second cyano group could not be readily derived from its spectrum. This ion was intense, because in contrast to the monocyanides it contained a cation-stabilizing cyano group.

During the analysis of the fatty acids of the extracts (see below), we found that trimethylsulfonium hydroxide not only formed methyl esters from fatty acids and esters but also methylated alcohols when the reaction took place at 50°C for 30 minutes. The resulting 1,3-dimethoxy compounds gave characteristic mass spectra, exhibiting ions at *m/z* = 103 ($CH_3OCH_2CH_2CHOCH_3^+$) and 339 ($C_{22}H_{44}OCH_3^+$) (Fig. 6C).

We also observed the occurrence of a GC peak in the underivatized extracts showing a very prominent base peak at *m/z* = 131 in its mass spectrum (Fig. 6D). This compound obviously was a reaction product of the diol with the GC phase, because the spectrum was consistent with 4-henicosyl-2,2-dimethyl-1,3-dioxa-2-silacyclohexane. The ion trace at 131 was enhanced after the peak until it reached the later-eluting parent diol. This behavior represented degradation of a compound on the column and was also observed after injection of synthetic diols. A standard BPX-5 (SGE) column containing dimethylsilane units was used, but this effect was also observed on similar phases from other suppliers. We have also encountered this phenomenon with other long-chain diols (22). To the best of our knowledge, this is one of the very few cases demonstrating the reaction of an apolar GC phase with a solute normally regarded as nonreactive.

*Other compounds*. Other silk components were fatty acids, wax-type esters, and 1-*O*-alkylglyceryl ethers. They were identified by their mass spectra or after derivatization with MSTFA or trimethylsulfonium hydroxide (23,24). The content of the fatty acids varied with the sample, but palmitic acid (16:0, 39% of all acids) was always the most prominent one, followed by 18:1 (17%), stearic acid (18:0, 15%), and 18:2 (8%) acids. Other acids found in lower amounts were 14:0 (3%), 13-Me-14:0 (1%), 15:0 (1%), 16:1 (4%), 17:0 (1%), 20:0 (2%), 22:0 (5%), and 24:0 (1%), while the acids 12:0, 13:0, 3-Me-13:0, 12-Me-13:0, 4-Me-14:0, 12-Me-14:0, 4-Me-15:0, 14-Me-14:0, 4-Me-16:0, 14-Me-16:0, 15-Me-16:0, 19:0, 21:0, and 23:0 occurred in amounts less than 1% of the total acids in the sample. Branching positions were determined by MS (11) and comparison of *I* values with those of authentic samples. The major wax-type ester was tetracosyl hexadecanoate, which was accompanied by tetracosyl octadecanoate. Both compounds always occurred in minor amounts. The three ether lipids 1-*O*-octadecyl-, 1-*O*-eicosyl-, and 1-*O*-docosylglycerol occurred as trace components.

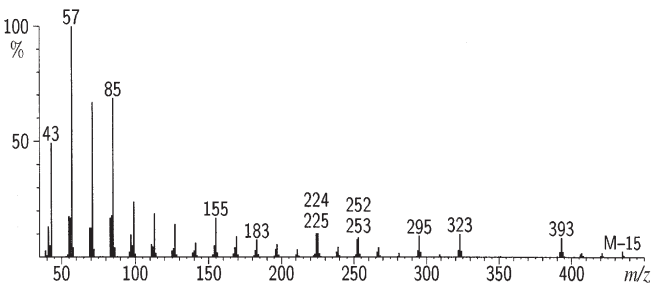


**FIG. 5.** Mass spectrum of the *Nephila clavipes* peak at *I* = 2950 consistent with 2,16,20,24- and/or 2,14,20,24- and/or 2,14,18,24-tetramethyloctacosane.

## DISCUSSION

The present study contains the first complete characterization of the silk lipids of a spider. Previously, only 1-methoxyalkanes from the silk of the linyphiid spider *Linyphia triangularis* had been characterized (5). The cuticular lipids of two spider species have also been analyzed. Although *Tegenaria*

**TABLE 3**
**Major Alkanes Identified in Web Extracts of *Nephila clavipes***

| | $C_{23}$ | $C_{24}$ | $C_{25}$ | $C_{26}$ | $C_{27}$ | $C_{28}$ | $C_{29}$ | $C_{30}$ | $C_{31}$ | $C_{32}$ | $C_{33}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Unbranched | **H1** | **H2** | **H3** | **H5** | **H7** | **H9** | **H11** | **H16** | | | |
| Methyl | | | **H4** | **H6** | **H8** | **H10** | **H14 H15** | **H18** | **H20** | **H26** | **H29** |
| | | | 3 | 2 | 3 | 2 | 2 3 | 2 | 9; 11; 13; 15 | 2 | 9; 11; 13; 15; 17 |
| | | | | | | | **H12** | | | | |
| | | | | | | | 5; 7; 9; 11; 13; 15 | | | | |
| Dimethyl | | | | | | **H11** | **H16** | **H19** | **H23** | **H27** | |
| | | | | | | 2,6[a]; 2,8[b]; 2,10[c]; | 3,7 | 2,6[e]; 2,8[f]; 2,10[g]; | 3,7 | 2,8[i]; 2,10[k]; 2,12[l]; | |
| | | | | | | 2,12[d]; 2,14; 2,16[d]; | H15 | 2,12[h]; 2,14[i]; 2,16[i]; | H22 | 2,14[m]; 2,16; 2,18[m]; | |
| | | | | | | 2,18[c]; 2,20[b]; 2,22[a]; | 5,9; 5,11; 5,13 | 2,18[h]; 2,20[g]; 2,22[f]; 2,24[e] | 5,11; 5,15; | 2,20[j]; 2,22[k]; 2,24[j] | |
| | | | | | | 2,24 | **H14** | | 7,11; 7,15 | | |
| | | | | | | | 7,11 | | | | |
| Trimethyl | | | | | | | **H17** | **H20** | **H24** | **H28** | **H31** |
| | | | | | | | 3,7,11 | 2,6,10[n]; 2,8,12[n]; 2,6,14[n]; | 3,7,11 | 2,6,14[n]; 2,6,16[n]; | 3,7,11 |
| | | | | | | | | 4,8,12 | | 2.8,16[n]; 2,10,14[n]; | |
| | | | | | | | | | **H23** | 2,10,16[n]; 2,12,16[n] | |
| | | | | | | | | | 5,9,13; | | |
| | | | | | | | | | 7,11,15 | | |
| Tetramethyl | | | | | | **H13** | | **H21** | **H25** | **H30** | |
| | | | | | | 2,6,10,14[n]; 2,16,20,24; | | 2,6,10,14[n]; 2,8,12,16[n]; | 3,7,11,15 | 2,6,10,14[n]; | |
| | | | | | | 2,14,20,24 | | 2,16,22,26; 2,18,22,26 | | 2,8,12,16[n] | |

[a]Bold numbers are compound assignments used in Figure 1, regular numbers indicate positions of methyl branches.
[b–n]Indices assign isomeric structures that would fit a given mass spectrum and retention time.
[o]Isomeric structure with n-1 methyl group instead C-2 methyl group also possible (see text).

*atrica* lipids are dominated by alkanes also found in insects (25–27), *Anelosimus eximus* contains unusual propyl esters of long-chain fatty acids together with alkanes (28). The current knowledge of cuticular and silk lipids of spiders has been reviewed (6). Previously we developed a method for identification of methyl ethers with up to two methyl branches (5). This method was improved and extended by replacing the final $LiAlD_4$ reduction step by substitution with cyanide (7). Comigrating alkanes are separated by this method from the resulting cyanides, which exhibit informative mass spectra.

The 1-methoxyalkanes, which are the major group of compounds in *N. clavipes* silk, contain up to four methyl branches in the chain. These methyl groups are located closer to the alkane end of the molecule and are most often arranged in a 1,5-pattern, with the exception of 1-methoxy-2,28-dimethyltriacontane (compound **17** in Table 1). This compound is also the only one with a methyl group at C-2, a prominent structural feature of the ethers from the silk of *L. triangularis* (5), which are less branched. No other ether occurs in both species, which are not closely related. The 1,5-arrangement of methyl substituents is the dominant pattern in di-, tri-, or tetramethyl alkanes from the insect cuticle (14,15,18).

The biosynthesis of the 1-methoxyalkanes, which have so far not been reported from organisms other than spiders, can be assumed to start from the alkyl end with the incorporation of methylmalonate instead of malonate units, leading to the methyl branches, as has been found in insects (16,18). The 4-methyl carboxylic acids present in traces might be suitable precursors for monomethyl-branched ethers. Elongation of these acids would lead to ethers containing a methyl group around C-20, a preferred location in the chain. Nevertheless, the putative precursors of the polymethyl-branched ethers, fatty acids with several methyl branches, could not be detected. Interestingly, α-oxidation or C-1 elongation seems to take place, because the structure of 3-methyltridecanoic acid cannot be explained by the known biosynthetic pathways (for a discussion on α-oxidation see Ref. 22). In the final steps, reduction and methylation furnish methyl ethers; a decarboxylation, which would lead to alkanes, does not occur. The unbranched, iso- and anteiso-branched fatty acids seem not to be involved in ether biosynthesis except for compounds **14** and **17**, which can be derived from anteiso-branched even-numbered fatty acids. The fact that methyl branches in the ethers always occur at even-numbered carbons points to an acetate starter unit for even-numbered ethers and a propionate starter for odd-numbered ethers, which do slightly prevail.

The hydrocarbons show a very broad range of structures: mono-, di-, tri-, and tetramethyl compounds. The predominating even-numbered 2-methylalkanes are formed most probably by a isobutyryl starter unit produced from valine (17). The other branches seem to be introduced relatively randomly, unlike in the ethers. These patterns indicate that the biosyntheses of hydrocarbon and ether chains are not closely related. Hydrocarbons with an odd number of carbon atoms in the chain usually predominate on the surface of arthropods (14,15,18). In contrast, major hydrocarbons of the silk lipids

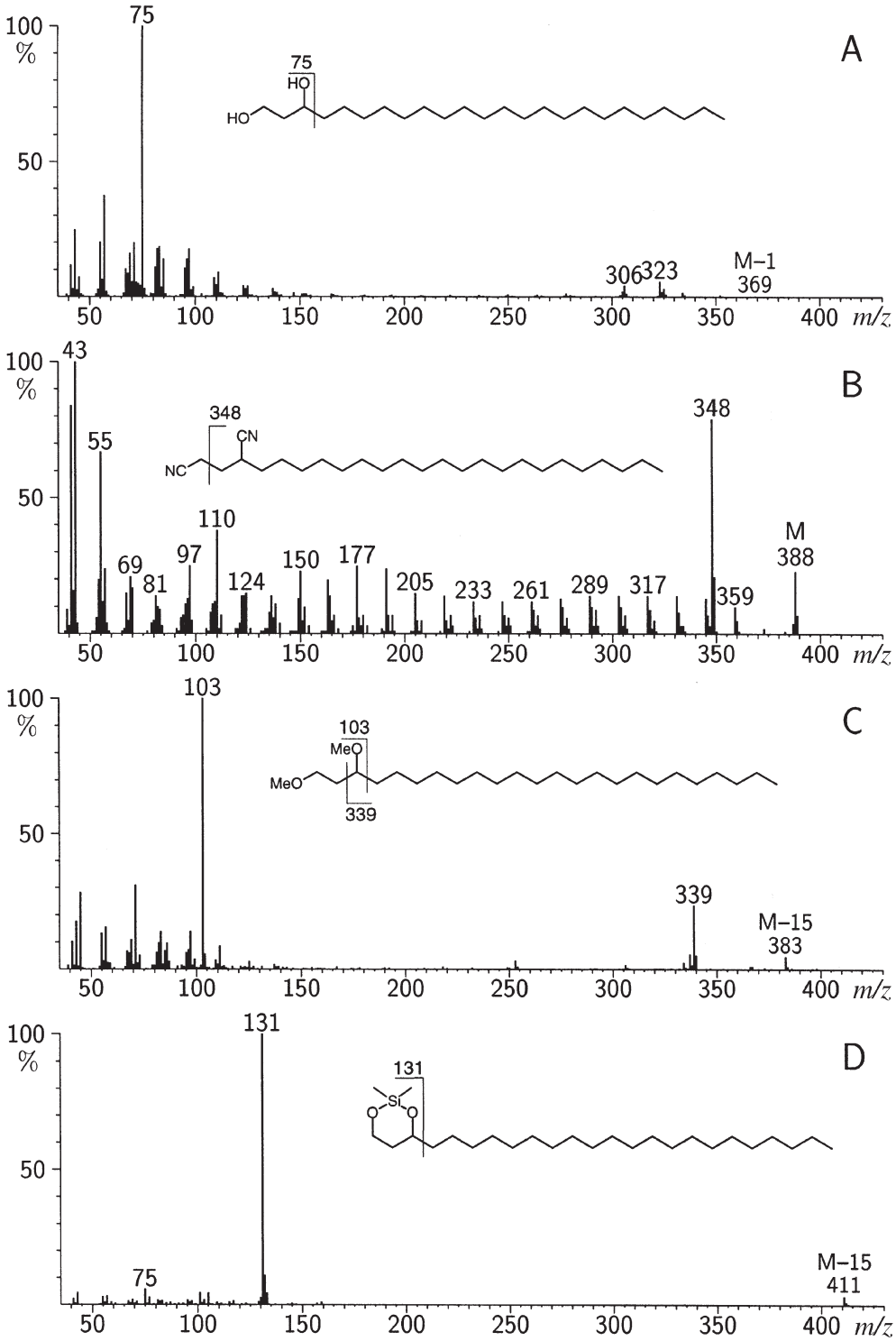


**FIG. 6.** Mass spectra of 1,3-tetracosanediol (A; compound **D2** in Fig. 1) from *Nephila clavipes*, its dicyanide derivative (B), the respective dimethyl ether (C), and its dimethylsiloxane derivative formed by reaction with the stationary phase (D).

of *N. clavipes* are even-numbered alkanes. This finding also points against a dietary uptake of the hydrocarbons, because the major alkanes of the flies used as food are odd-numbered 2-methyl and other alkanes (29,30).

The identified alcohols are not structurally related to the ethers. While the 1-alkanols have often been found in arthropod cuticle (18), the 1,3-diols are not known from any other arthropod so far. They have been recently identified in *Pa-*

*paver* leaf wax (31). Fatty acids and wax esters frequently occur in the cuticle of arthropods, while glycerol ether lipids have, to the best of our knowledge, not been reported before from such sources.

The function of the silk lipids remains unknown. Most of the lipids are likely to be present on the silk fiber, but the fact that prolonged extraction yields larger amounts of lipids may indicate involvement in the core fiber structure. The primary function may be regulation of the water content of the silk or protection of the protein-rich silk against degradation, e.g., by microorganisms or chemical agents. The lipids may also be used for communication, because males can recognize the webs of females by contact (32). Which one of the different silk types produced by *N. clavipes* (33) contains the most lipids is unknown, as is the origin of the lipids. Freshly drawn dragline silk from another *Nephila* species contains only relatively low amounts of lipids (Vollrath, F., and Schulz, S., unpublished data), suggesting that most of it is present in other silk types or is deposited from the spider while walking on the web. On the other hand, the cuticle lipids of the spider contain only small amounts of ethers and are dominated by hydrocarbons. Therefore, the possibility exists that the hydrocarbons are transferred from the cuticle during walking, while the ethers originate from another source, e.g., glands. 1-Methoxyalkanes have now been found in silk from three spider families: Araneidae, Linyphiidae, and Theridiidae (6). The reason for the production of more elaborate ethers as lipids instead of the more widespread alkanes is not clear. Probably some degree of polarity on one end of the molecule is needed for good interaction with the proteinaceous fiber or other silk compounds. The polarity of the head group of the long-chain compounds decreases from diol to 1-alkanol to ether to alkane.

The conversion of methyl ethers into cyanides proved to be an important tool for compound identification in this study. In addition to the ethers, primary and secondary alcohols were also cleanly transformed into cyanides. Esters of fatty acids can be transformed into alcohols prior to derivatization, as we have shown recently (28). On the other hand, attempts to determine the position of double bonds in unsaturated alcohols or ethers failed. No characteristic ions useful for the location of the double bond could be detected in the mass spectra of synthetic (*Z*)-9- and (*Z*)-11-octadecenyl cyanides.

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# Fullerenoid Lipids: First Synthesis of Structured Triacylglycerols Containing an Aza-[60]fullerene Unit

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**ABSTRACT:** Some 1,2- and 1,3-diacyl glycerols (with acyl groups as stearyl, oleyl, linoleyl, or stearolyl) were synthesized by conventional methods. The diacyl glycerols were esterified with 6-bromo-hexanoic acid to give the corresponding bromo-triacylglycerols (of the type AAB and ABA containing a bromo group at the distal part of the hexanoate chain). The bromo function was transformed to an azide group by reaction of the bromo-triacylglycerols with sodium azide. The resulting azido-triacyl-glycerols were then reacted with [60]fullerene to give the requisite aza-fullerenoid triacylglycerol of the type ABA or AAB (45–62% yield based on the amount of [60]fullerene reacted). The nitrogen atom attached to the carbon cage formed a "[5,6]-open" type aza substructure, which was confirmed by the appearance of 31–32 signals in the region of $\delta_C$ 133–148 (carbon shifts of $sp^2$ carbons of the cage) in the $^{13}C$ nuclear magnetic resonance spectra. The spectroscopic and mass spectrometric properties of these novel fullerenoid triacylglycerols are reported.

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In the past 15 yr, the chemistry of the [60]fullerenes ("bucky balls") has been one of the most exciting challenges in chemical research (1–6). A number of potential medical applications of derivatives of [60]fullerene have been reported (7). For example: *p,p′*-bis(2-aminoethyl)-diphenyl-[60]fullerene is found to be a potent HIV protease inhibitor (8); a [60]fullerene derivative containing three methano-dicarboxylic acid groups is a strong antioxidant with neuroprotective abilities which is being targeted for controlling amyotrophic lateral sclerosis (Lou Gehrig's disease) (9).

Polyethylene glycol derivatives of [60]fullerene are potential photodynamic therapy agents, which are shown to cure mice induced with fibrosarcoma tumors (10). Most researchers have concentrated their efforts on the production of water-soluble [60]fullerene derivatives. Only a few have described [60]fullerene derivatives containing a fatty acid or ester moiety, such as the preparation of amphiphilic $C_{60}$ [(ethoxycarbonyl)decylene]fullerene and bis[(ethoxycarbonyl)decylene]-fullerene (11) and [60]fullerene bearing a triple-chain lipid (12). The structural and dynamic effects of some lipophilic [60]fullerene derivatives in phospholipid bilayers have also been reported (13).

We recently reported the syntheses of a number of [60]fullerenoid lipids: *viz*. dialkyl 1,2-[6,6]methano-[60]-fullerene dicarboxylates (14) and several fullerenoid-lipid molecules containing a triester system (15).

This paper reports the synthesis of the first structured triacylglycerols of the type ABA (compounds **1a–1d**) and AAB (compounds **2a–2d**), where the letter B represents an acyl group containing an aza-[60]fullerene cage and the letter A represents acyl groups derived from either stearic acid, oleic acid, linoleic acid, or stearolic acid (Schemes 1 and 2).

## MATERIALS AND METHODS

Infrared (IR) spectra were recorded on a Bio-Rad FTS-165 FT-IR spectrometer (Bio-Rad Inc., Hercules, CA). Samples were run as neat films for liquids on KBr discs. Ultraviolet (UV) spectra were recorded on a Hewlett-Packard Diode Array Spectrophotometer, model 8452A (Hewlett-Packard, Palo Alto, CA) of solutions in dichloromethane. 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 (TMS) as the internal reference standard. Chemical shifts are given in δ-values in ppm downfield from TMS ($\delta_{TMS}$ = 0 ppm). Mass spectral analyses [atmospheric pressure chemical ionization (APCI)] were carried out on Finnigan MAT-LCQ (Finnigan Corp., San Jose, CA).

Stearic acid, oleic acid, linoleic acid and 6-bromo-hexanoic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Stearolic acid (9-octadecynoic acid) was prepared by bromination-dehydrobromination reaction of oleic acid as described elsewhere (16). [60]Fullerene was purchased from Materials and Electrochemical Research Corp. (Tucson, AZ).

*Synthesis of triacylglycerols (**1a–1d**) of type ABA containing an aza-fullerenoid acyl group at the β-position of the glycerol as exemplified by the synthesis of compound **1a** (Scheme 1).* 1,3-Dioctadecanoate glycerol (**4**) was synthesized starting from 1,3-dihydroxy-propan-2-one (**3**) as described by Jackson and Lundberg (17).

A mixture of compound **4** (2.0 g, 3.5 mmol), 6-bromo-hexanoic acid (0.67 g, 3.6 mmol), *N,N*-dicyclohexylcarbodiimide (DCC) (0.71 g, 3.67 mmol) and 4-dimethylaminopyridine

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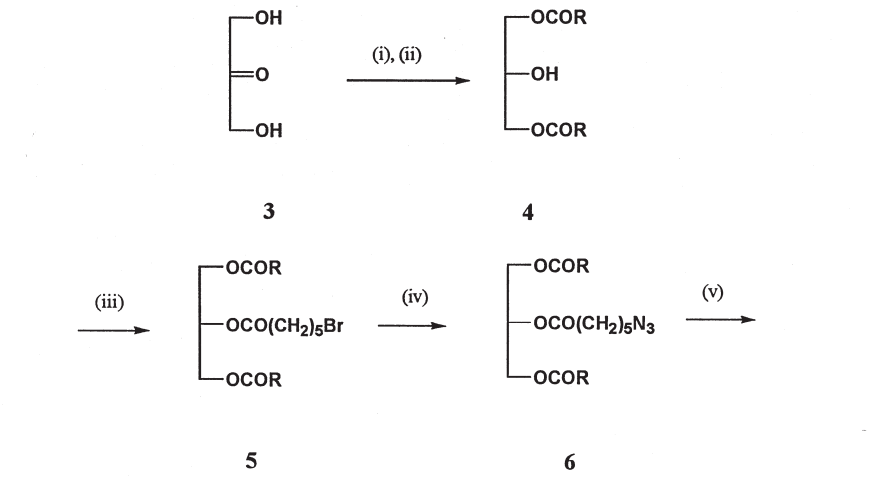
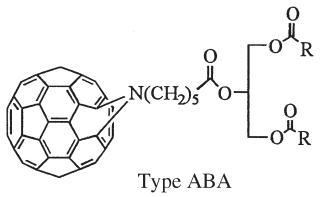
Abbreviations: APCI, atmospheric pressure chemical ionization; DCC, *N,N*-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMSO, dimethylsulfoxide; IR, infrared; MS, mass spectrometry, NMR, nuclear magnetic resonance; PPTS, pyridinium-*p*-toluenesulfonate; TLC, thin-layer chromatography; TMS, tetramethylsilane; UV, ultraviolet.

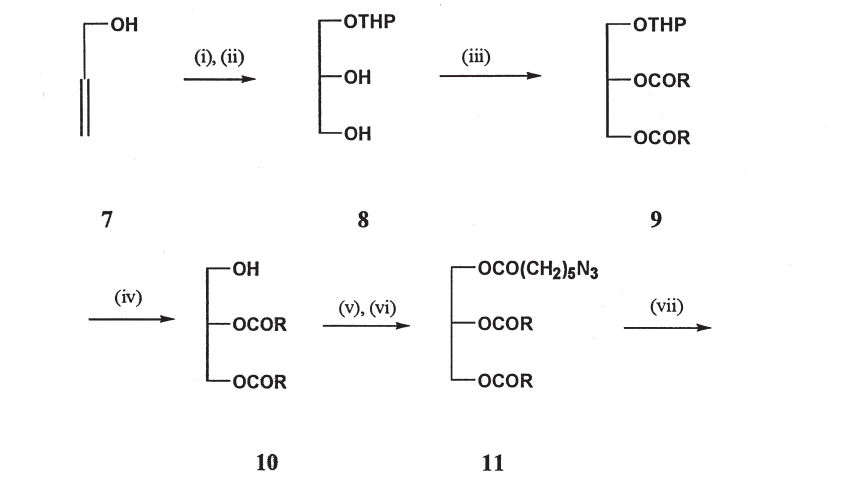


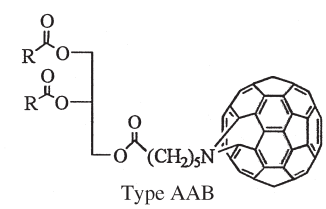




| Compound | R |
|---|---|
| **1 a** | $(CH_2)_{16}CH_3$ |
| **1 b** | $(CH_2)_7CH{=}CH(CH_2)_7CH_3$ |
| **1 c** | $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$ |
| **1 d** | $(CH_2)_7C{\equiv}C(CH_2)_7CH_3$ |

Reagents and conditions: (i) DCC, DMAP, $CH_2Cl_2$, 25°C, 4 h, RCOOH; (ii) $NaBH_4$, tetrahydrofuran, $H_2O$, 0–5°C, 30 min; (iii) DCC, DMAP, $Br(CH_2)_5COOH$, $CH_2Cl_2$, 25°C, 4 h; (iv) $NaN_3$, DMSO, 70°C, 4 h; (v) [60]fullerene, chlorobenzene, reflux 12 h.

**SCHEME 1**




| Compound | R |
|---|---|
| **2 a** | $(CH_2)_{16}CH_3$ |
| **2 b** | $(CH_2)_7CH{=}CH(CH_2)_7CH_3$ |
| **2 c** | $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$ |
| **2 d** | $(CH_2)_7C{\equiv}C(CH_2)_7CH_3$ |

Reagents and conditions: (i) 3,4-dihydro-2*H*-puran, pyridinium-*p*-toluene sulfonate (PPTS), $CH_2Cl_2$, 25°C, 4 h; (ii) $KMnO_4$, 0–5°C, 2 h at room temperature and heated for 1 h at 100°C; (iii) DCC, DMAP, RCOOH, $CH_2Cl_2$, 25°C, 4 h; (iv) PPTS, EtOH, 55°C, 4 h; (v) DCC, DMAP, $Br(CH_2)_5COOH$, $CH_2Cl_2$, 25°C, 4 h; (vi) $NaN_3$, DMSO, 70°C, 4 h; (vii) [60]fullerene, chlorobenzene, reflux 12 h.

**SCHEME 2**

(DMAP) (50 mg) in $CH_2Cl_2$ (50 mL) was stirred for 4 h. The filtrate was evaporated under reduced pressure. *n*-Hexane (50 mL) was added to the residue, and the precipitate was filtered off. The filtrate was evaporated under reduced pressure. The residue was chromatographed on a silica gel column (100 g) using a mixture of diethyl ether/*n*-hexane (8:92, vol/vol, 250 mL) as eluent to give 2-(6-bromo-hexanoate)-1,3-dioctadecanoate glycerol (**5**, 1.9 g, 80%). Compound **5** (200 mg, 0.25 mmol) was stirred with sodium azide (24 mg, 0.36 mmol) in dimethylsulfoxide (DMSO: 20 mL) at 70°C for 4 h. The reaction mixture was cooled, and water (50 mL) was added. The mixture was extracted with diethyl ether (2 × 30 mL). The ethereal layer was washed with brine (30 mL) and water (30 mL) and then dried over $Na_2SO_4$. The filtrate was evaporated under reduced pressure to give 2-(6-azido-hexanoate)-1,3-dioctadecanoate glycerol as a colorless oil (**6**, 175 mg, 90%).

A mixture of compound **6** (83 mg, 0.11 mmol) and [60]fullerene (72 mg, 0.1 mmol) in chlorobenzene (90 mL) was refluxed in an oil bath for 12 h under an atmosphere of argon. The mixture was cooled, and the chlorobenzene was evaporated under reduced pressure. The residue was dissolved in toluene (10 mL) and separated by silica gel (120 g) column chromatography using *n*-hexane/toluene (1:1, vol/vol, 400 mL) to remove the unreacted [60]fullerene (50 mg). This was followed by elution with toluene (400 mL) to give the requisite compound **1a** as a viscous dark brown oil (24.5 mg, 60% based on the amount of [60]fullerene consumed). The yield of **1a–1d** in the last reaction sequence was 45–60% based on the amount of [60]fullerene reacted.

*Synthesis of triacylglycerols (**2a–2d**) of type AAB containing an aza-fullerenoid acyl group at the α-position of the glycerol as exemplified by the synthesis of compound **2a** (Scheme 2).* 1,2-Dioctadecanoate glycerol (**10**) was synthesized starting from allyl alcohol (**7**) as described by Mattson and Volpenhein (18)

Esterification of the diacylglycerol (**10**) with 6-bromohexanoic acid, followed by azidonation gave the corresponding azido-containing triacylglycerol (**11**). The latter compound was reacted with [60]fullerene as by the general method described above for the synthesis of compounds **1a–1d**. The yield of the requisite triacylglycerols, **2a–2d**, in the last step of the reaction sequence, ranged from 51–62% based on the amount of [60]fullerene reacted.

*2-(6-[2′,3′;5,6]-Aza-[60]fullerene-hexanoate)-1,3-dioctadecanoate glycerol (**1a**).* Dark brown oil, 60% yield (based on amount of [60]fullerene reacted); thin-layer chromatography (TLC) ($R_f$) = 0.2 (toluene); UV ($CH_2Cl_2$, $\lambda_{max}$ nm): 238, 260, 330; IR ($cm^{-1}$): 1743 (C=O, ester str.), 525 ($C_{60}$ cage); $^1H$ NMR ($CDCl_3$, $\delta_H$, *J*/Hz): 5.28 (*m*, β-H of glycerol, 1H), 4.13–4.36 (*m*, α-H of glycerol, 4H), 3.81 (*t, J* = 7.3, 6-*H* of fullerenoid β-acyl, 2H), 2.45 (*t, J* = 7.3, 2-*H* of fullerenoid β-acyl, 2H), 2.33 (*t, J* = 7.6, 2-*H* of 18:0, 4H), 2.05 (*qn, J* = 7.3, 5-*H* of fullerenoid β-acyl, 2H), 1.70–1.89 (*m*, 3-*H*, 4-*H* of fullerenoid β-acyl, 4H), 1.58–1.62 (*m*, 3-*H* of α-acyl 4H), 1.26–1.33 (*m*, $CH_2$, 56H), 0.88 (*t, J* = 6.7, $CH_3$, 6H); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 173.28 (C-1 of 18:0 at α-acyl), 172.61 (C-1 of fullerenoid β-acyl), [147.79, 146.77, 145.03, 144.72, 144.56, 144.46, 144.32, 144.28, 144.15, 144.11, 143.84, 143.64, 143.53, 143.39, 143.21, 143.10, 142.90, 142.79, 142.70, 142.64, 141.42, 140.78, 140.74, 139.21, 138.50, 138.03, 137.84, 137.27, 137.19, 136.20, 135.79, 133.71 ($sp^2$ C of cage)], 69.08 (β-C of glycerol), 62.07 (α-C of glycerol), 51.41 ($NCH_2$), 34.12 (C-2 of fullerenoid β-acyl), 34.08 (C-2 of 18:0 at α-acyl), 31.94 (C-16 of α-acyl), [29.73, 29.68, 29.52, 29.38, 29.32, 29.26, 29.16 ($CH_2$)], 26.74 (C-4 of fullerenoid β-acyl), 24.90 (C-3 of 18:0 at α-acyl), 24.73 (C-3 of fullerenoid β-acyl), 22.71 (C-17 of α-acyl ), 14.15 ($CH_3$); mass spectrometry (MS) (APCI) (*m/z* , relative intensity in parentheses, calc. $M^+$ *m/z* = 1456.835): 1458.1 ($M^+$, 100), 1430.0 (25), 722.2 (10).

*2-(6-[2′,3′;5,6]-Aza-[60]fullerene-hexanoate)-1,3-di-9Z-octadecenoate glycerol (**1b**).* Dark brown oil, 45% yield (based on amount of [60]fullerene reacted); TLC ($R_f$) = 0.2 (toluene); UV ($CH_2Cl_2$, $\lambda_{max}$ nm): 234, 262, 328. IR ($cm^{-1}$): 3004 (C-H str., olefin), 1744 (C=O, ester str.), 526 ($C_{60}$ cage); $^1H$ NMR ($CDCl_3$, $\delta_H$, *J*/Hz): 5.30–5.40 (*m*, C*H*=C*H*, 4H), 5.29 (*m*, β-H of glycerol, 1H), 4.14–4.36 (*m*, α-H of glycerol, 4H), 3.81 (*t, J* = 7.2 Hz, 6-*H* of fullerenoid β-acyl, 2H), 2.45 (*t, J* = 7.2 Hz, 2-*H* of fullerenoid β-acyl, 2H), 2.33 [*t, J* = 7.6 Hz, 2-*H* of 18:1 (9*Z*), 4H], 2.00–2.07 (*m*, $CH_2$CH=CH and 5-*H* of β-acyl, 10H), 1.70–1.89 (*m*, 3-*H*, 4-*H* of fullerenoid β-acyl, 4H), 1.58–1.62 (*m*, 3-*H* of α-acyl, 4H), 1.26–1.33 (*m*, $CH_2$, 40H), 0.88 (*t, J* = 6.7 Hz, $CH_3$, 6H); $^{13}C$ NMR ($CDCl_3$, $\delta_C$): 173.35 (C-1 of 18:1 at α-acyl), 172.71 (C-1 of fullerenoid β-acyl), [147.89, 146.86, 145.13, 144.83, 144.65, 144.56, 144.42, 144.38, 144.25, 144.21, 143.94, 143.75, 143.63, 143.50, 143.32, 143.21, 143.01, 142.89, 142.80, 142.74, 141.51, 140.87, 140.85, 139.32, 138.60, 138.14, 137.94, 137.37, 137.28, 136.31, 135.89, 133.81 ($sp^2$ C of cage)], 130.13, 129.81 (*C*H=*C*H), 69.18 (β-C of glycerol), 62.18 (α-C of glycerol), 51.50 ($NCH_2$), 34.12 (C-2 of fullerenoid β-acyl), 34.05 (C-2 of 18:1 at α-acyl), 32.01 (C-16 of α-acyl), [29.88, 29.83, 29.64, 29.44, 29.43, 29.35, 29.31, 29.24, 29.22 ($CH_2$)], 27.22 ($CH_2$CH=CH), 26.74 (C-4 of fullerenoid β-acyl), 24.87 (C-3 of 18:1 at α-acyl), 24.73 (C-3 of fullerenoid β-acyl), 22.79 (C-17 of α-acyl), 14.24 ($CH_3$); MS (APCI) (*m/z*, relative intensity in parentheses, calc. $M^+$ *m/z* = 1452.797): 1454.1 ($M^+$, 100), 1002.1 (15), 834.4 (15), 721.9 (20).

*2-(6-[2′,3′;5,6]-Aza-[60]fullerene-hexanoate)-1,3-di-9Z,12Z-octadecadienoate glycerol (**1c**).* Dark brown oil, 45% yield (based on amount of [60]fullerene reacted); TLC ($R_f$) = 0.2 (toluene); UV ($CH_2Cl_2$, $\lambda_{max}$ nm): 232, 262, 330. IR ($cm^{-1}$): 3009 (C-H olefin, str.), 1744 (C=O, ester str.), 526 (C=C, cage); $^1H$ NMR ($CDCl_3$, $\delta_H$, *J*/Hz): 5.30–5.40 (*m*, C*H*=C*H*, 8H), 5.28 (*m*, β-H of glycerol, 1H), 4.14–4.36 (*m*, α-H of glycerol, 4H), 3.80 (*t, J* = 7.2 Hz, 6-*H* of fullerenoid β-acyl, 2H), 2.77 (*t, J* = 5.8 Hz, skipped methylene carbon, 4H), 2.46 (*t, J* = 7.3 Hz, 2-*H* of fullerenoid β-acyl, 2H), 2.33 [*t, J* = 7.4 Hz, 2-*H* of 18:2 (9*Z*,12*Z*), 4H], 2.01–2.06 (*m*, $CH_2$CH=CH of 18:2 (9*Z*,12*Z*) and 5-*H* of β-acyl, 10H), 1.70–1.86 (*m*, 3-*H*, 4-*H* of fullerenoid β-acyl, 4H), 1.58–1.62 (*m*, 3-*H* of α-acyl, 4H), 1.27–1.33 (*m*, $CH_2$, 28H), 0.88 (*t, J* =

6.7 Hz, C*H*$_3$, 6H); $^{13}$C NMR (CDCl$_3$, $\delta_C$): 173.24 (C-1 of 18:2 at α-acyl), 172.62 (C-1 of fullerenoid β-acyl), [147.80, 146.76, 145.04, 144.74, 144.57, 144.47, 144.33, 144.30, 144.17, 144.12, 143.85, 143.66, 143.55, 143.41, 143.23, 143.12, 142.92, 142.80, 142.72, 142.65, 141.43, 140.79, 140.76, 139.23, 138.52, 138.05, 137.86, 137.29, 137.19, 136.22, 135.81, 133.72 (*sp*$^2$ C of the cage)], [130.24, 130.01, 128.09, 127.90 (*C*H=*C*H)], 69.10 (β-C of glycerol), 62.09 (α-C of glycerol), 51.41 (N*C*H$_2$), 34.12 (C-2 of fullerenoid β-acyl), 34.05 (C-2 of 18:2 at α-acyl), 31.54 (C-16 of α-acyl), [29.70, 29.64, 29.36, 29.26, 29.22, 29.15, 29.12, 28.92 (CH$_2$)], 27.22 (*C*H$_2$CH=CH), 26.74 (C-4 of fullerenoid β-acyl), 25.66 (skipped methylene carbon), 24.87 (C-3 of 18:2 at α-acyl), 24.73 (C-3 of fullerenoid β-acyl), 22.97 (C-17 of α-acyl), 14.11 (CH$_3$); MS (APCI) (*m/z*, relative intensity in parentheses, calc. M$^+$ *m/z* = 1448.765): 1449.3 (M$^+$, 100), 1000.9 (15), 791.9 (85) and 722.2 (75).

*2-(6-[2′,3′;5,6]-Aza-[60]fullerene-hexanoate)-1,3-di-9-octadecynoate glycerol (**1d**).* Dark brown oil, 52% yield (based on amount of [60]fullerene reacted); TLC ($R_f$) = 0.2 (toluene); UV (CH$_2$Cl$_2$, $\lambda_{max}$ nm): 232, 262, 330. IR (cm$^{-1}$): 1743 (C=O, ester str.), 526 (C$_{60}$ cage); $^1$H NMR (CDCl$_3$, $\delta_H$, *J*/Hz): 5.29 (*m*, β-H of glycerol, 1H), 4.13–4.36 (*m*, α-H of glycerol, 4H), 3.81 (*t, J* = 7.2 Hz, 6-*H* of fullerenoid β-acyl, 2H), 2.45 (*t, J* = 7.3 Hz, 2-*H* of fullerenoid β-acyl, 2H), 2.33 [*t, J* = 7.6 Hz, 2-*H* of 18:1 (9*A*), 4H], 2.13 (*m*, C*H*$_2$C≡C, 8H), 2.05 (*qn, J* = 7.3 Hz, 5-*H* of fullerenoid β-acyl, 2H), 1.70–1.88 (*m*, 3-*H*, 4-*H* of fullerenoid β-acyl, 4H), 1.58–1.62 (*m*, 3-*H* of α-acyl, 4H), 1.27–1.33 (*m*, C*H*$_2$, 40H), 0.88 (*t, J* = 6.7 Hz, C*H*$_3$, 6H); $^{13}$C NMR (CDCl$_3$, $\delta_C$): 173.20 (C-1 of 18:1 at α-acyl), 172.60 (C-1 of fullerenoid β-acyl), [147.80, 146.78, 145.04, 144.74, 144.57, 144.47, 144.33, 144.30, 144.16, 144.12, 143.85, 143.66, 143.55, 143.41, 143.23, 143.12, 142.92, 142.80, 142.72, 142.65, 141.43, 140.79, 140.76, 139.23, 138.52, 138.06, 137.86, 137.31, 137.19, 136.23, 135.82, 133.73 (*sp*$^2$ C of the cage)], [80.38, 80.07 (*C*≡*C*)], 69.11 (β-C of glycerol), 62.09 (α-C of glycerol), 51.44 (N*C*H$_2$), 34.12 (C-2 of fullerenoid β-acyl), 34.03 (C-2 of 18:1 at α-acyl), 31.86 (C-16 of α-acyl), [29.70, 29.27, 29.24, 29.19, 29.15, 29.12, 29.04, 28.91, 28.83, 28.69, 28.68 (CH$_2$)], 26.75 (C-4 of fullerenoid β-acyl), 24.84 (C-3 of 18:1 at α-acyl), 24.73 (C-3 of fullerenoid β-acyl), 22.68 (C-17 of α-acyl), [18.80, 18.78 (*C*H$_2$C≡C)], 14.14 (*C*H$_3$); MS (APCI) (*m/z*, relative intensity in parentheses, calc. M$^+$ *m/z* = 1448.765): 1449.3 (M$^+$, 15), 1001.2 (15), 730.7 (100), 722.4 (55).

*3-(6-[2′,3′;5,6]-Aza-[60]fullerene-hexanoate)-1,2-dioctadecanoate glycerol (**2a**).* Dark brown oil, 58% yield (based on amount of [60]fullerene reacted); TLC ($R_f$) = 0.2 (toluene); UV (CH$_2$Cl$_2$, $\lambda_{max}$ nm): 236, 262, 332; IR (cm$^{-1}$): 1744 (C=O, ester str.), 525 (C$_{60}$ cage); $^1$H NMR (CDCl$_3$, $\delta_H$, *J*/Hz): 5.28 (*m*, β-H of glycerol, 1H), 4.13–4.36 (m, α-H of glycerol, 4H), 3.81 (*t, J* = 7.3 Hz, 6-*H* of fullerenoid α-acyl, 2H), 2.45 (*t, J* = 7.3 Hz, 2-*H* of fullerenoid α-acyl, 2H), 2.34 (*t, J* = 7.6 Hz, 2-*H* of 18:0 at β-acyl, 2H), 2.32 (*t, J* = 7.6 Hz, 2-*H* of 18:0 at α-acyl, 2H), 2.05 (*qn, J* = 7.3 Hz, 5-*H* of fullerenoid α-acyl, 2H), 1.70–1.86 (*m*, 3-*H*, 4-*H* of fullerenoid α-acyl, 4H), 1.58–1.62 (*m*, 3-*H* of 18:0, 4H), 1.26–1.33 (*m*, C*H*$_2$, 56H), 0.88 (*t, J* = 6.7 Hz, C*H*$_3$, 6H); $^{13}$C NMR (CDCl$_3$, $\delta_C$): 173.30 (C-1 of 18:0 at α-acyl), 172.89 (C-1 of 18:0 at β-acyl), 173.04 (C-1 of fullerenoid α-acyl), [147.82, 146.79, 145.04, 144.73, 144.56, 144.47, 144.33, 144.29, 144.14, 143.85, 143.66, 143.54, 143.47, 143.22, 143.11, 142.91, 142.81, 142.71, 142.65, 141.43, 140.80, 140.76, 139.23, 138.51, 138.05, 137.86, 137.29, 137.21, 136.22, 135.82, 133.73 (*sp*$^2$ C of the cage)], 68.88 (β-C of glycerol), 62.27 (α-C of fullerenoid glycerol), 62.11 (α-C of glycerol)], 51.42 (N*C*H$_2$), 34.26 (C-2 of β-acyl), 34.07 (C-2 of α-acyl), 33.98 (C-2 of fullerenoid α-acyl), 31.94 (C-16 of 18:0), [29.70, 29.55, 29.52, 29.38, 29.31, 29.26, 29.15, 29.12, 28.93 (CH$_2$)], 26.79 (C-4 of fullerenoid α-acyl), 24.95 (C-3 of 18:0 at β-acyl), 24.89 (C-3 of 18:0 at α-acyl), 24.71 (C-3 of fullerenoid at α-acyl), 22.71 (C-17 of 18:0), 14.15 (*C*H$_3$); MS (APCI) (*m/z*, relative intensity in parentheses, calc. M$^+$ *m/z* = 1456.835): 1457.3 (M$^+$, 25), 738.3 (100), 722.2 (5).

*3-(6-[2′,3′;5,6]-Aza-[60]fullerene-hexanoate)-1,2-di-9Z-octadecenoate glycerol (**2b**).* Dark brown oil, 62% yield (based on amount of [60]fullerene reacted); TLC ($R_f$) = 0.2 (toluene); UV (CH$_2$Cl$_2$, $\lambda_{max}$ nm): 240, 260, 326. IR (cm$^{-1}$): 3004 (C-H, olefin str.), 1744 (C=O, ester str.), 526 (C$_{60}$ cage); $^1$H NMR (CDCl$_3$, $\delta_H$, *J*/Hz): 5.30–5.38 (*m*, C*H*=C*H*, 4H), 5.29 (*m*, β-H of glycerol, 1H), 4.14–4.36 (*m*, α-H of glycerol, 4H), 3.81 (*t, J* = 7.2 Hz, 6-*H* of fullerenoid α-acyl, 2H), 2.45 (*t, J* = 7.2 Hz, 2-*H* of fullerenoid α-acyl, 2H), 2.34 [*t, J* = 7.6 Hz, 2-*H* of 18:1 (9*Z*) at β-acyl, 2H], 2.32 [t, *J* = 7.6 Hz, 2-*H* of 18:1 (9*Z*) at α-acyl, 2H], 2.00-2.07 [*m*, C*H*$_2$CH=CH of 18: 1 (9*Z*) and 5-*H* of fullerenoid α-acyl, 10H], 1.70–1.89 (m, 3-*H*, 4-*H* of fullerenoid α-acyl, 4H), 1.58–1.62 [*m*, 3-*H* of 18:1(9*Z*), 4H], 1.26–1.33 (*m*, C*H*$_2$, 40H), 0.88 (*t, J* = 6.7 Hz, C*H*$_3$, 6H); $^{13}$C NMR (CDCl$_3$, $\delta_C$): 173.27 (C-1 of 18:1 at α-acyl), 173.03 (C-1 of fullerenoid α-acyl), 172.86 (C-1 of 18:1 at β-acyl), [147.82, 146.79, 145.05, 144.73, 144.56, 144.47, 144.33, 144.29, 144.17, 144.14, 143.86, 143.66, 143.40, 143.22, 143.11, 142.91, 142.81, 142.71, 142.65, 141.43, 140.80, 140.76, 139.23, 138.51, 138.05, 137.86, 137.29, 137.21, 136.22, 135.82, 133.73 (*sp*$^2$ C of the cage)], [130.06, 130.03, 129.72, 129.69 (*C*H=*C*H of 18:1)], 68.88 (β-C of glycerol), 62.26 (α-C of fullerenoid glycerol), 62.11 (α-C of glycerol), 51.41 (N*C*H$_2$), 34.23 (C-2 of 18:1 at β-position), 34.05 (C-2 of 18:1 at α-acyl), 33.98 (C-2 of fullerenoid α-acyl), 31.92 (C-16 of 18:1), [29.79, 29.75, 29.73, 29.55, 29.34, 29.26, 29.24, 29.20, 29.17, 29.14, 29.11, 29.09 (CH$_2$)], [27.24, 27.21 (*C*H$_2$CH=CH)], 26.79 (C-4 of fullerenoid α-acyl), 24.93 (C-3 of 18:1 at β-acyl), 24.86 (C-3 of 18:1 at α-acyl), 24.71 (C-3 of fullerenoid α-acyl), 22.70 (C-17 of 18:1), 14.15 (*C*H$_3$); MS (APCI) (*m/z*, relative intensity in parentheses, calc. M$^+$ *m/z* = 1452.797): 1453.3 (M$^+$, 100), 734.8 (100), 722.6 (45).

*3-(6-[2′,3′;5,6]-Aza-[60]fullerene-hexanoate)-1,2-di-9Z,12Z-octadecadienoate glycerol (**2c**).* Dark brown oil, 53% yield (based on amount of [60]fullerene reacted); TLC ($R_f$) = 0.2 (toluene); UV (CH$_2$Cl$_2$, $\lambda_{max}$ nm): 232, 262, 330; IR (cm$^{-1}$): 3009 (C-H olefin, str.), 1744 (C=O, ester str.), 526 (C$_{60}$ cage); $^1$H NMR (CDCl$_3$, $\delta_H$, *J*/Hz): 5.30–5.40 (*m*,

C*H*=C*H*, 8H), 5.28 (*m*, β-H of glycerol, 1H), 4.14–4.36 (*m*, α-H of glycerol, 4H), 3.80 (*t, J* = 7.2 Hz, 6-*H* of fullerenoid α-acyl, 2H), 2.77 (*t, J* = 5.8 Hz, skipped methylene carbon, 4H), 2.46 (*t, J* = 7.3, 2-*H* of fullerenoid α-acyl, 2H), 2.34 [*t, J* = 7.6 Hz, 2-*H* of 18:2 (9*Z*,12*Z*) at β-acyl, 2H], 2.32 [*t, J* = 7.4 Hz, 2-*H* of 18:2 (9*Z*,12*Z*) at α-acyl, 2H], 2.01–2.06 [*m*, $CH_2$CH=CH of 18:2 (9*Z*,12*Z*) and 5-*H* of fullerenoid α-acyl, 10H], 1.70–1.86 (*m*, 3-*H*, 4-*H* of fullerenoid α-acyl, 4H), 1.58–1.62 [*m*, 3-*H* of 18:2 (9*Z*,12*Z*), 4H], 1.27–1.33 (*m*, $CH_2$, 28H), 0.88 (*t, J* = 6.7 Hz, $CH_3$, 6H); $^{13}$C NMR ($CDCl_3$, $\delta_C$): 173.26 (C-1 of 18:2 at α-position), 173.03 (C-1 of fullerenoid α-acyl), 172.85 (C-1 of 18:2 at β-position), [147.81, 146.80, 145.04, 144.74, 144.57, 144.47, 144.33, 144.29, 144.16, 144.13, 143.85, 143.66, 143.40, 143.22, 143.12, 142.92, 142.80, 142.72, 142.65, 141.43, 140.80, 140.76, 139.23, 138.51, 138.05, 137.85, 137.29, 137.21, 136.22, 135.82, 133.73 ($sp^2$ C of the cage)], [130.26, 130.24, 130.02, 129.99, 128.11, 128.08, 127.90, 127.89 (*C*H=*C*H)], 68.89 (β-C of glycerol), 62.26 (α-C of fullerenoid glycerol), 62.10 (α-C of glycerol), 51.41 (N$CH_2$), 34.22 (C-2 of 18:2 at β-acyl), 34.04 (C-2 of 18:2 at α-acyl), 33.97 (C-2 of fullerenoid α-acyl), 31.54 (C-16 of 18:2), [29.65, 29.63, 29.36, 29.26, 29.24, 29.20, 29.17, 29.14, 29.11, 29.08 ($CH_2$)], 27.22 (*C*$H_2$CH=CH), 26.79 (C-4 of fullerenoid α-acyl), 25.66 (skipped methylene carbon of 18:2), 24.92 (C-3 of 18:2 at β-acyl), 24.86 (C-3 of 18:2 at α-acyl), 24.70 (C-3 of fullerenoid α-acyl), 22.60 (C-17 of 18:2), 14.11 (*C*$H_3$); MS (APCI) (*m/z*, relative intensity in parentheses, calc. $M^+$ *m/z* = 1448.765): 1449.2 ($M^+$, 5), 812.6 (25), 730.9 (100), 722.1 (15).

*3-(6-[2′,3′;5,6]-Aza-[60]fullerene-hexanoate)-1,2-di-9-octadecynoate glycerol (**2d**).* Dark brown oil, 51% yield (based on amount of [60]fullerene reacted); TLC ($R_f$) = 0.2 (toluene); UV ($CH_2Cl_2$, $\lambda_{max}$ nm): 236, 262, 330; IR ($cm^{-1}$): 1740 (C=O, ester str.), 526 ($C_{60}$ cage); $^1$H NMR ($CDCl_3$, $\delta_H$, *J*/Hz): 5.29 (*m*, β-H of glycerol, 1H), 4.13–4.36 (*m*, α-H of glycerol, 4H), 3.81 (*t, J* = 7.2 Hz, 6-*H* of fullerenoid α-acyl, 2H), 2.45 (*t, J* = 7.3 Hz, 2-*H* of fullerenoid α-acyl, 2H), 2.34 [*t, J* = 7.6 Hz, 2-*H* of 18:1 (9*A*) at β-acyl, 2H], 2.32 [*t, J* = 7.6 Hz, 2-*H* of 18:1 (9*A*) at α-acyl, 2H], 2.13 (*m*, $CH_2$C≡C, 4H), 2.05 (*qn, J* = 7.3 Hz, 5-*H* of fullerenoid α-acyl, 2H), 1.70–1.88 (*m*, 3-*H*, 4-*H* of fullerenoid α-acyl, 4H), 1.52–1.62 (*m*, 3-*H* of 18:1, 4H), 1.27–1.33 (*m*, $CH_2$, 40H), 0.88 (*t, J* = 6.7 Hz, $CH_3$, 6H); $^{13}$C NMR ($CDCl_3$, $\delta_C$): 173.23 (C-1 of 18:1 at α-acyl), 173.03 (C-1 of fullerenoid α-acyl), 172.83 (C-1 of 18:1 at β-acyl), [147.83, 146.81, 145.06, 144.75, 144.58, 144.48, 144.35, 144.31, 144.17, 144.14, 143.87, 143.67, 143.42, 143.24, 143.13, 142.93, 142.82, 142.73, 142.67, 141.45, 140.82, 140.77, 139.24, 138.53, 138.07, 137.87, 137.33, 137.22, 136.25, 135.85, 133.75 ($sp^2$ C of the cage)], [80.40, 80.37, 80.07, 80.05 (*C*≡*C*)], 68.93 (β-C of glycerol), 62.26 (α-C of fullerenoid glycerol), 62.12 (α-C of glycerol), 51.44 (N$CH_2$), 34.20 (C-2 of 18:1 at β-position), 34.03 (C-2 of 18:1 at α-acyl), 33.98 (C-2 of fullerenoid α-acyl), 31.94 (C-16 of 18:1), [29.70, 29.37, 29.25, 29.19, 29.15, 29.03, 29.00, 28.91, 28.85, 28.82, 28.71, 28.68 (*C*$H_2$)], 26.80 (C-4 of fullerenoid at α-acyl), 24.89 (C-3 of 18:1 at β-acyl), 24.83 (C-3 of 18:1 at α-acyl), 24.71 (C-3 of fullerenoid α-acyl), 22.70 (C-17 of 18:1), 18.79 (*C*$H_2$C≡C), 14.12 (*C*$H_3$); MS (APCI) (*m/z*, relative intensity in parentheses, calc. $M^+$ *m/z* = 1448.765): 1448.3 ($M^+$, 40), 1000.3 (5), 803.1 (30), 730.7 (100), 722.4 (25).

## DISCUSSION

The synthesis strategies and the experimental conditions of the desired fullerenoid triacylglycerols (**1a–1d** and **2a–2d**) are shown in Schemes 1 and 2. The yield of the product obtained in the last step of the reaction sequence, i.e., between the corresponding key azido-containing triacylglycerols with [60]fullerene, was rather low (15–18%) when the calculation was based on the amount of [60]fullerene employed. However, in fullerene chemistry the yield of fullerenoid derivatives is normally calculated on the net amount of [60]-fullerene consumed during the reaction (19). Based on the net amount of [60]fullerene reacted, the yields of the final reactions described in Schemes 1 and 2 ranged from 45 to 62% and are considered to be reasonable and good.

The fullerenoid triacylglycerols (**1a–1d, 2a–2d**) obtained were viscous brown oils. Unlike [60]fullerene itself, these fullerenoid lipids dissolved readily in a wide range of organic solvents, including diethyl ether, *n*-hexane, chloroform, tetrahydrofuran, toluene, chlorobenzene, benzene, and tetrachloroethane. Compounds **1a** and **2a** did not solidify at room temperature despite the presence of two saturated acyl chains (18:0), which are esterified to the glycerol "backbone." Attempts to crystallize these derivatives from organic solvents have failed. The choice to use a short C-6 acyl group to link to a [60]fullerene unit *via* a nitrogen bridge was twofold. Firstly, this would ensure that the total length of this fullerene-containing acyl group was about the same as that of a medium- to long-chain fatty acyl group for future biological studies. Secondly, the $C_6$ acyl group could be readily identified by NMR spectroscopy, which would allow it to serve as an internal marker to facilitate the interpretation of the various spectra.

The presence of an aza-[60]fullerene unit in compounds **1a–1d, 2a–2d** was evident from the individual mass value of the molecular ion ($M^+$) and the appearance of the characteristic peak at *m/z* = 722 (ion fragment of a $C_{60}$) in the mass spectra (APCI). The [60]fullerene cage unit in these compounds gave rise to absorption bands in the UV spectra (bands at about 230, 260, and 330 nm from the conjugated C=C double bonds) and also by the appearance of a characteristic absorption band at 525 $cm^{-1}$ in the IR spectra. The TLC analysis showed a brown spot at $R_f$ = 0.2 when toluene was used as the developer (triolein gave an $R_f$ of 0.6 under same condition). The proton NMR confirmed the glycerol backbone structure of the compounds from the multiplets at $\delta_H$ 5.3 (methine proton) and $\delta_H$ 4.2 (methylene protons). In the case of compounds **1b**, **2b,** and **1c**, **2c**, which contain oleyl and linoleyl acyl groups, respectively, the shifts of the olefinic protons appeared at $\delta_H$ 5.3 as multiplets.

The $^{13}$C NMR spectroscopic analyses allowed the two types (ABA and AAB) of triacylglycerols to be clearly dis-

tinguished. In the case of compounds **1a–1d**, the ABA arrangement of acyl groups on the glycerol backbone was confirmed by the appearance of a pair of signals for the C-1 (in the region of $\delta_C$ 172–173), a pair of signals for the C-2 (at $\delta_C$ 34), and another pair of signals for C-3 (at $\delta_C$ 24) carbon atoms of the acyl groups. The more intense signal of these pairs of signals was attributed to the shifts of the carbon atoms of the two identical α-acyl groups. To support the ABA arrangement of acyl groups in compounds **1a–1d** further, the spectra showed two signals for the shifts of the methylene (at $\delta_C$ 62) and methine (at $\delta_C$ 68) carbon atoms of the glycerol. These pairs of signals confirm the ABA arrangement of the acyl group in compounds **1a–1d**.

The spectra of compounds **2a–2d**, which are of the type AAB, showed three signals of equal intensity for the C-1 (at $\delta_C$ 172–173), three signals for the C-2 (at $\delta_C$ 33–34), and three signals for the C-3 (at $\delta_C$ 24) carbon atoms of the acyl groups. Three distinct signals (at $\delta_C$ 68.8, 62.2 and 62.1) were also observed for the shifts of the methylene and methine carbon atoms of the glycerol structure. The pattern of these spectra was in agreement with carbon shift data for conventional structured triacylglycerols of the type ABA and AAB reported by Lie Ken Jie *et al.* (20,21).

Of great importance was the appearance of 31–32 signals in the region of $\delta_C$ 130–150 of the $^{13}C$ NMR spectra, which denoted the presence of a "[5,6]-open" aza substructure of the carbon cage. Similar "[5,6]-open" aza-fullerenes (also showing 31–32 signals in the carbon NMR spectra) were reported by Prato *et al.* (22) and Hawker *et al.* (23), when [60]fullerene was reacted with organic azides. The absence of signals in the $\delta_C$ 70–90 region of the $^{13}C$ NMR spectra (shift region for $sp^3$ hybridized carbon atoms when present in the [60]fullerene cage) supported the structure of compounds **1a–1d** and **2a–2d** as containing a [60]fullerene cage with a "[5,6]-open" aza substructure.

In this work we have demonstrated that structured triacylglycerols of the type ABA and AAB can be successfully synthesized to include an acyl chain with a "[5,6]open" aza-[60]fullerenoid unit tethered to the distal end of one of the acyl groups.

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REVIEW

# Studies on Effects of Dietary Fatty Acids as Related to Their Position on Triglycerides

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**ABSTRACT:** This article reviews published literature on how the stereospecific structure of dietary triglycerides may affect lipid metabolism in humans. Animal studies have shown enhanced absorption of fatty acids in the *sn*-2 position of dietary triglycerides. Increasing the level of the saturated fatty acid palmitic acid in the *sn*-2 position (e.g., by interesterification of the fat to randomize the positions of the fatty acids along the glycerol backbone) has been shown in rabbits to increase the atherogenic potential of the fat without impacting levels of blood lipids and lipoproteins. In contrast, enhancing the level of stearic acid in the *sn*-2 position has not been found to affect either atherogenic potential or levels of blood lipids and lipoproteins in rabbits. Fatty acids other than palmitic and stearic have not been studied systematically with respect to possible positional effects. A limited number of human studies have shown no significant effects of interesterified fats on blood lipid parameters. However, it is unknown whether modifying the stereospecific structure of dietary triglycerides would affect atherogenicity or other long-term health conditions in humans. It is possible that incorporation of palmitic acid into the *sn*-2 position of milk fat is beneficial to the human infant (as a source of energy for growth and development) but not to human adults. Additional research is needed to determine whether processes like interesterification, which can be used to alter physical parameters of dietary fats (e.g., melting characteristics), may result in favorable or unfavorable long-term effects in humans.

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The purpose of this review is to determine, based on published literature, our current level of understanding about how the stereospecific structure of dietary triglycerides may affect lipid metabolism in humans. Of particular interest is whether altering the position of fatty acids on dietary triglycerides has an effect on blood lipid and lipoprotein levels. This review first highlights relevant experimental animal feeding trials as background and then focuses on recent human clinical studies that address this issue.

Human and animal studies conducted during the past 40–50 yr have established that different categories of dietary fatty acids have different effects on blood lipid and lipoprotein levels (1–5). Unsaturated fatty acids found primarily in fats derived from vegetable sources tend to lower total and low density lipoprotein (LDL) cholesterol when compared with saturated fatty acids. On the other hand, saturated fatty acids found primarily in animal fats tend to raise total and LDL-cholesterol when compared with unsaturated fatty acids. For example, linoleic acid, which is abundant in most liquid vegetable oils (e.g., soybean, corn, and safflower), has been associated with blood cholesterol lowering, whereas lauric, myristic, and palmitic acids, common in animal fats and certain vegetable oils (palm and coconut), have been associated with blood cholesterol raising. The saturated fatty acid stearic acid has been reported to have a neutral or lowering effect on total and LDL-cholesterol compared with other saturated fatty acids (6). The monounsaturated fatty acid oleic acid, compared with saturated fatty acids, appears to lower LDL-cholesterol level but to retain high density lipoprotein (HDL) cholesterol (7).

Despite these widely accepted generalizations, studies focusing on feeding high levels of specific saturated or unsaturated fatty acids can be difficult to interpret, because fats having similar ratios of unsaturated to saturated fatty acids may have dramatically different fatty acid compositions. For example, cocoa butter and milk fat have similar levels of saturation; however, the saturated fatty acids of cocoa butter (expressed as a percentage of total saturated fatty acids) are primarily palmitic (42.5%) and stearic (55%) acids, whereas milk fat contains a wider variety of saturated fatty acids including stearic (19%), palmitic (42%), myristic (16%), and medium- (MCFA) and short-chain fatty acids (SCFA) (≤ 12:0 = 23%). Other examples are beef tallow and palm oil, both of which contain about 51% saturated fatty acids. As a percentage of total saturated fatty acids, beef tallow contains about 38% stearic and 50% palmitic acids, whereas palm oil contains about 9% stearic and 88% palmitic acids (8).

In addition to overall fatty acid composition, the stereospecific distribution of fatty acids in a particular fat also should be considered when fatty acid effects are examined. Fatty acids can occupy any of three positions on the glycerol backbone, designated as *sn*-1, *sn*-2, and *sn*-3 (*"sn"* stands for "stereospecific numbering"). Table 1 (9–13) illustrates the positional distribution of fatty acids in the triacylglycerols of several natural oils and fats. Among animal fats, bovine milk fat and lard (pork fat) contain mainly saturated fatty acids in the *sn*-2 position, whereas tallow contains saturated fatty acids primarily in the *sn*-1 and *sn*-3 positions. Oils and fats of plant origin, such as soybean oil and cocoa butter, contain unsaturated fatty acids in the *sn*-2 position and saturated fatty acids in the *sn*-1 and *sn*-3 positions. In lard, oleic acid is mostly in the *sn*-1 and *sn*-3 positions, whereas in cocoa butter, oleic acid is almost entirely in the *sn*-2 position. In peanut oil and olive oil, oleic acid is more evenly distributed among all three positions. Estimates of the major triacylglycerol species found in a

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Abbreviations: AHA, American Heart Association; apo, apolipoprotein; CHO, cholesterol; HDL, high density lipoprotein; HOSO, high-oleic sunflower oil; LDL, low density lipoprotein; LMP, lauric, myristic, and palmitic fatty acids; MCFA, medium-chain fatty acid; MCT, medium-chain triglycerides; NCEP, National Cholesterol Education Program; O, oleic acid; OL, oleic acid diet; P, palmitic acid; RBD, refined, bleached, deodorized; S, stearic acid; SCFA, short-chain fatty acid; STE; stearic acid diet; TFA, *trans* fatty acids; VOO, virgin olive oil.

**TABLE 1**
**Positional Distribution of Fatty Acids in TG of Natural Fats and Oils[a]**

| Source | Position | Fatty acid (mol%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 22:0 |
| Cow's milk | 1 | 11 | 36 | 15 | 21 | 1 | | | |
| | 2 | 20 | 33 | 6 | 14 | 3 | | | |
| | 3 | 7 | 10 | 4 | 15 | <1 | | | |
| Pig (outer back) | 1 | 1 | 10 | 30 | 51 | 6 | | | |
| | 2 | 4 | 72 | 2 | 13 | 3 | | | |
| | 3 | — | — | 7 | 73 | 8 | | | |
| Beef (depot) | 1 | 4 | 41 | 17 | 20 | 4 | 1 | | |
| | 2 | 9 | 17 | 9 | 41 | 5 | 1 | | |
| | 3 | 1 | 22 | 24 | 37 | 5 | 1 | | |
| Cocoa butter | 1 | | 34 | 50 | 12 | 1 | | | |
| | 2 | | 2 | 2 | 87 | 9 | | | |
| | 3 | | 37 | 53 | 9 | — | | | |
| Peanut | 1 | | 14 | 5 | 59 | 18 | | 1 | — |
| | 2 | | 1 | <1 | 58 | 39 | | — | — |
| | 3 | | 11 | 5 | 57 | 10 | | 4 | 6 |
| Corn | 1 | | 18 | 3 | 27 | 50 | 1 | | |
| | 2 | | 2 | <1 | 26 | 70 | <1 | | |
| | 3 | | 13 | 3 | 31 | 51 | 1 | | |
| Soybean | 1 | | 14 | 6 | 23 | 48 | 9 | | |
| | 2 | | 1 | <1 | 21 | 70 | 7 | | |
| | 3 | | 13 | 6 | 28 | 45 | 8 | | |
| Olive | 1 | | 13 | 3 | 72 | 10 | <1 | | |
| | 2 | | 1 | — | 83 | 14 | 1 | | |
| | 3 | | 7 | 4 | 74 | 5 | 1 | | |

[a]In this table, not all fatty acids are listed for each fat source. Also, each line represents the mol% of each fatty acid in position 1, 2, or 3. Most lines sum to *ca.* 100%; columns do not sum to 100%. The total percentage of a specific fatty acid at a specific position can be determined by dividing the mol% at that position by the total mol% for all three positions. For example, in cow's milk, the percentage of total 16:0 in position 1 is 36 mol%/79 mol% = 46%. References: 9–13. TG = triglyceride.

variety of natural fats and oils are listed in Table 2 (14). Most of these triacylglycerols have not been fully analyzed, and the specific stereoisomers have not been reported.

The stereospecific position of fatty acids on triglycerides plays a major role in the functionality of fats in food products. For example, in the case of lard, the presence of palmitic acid in the *sn*-2 position contributes to desirable flakiness of pie crusts when lard is used as a baking shortening. Also, in the case of cocoa butter, the unique positioning of palmitic, oleic, and stearic acids in two predominant triglyceride forms gives cocoa butter a sharp melting point just below body temperature. The way cocoa butter melts is one of the reasons for the pleasant eating quality of chocolate. The unique functionalities attributed to the location of specific fatty acids in fats, such as lard or cocoa butter, are difficult to duplicate in fat substitutes.

**TABLE 2**
**Principal TG Species of Natural Fats and Oils[a]**

| Fat or oil | Major TG species | | |
|---|---|---|---|
| Butterfat | PPB | PPC | POP |
| Lard | SPO | OPL | OPO |
| Beef tallow | POO | POP | POS |
| Cocoa butter | POS | SOS | POP |
| Coconut oil | DDD | CDD | CDM |
| Palm oil | POP | POO | POL |
| Cottonseed oil | PLL | POL | LLL |
| Peanut oil | OOL | POL | OLL |
| Corn oil | LLL | LOL | LLP |
| Soybean oil | LLL | LLO | LLP |
| Olive oil | OOO | OOP | OLO |
| Canola oil | OOO | LOO | OOLn |

[a]Specific TG have been estimated from stereospecific fatty acid analyses. In many cases the stereospecific composition is uncertain. Abbreviations for acyl chains of TG: B = butyric (4:0); C = capric (10:0); D = dodecanoic (12:0); M = myristic (14:0); P = palmitic (16:0); S = stearic (18:0); O = oleic (18:1n-9); L = linoleic (18:2n-6); Ln = linolenic (18:3n-3). Reference: 14. See Table 1 for other abbreviation.

The relationship between triglyceride structure and metabolic behavior has been reviewed in several recent articles (8,9,14–16). The stereospecific position of fatty acids is important because it determines how triglycerides are digested. The process by which triglycerides are broken down *in vivo* was established by Mattson and Volpenhein (17). These investigators showed that during digestion in the gastrointestinal tract, pancreatic lipase, an enzyme highly specific for the *sn*-1 and *sn*-3 esters, catalyzes the formation of *sn*-2 monoglycerides and free fatty acids that are absorbed in the small intestine. The 2-monoglycerides are reacylated into new triglycerides that enter the lymph chylomicrons.

Fatty acids released from the *sn*-1 and *sn*-3 positions often have different metabolic fates than fatty acids retained in the *sn*-2 position. These metabolic fates depend on the fatty acid chain-length and stereospecific location on the triglyceride. SCFA and MCFA (≤10 carbon atoms) can be solubilized in the aqueous phase of the intestinal contents, where they are absorbed, bound to albumin, and transported to the liver by the portal vein. Longer-chain fatty acids, such as palmitic and stearic, have low coefficients of absorption because of melting points above body temperature and because of their ability to form calcium soaps.

**TABLE 3**
**Positional Distribution of Fatty Acids in Native and Interesterified Peanut Oil[a]**

| | | Fatty acid (mol%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Source | Position | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 20:0 | 22:0 | 24:0 |
| Native peanut oil | 1 | 19.9 | 0.1 | 3.5 | 47.5 | 26.1 | 0.3 | 0.1 | |
| | 2 | 2.2 | 0.1 | 1.0 | 50.8 | 46.2 | | 0.1 | |
| | 3 | 12.8 | 0.2 | 3.7 | 54.0 | 11.3 | 4.0 | 7.3 | 3.3 |
| Interesterified peanut oil | 1 | 11.8 | 0.1 | 2.7 | 50.8 | 27.8 | 1.4 | 2.6 | 1.1 |
| | 2 | 11.9 | 0.1 | 3.3 | 50.9 | 26.8 | 1.2 | 2.6 | 0.8 |
| | 3 | 11.9 | 0.1 | 2.1 | 50.3 | 27.9 | 1.4 | 2.6 | 1.6 |

[a]Each line represents the mol% of each fatty acid in position 1, 2, or 3. Most lines sum to *ca.* 100%; columns do not sum to 100%. The total percentage of a specific fatty acid at a specific position can be determined by dividing the mol% at that position by the total mol% for all three positions. Reference: 18.

Thus, fats with long-chain fatty acids in the *sn*-1 and *sn*-3 positions of triacylglycerols may exhibit different absorption patterns from fats with these fatty acids in the *sn*-2 position.

The role of stereospecific location of dietary fatty acids can be studied using a process called interesterification (sometimes referred to as randomization), in which the positions of the fatty acids on the glycerol backbone are rearranged. Interesterification is accomplished by catalytic methods at relatively low temperature and permits the exchange of fatty acids both within and between triacylglycerols. The process may be enzymatic (i.e., highly controlled) or chemical (i.e., relatively uncontrolled). The food industry uses interesterification (largely chemical) to modify the melting and crystallization behavior of fats. Also, the hardening of a liquid oil by interesterifying it with a solid fat offers an alternative to the use of partial hydrogenation in the manufacture of margarines and spreads. This exchange of fatty acids results in their randomization among all three stereospecific positions. Thus, a naturally occurring fat having oleic acid primarily in the *sn*-2 position after interesterification could have oleic acid roughly equally distributed among the *sn*-1, *sn*-2, and *sn*-3 positions.

The interesterification (randomization) process does not change the degree of unsaturation or the isomeric state of the fatty acids, as they transfer in their entirety from one position to another. Interesterification thus allows investigators to compare nutritional effects of diets that are identical in fatty acid composition but different in triacylglycerol composition. Randomization of naturally occurring fats could have implications regarding cardiovascular health. This is because randomization alters not only physical properties of fats (e.g., melting characteristics) but also their absorption properties. Table 3 (18) illustrates the percentage of fatty acids in the *sn*-1, *sn*-2, and *sn*-3 positions in native and interesterified peanut oil. Interesterification resulted in a roughly equal distribution of all fatty acids at all three positions of the triglyceride.

## BACKGROUND

*Fat absorption and metabolic clearance studies involving interesterified fats, structured triglycerides, and natural fats.* Studies with interesterified fats have found that altering the fatty acid composition at the *sn*-2 position affects the amount of fat absorbed. Filer *et al*. (19) reported that infants fed formulas containing randomized lard (i.e., having less palmitic acid in the *sn*-2 position than in native lard) excreted six times as much fat as infants fed native lard. This meant that the interesterified lard was less readily absorbed than its native counterpart. Lien and coworkers (20) fed rats combinations of coconut oil and palm olein either in their native state or interesterified. In a mixture of 25:75 coconut oil/palm olein, 93% of the palmitic acid was in the *sn*-1 and *sn*-3 positions in the native fats, and 65% of the palmitic acid was in the *sn*-1 and *sn*-3 positions in the interesterified fats. Interesterification significantly decreased fecal excretion of saturated fatty acids, suggesting that increasing the amount of palmitic acid in the *sn*-2 position could increase its absorption. Renaud and coworkers (21) fed rats diets containing either palm oil (58% of palmitic acid at *sn*-1 and *sn*-3), lard (65% of palmitic acid at *sn*-2), or their interesterified counterparts. Fecal excretion of palmitic acid was greatest in the diets containing high amounts of palmitic acid at the *sn*-1 and *sn*-3 positions (native palm oil and interesterified lard). Interesterification of lard decreased the incorporation of palmitic acid into plasma lipids and decreased total plasma triglycerides, consistent with reduced absorption of palmitic acid.

The specific positioning of 16:0 at the 2-position of human milk triglycerides has been suggested as one of the reasons for the high efficiency of absorption of fat from human milk (19,22). Support for this hypothesis has been provided by studies showing better absorption of total dietary fat or 16:0 by infants fed formula with 16:0 esterified to the *sn*-2 position rather than to the *sn*-1 or *sn*-3 positions of the dietary triglycerides (19,23). However, because of the proposed role of the milk enzyme bile salt-stimulated lipase, which in newborn infants is apparently responsible for the hydrolysis of 2-monoglycerides to free glycerol and fatty acid (24), the contribution of *sn*-2 16:0 to the total amount of milk fat being absorbed remains uncertain.

All of these studies demonstrated that modifying the dietary triglyceride structure to increase the level of palmitic acid in the *sn*-2 position led to increased absorption of palmitic acid.

The chain length of a fatty acid affects its transport and metabolism. Triglycerides containing fatty acids having a chain length of more than 10 carbon atoms are transported by the lymphatic system, whereas MCFA (having 10 or fewer carbon atoms) are transported by the portal vein to the liver, where they are metabolized rapidly. Carvajal *et al.* (25) recently conducted a study to determine if the positional distribution of MCFA in a synthetic dietary fat influences the lymphatic transport of dietary triglycerides and the chemical composition of chylomicrons in rats. In this study, rats with

cannulated thoracic ducts were fed diets containing one of four types of synthetic triglycerides: (i) *sn*-1(3) MCFA-*sn*-2 linoleic acid, (ii) interesterified *sn*-1(3) MCFA-*sn*-2 linoleic acid, (iii) *sn*-2 MCFA-*sn*-1(3) linoleic acid, or (iv) interesterified *sn*-2 MCFA-*sn*-1(3) linoleic acid. Lymph was collected, and lymph chylomicron triglycerides were analyzed for total fatty acid composition and triacylglycerol structure.

The positional distribution of MCFA in the dietary fat had no significant effect on lymph flow, triglyceride output, or lipid composition of the chylomicrons. The positional distribution of *sn*-2 fatty acids in the synthetic triglycerides was largely maintained in the chylomicron triglycerides. These results indicated that fatty acids with chain lengths <10 in the dietary triglycerides are transported by the lymphatic system, and the positional distribution is preserved in chylomicron triglycerides.

Kubow pointed out in his review (9) that although animal and human infant data suggest the role of molecular structure of triacylglycerols in influencing saturated fatty acid absorption, there are questions in the human adult regarding the relevance of positional distribution of saturated fatty acids on their absorption. It has been suggested that human adults can absorb efficiently most dietary fatty acids whether in nonesterified or monoacyl form, despite the apparent limited capacity of newborn infants to absorb saturated fatty acids in their nonesterified form. Kubow noted that studies on the efficiency of absorption of free fatty acids by human adults have been inconsistent and that the role of positional distribution in this regard needs further investigation.

Early events in the metabolic processing of dietary triglycerides may have an important impact on the risk of chronic diseases such as coronary heart disease. By using structured triglycerides containing predominantly stearic and oleic acids, Summers *et al.* (26) assessed the possibility that the initial removal of fatty acids from chylomicron triglycerides might be fatty acid-specific. The hypothesis was that a stearoyl chain at the *sn*-2 position of chylomicron triglyceride might hinder lipoprotein lipase action and thus clearance of stearic acid. A group of 14 healthy women (aged 29–70 yr, median age 49 yr) consumed meals in random order containing 60 g of either of two structured triglycerides containing predominantly stearic or oleic acids at the *sn*-2 position, i.e., SOO or OSO (S = stearic acid, O = oleic acid). Systemic concentrations and arteriovenous differences across adipose tissue were measured for plasma triglyceride and nonesterified fatty acids for up to 6 h after the meals.

The stereospecific structure of the ingested triglyceride was largely preserved in chylomicron triglycerides. Systemic concentrations of total and individual nonesterified fatty acids were not significantly different after ingestion of the two fats. The composition of nonesterified fatty acids released from adipose tissue changed after the meal to reflect the composition of the ingested triglyceride; however, no significant differences were observed between the two test meals. No detectable monoglycerides were released from adipose tissue after either test meal. The investigators concluded that there is no release of monoglyceride nor preferential uptake or release of fatty acids from chylomicron triglycerides according to the nature or position of the fatty acid within the triglyceride. Thus the data suggest that lipoprotein lipase processes *sn*-2 saturated fatty acids and *sn*-2 unsaturated fatty acids in a similar fashion.

A similar study using vegetable oils instead of structured triglycerides was conducted by Abia *et al.* (27). The purpose of this study was to determine whether two oils with equal levels of oleic acid but with different compositions of other fatty acids and triglyceride molecular species could produce different responses in the triglyceride molecular species of the "triacylglycerol-rich lipoprotein" fraction in the postprandial state. (The "triacylglycerol-rich lipoprotein" particles referred to by Abia *et al*. included chylomicrons, very low density lipoprotein, and their remnants.) Normolipidemic men ($n$ = 8; average age = 27 yr) consumed three meals in random order on separate occasions: control meal, control meal plus 70 g virgin olive oil (VOO), and control meal plus 70 g high-oleic sunflower oil (HOSO). Plasma total triglyceride and triacylglycerol-rich lipoprotein levels were measured for 7 h after ingestion. Triglyceride molecular species and *sn*-2 positional fatty acids within these molecular species were analyzed in the triacylglycerol-rich lipoprotein fraction.

Plasma total triglyceride concentrations in response to both oils were the same. Triglyceride molecular species from the triacylglycerol-rich lipoprotein fraction derived from VOO were found to disappear from the circulation more quickly than those from the triacylglycerol-rich lipoprotein fraction derived from HOSO. On the other hand, the triacylglycerol-rich lipoprotein triglycerides derived from either VOO or HOSO ingestion were reported to contain unexpectedly high percentages of the saturated fatty acids palmitic and stearic acids in the *sn*-2 position. The triacylglycerol-rich lipoprotein triglycerides derived from VOO contained 22% palmitic acid and 12% stearic acid (expressed as a percentage of total fatty acids) in the *sn*-2 position; those derived from HOSO contained 32% palmitic acid and 28% stearic acid in the *sn*-2 position. These results seem highly unlikely considering that natural vegetable oils such as olive oil typically have less than 2% saturated fatty acids in the *sn*-2 position (Table 1) and that *sn*-2 fatty acids are largely preserved in chylomicron triglycerides (9,14,26). Abia *et al*. (27) offered no explanation as to how the *sn*-2 position of the triacylglycerol-rich lipoprotein triglycerides they analyzed could have been substantially enriched with saturated fatty acids. As a result, the conclusion of Abia *et al.* (27) that the *sn*-2 positional distribution of saturated stearic and palmitic acids into the triglyceride molecules of the triacylglycerol-rich lipoprotein fraction may be important determinants of postprandial lipemia in normolipidemic men does not seem justified.

In contrast to the results of Abia *et al.*, Summers *et al*. (26) reported that the *sn*-2 fatty acid composition of triglyceride molecular species of the chylomicron triglyceride fractions they evaluated was largely unaffected by the nature or the position of stearic and oleic acids within the dietary triglycerides.

*Experimental animal studies involving interesterified fats*. Modifying the structure of dietary fats by interesterification has been found to affect not only the absorption characteristics of certain fats but also their atherogenic potential in ani-

mals. It should be noted, however, that while several different native and interesterified fats have been compared in animal studies, only two fatty acids have been studied for their possible positional effects, primarily palmitic, and to a lesser extent, stearic. Relevant studies are highlighted below.

Kritchevsky and Tepper (28) used randomized fats to compare the atherogenic potential of specific saturated fatty acids in rabbits. Special fats were prepared by randomizing corn oil with pure trilaurin, trimyristin, tripalmitin, and tristearin, yielding oils of similar iodine value but differing in the predominating saturated fatty acids. Rabbits were fed for 8 wk on diets consisting of chow augmented with 2% cholesterol and 6% special fats. Other groups of rabbits were fed diets in which corn oil or randomized corn oil was used as the cholesterol vehicle. In four experiments with cholesterol-containing diets, the four different saturated fats were more atherogenic than either native or randomized corn oil. The palmitic acid-rich fat was somewhat more atherogenic than the other special fats. Average serum cholesterol levels were lower in the special fat groups than they were in the rabbits fed corn oil. The investigators suggested that the presence in the diets of relatively large amounts of unsaturated fatty acids may have masked the specific hypercholesterolemic effects of the saturated fatty acids. This study did not compare the atherogenic potential of native vs. randomized fats but rather used randomization to enhance the absorption of specific saturated fatty acids.

In a subsequent study, Kritchevsky and coworkers (29) fed rabbits diets containing 2% cholesterol and 6% specific fat for 8 wk. The special fats included peanut oil, randomized peanut oil, and corn oil. In two of three experiments, the average serum cholesterol level of the randomized peanut oil group was greater than those of the other two groups, and in one experiment it was lower than those of the other two groups. Nevertheless, the average serum cholesterol values for the three experiments were very similar among the three treatment groups (Table 4). Despite this similarity in serum cholesterol levels among the three groups, the native peanut oil group had consistently more atherosclerosis than the randomized peanut oil or corn oil groups (Table 4).

While randomization of peanut oil reduced significantly its atherogenic properties, Kritchevsky *et al.* (30) indicated that native and randomized peanut oil have similar rates of lipolysis and that rats fed the two oils absorb and transport lipids in a similar manner. These authors noted further that peanut oil differs from other oils in having a relatively high lectin content and that the randomization process markedly reduces the lectin content as well. (Lectins are glycoproteins with high affinity binding to cellular carbohydrate residues.) A followup study by Kritchevsky *et al.* (30) suggested that peanut oil's endogenous lectin (rather than its triglyceride structure) may contribute significantly to its atherogenic properties in rabbits.

Kritchevsky and coworkers conducted a series of experiments in which native and randomized fats were compared for their effects on blood lipids and atherosclerosis. In one study (31) rabbits were fed diets containing 14% native or randomized lard or tallow and 0.5% cholesterol for 60 d to determine if

**TABLE 4**
**Blood Lipid and Atherosclerosis Data in Rabbits or Hamsters Fed Native vs. Interesterified Fats**

| Study | Feeding period | Dietary fat type | Total serum or plasma cholesterol (mg/dL) | Total plasma TG (mg/dL) | Average atherosclerosis[c] |
|---|---|---|---|---|---|
| Kritchevsky *et al.* (Ref. 29; rabbits; data represent averages of three experiments) | 8 wk | Peanut oil | 1873 | NR[b] | 1.88** |
| | | I-Peanut oil[a] | 1833 | NR | 1.18 |
| | | Corn oil | 1678 | NR | 1.17 |
| Kritchevsky *et al.* (Ref. 31; rabbits) | 60 d | Lard | 926 | 175 | 2.22* |
| | | I-Lard | 834 | 58* | 1.10 |
| | | Tallow | 1177 | 144 | 1.04 |
| | | I-Tallow | 1189 | 223* | 1.15 |
| Kritchevsky *et al.* (Ref. 32; rabbits) | 90 d | Cottonseed oil | 546 | 57 | 0.71 |
| | | I-Cottonseed oil | 542 | 60 | 2.09* |
| Kritchevsky *et al.* (Ref. 34; rabbits; study used synthetic TG: S = stearate, O = oleate, P = palmitate) | 20 wk | SOS | 328 | 68 | 1.35 |
| | | SSO | 272 | 83 | 0.97 |
| | | POP | 308 | 94 | 0.83 |
| | | PPO | 415 | 81 | 1.80* |
| Nicolosi *et al.*, unpublished[d] (hamsters) | 6 wk | Peanut oil | 445 | 511 | 30.1 |
| | | I[a]-Peanut oil | 554 | 612 | 34.4 |
| | | Lard | 529 | 679 | 29.9 |
| | | I-Lard | 512 | 887 | 32.0 |
| | | Tallow | 588 | 1021 | 27.5 |
| | | I-Tallow | 550 | 1030 | 28.8 |

[a]I = interesterified (randomized) oil.
[b]NR = not reported.
[c]In the studies by Kritchevsky *et al.,* average atherosclerosis represents the severity of atherosclerosis [(aortic arch + thoracic aorta)/2] graded visually on a 0–4 scale. In the study by Nicolosi *et al.* (footnote *d*), average atherosclerosis represents aortic fatty streak area expressed as $\mu m^2/mm^2 \times 100$.
[d]Unpublished data of R.J. Nicolosi, T. Wilson, and C. Lawton, used with permission. For a given study, the absence of superscripts for total cholesterol, total TG, or atherosclerosis values indicates these respective values were not significantly different from each other: *Significantly different from other groups, $P < 0.05$. **Significantly different from other groups, $P < 0.01$. To convert serum cholesterol units from mg/dL to mM, divide by 38.67. To convert serum TG units from mg/dL to mM, divide by 88.54. See Table 1 for other abbreviation.

these two animal fats have different atherogenic potentials and if randomization of the fats might affect atherogenesis. Lard contains more linoleic acid and less stearic acid than tallow. Both lard and tallow contain *ca.* 24% palmitic acid (16:0). However, in lard over 90% of the palmitic acid is in the *sn*-2 position of the triglyceride, whereas only 15% of the palmitic acid in tallow is in the *sn*-2 position. The fats were randomized so that every component fatty acid was present at each triglyceride carbon at *ca.* one-third of its total concentration. The randomized tallow and lard had 8.5 and 7.6% of their component 16:0 (i.e., about one-third of their total 16:0 concentration) at the *sn*-2 position, respectively. Total cholesterol levels were not significantly different among the four groups. However, rabbits fed native lard exhibited significantly more atherosclerosis than those rabbits fed native tallow (Table 4). The atherogenicity of diets containing either randomized lard or tallow was virtually the same and less than that of the diet containing native lard. Randomization of tallow led to somewhat more palmitic acid in the *sn*-2 position and slightly (but not significantly) increased the severity of atherosclerosis compared to native tallow. On the other hand, randomization of lard caused reduction in the amount of palmitic acid in the *sn*-2 position and a significant decrease in the severity of atherosclerosis. This study showed that the level of 16:0 at the *sn*-2 position of a triglyceride influences its atherogenicity in rabbits.

Another study compared the atherogenic potential in rabbits of feeding native and randomized cottonseed oil (32). Cottonseed oil contains about 24% palmitic acid, of which only 2% is present at the *sn*-2 position. After randomization, one-third of the palmitic acid of cottonseed oil was at the *sn*-2 position. Rabbits were fed semipurified diets containing 14% native or randomized cottonseed oil and 0.10% cholesterol for 90 d. The rabbits fed the randomized cottonseed oil had significantly more severe atherosclerosis than those rabbits fed the native cottonseed oil. Plasma cholesterol and triglyceride levels were similar in the two groups (Table 4). It was suggested that the increased atherogenicity of the randomized cottonseed oil may reflect the increased absorbability of fats with palmitic acid at the *sn*-2 position.

A further study by Kritchevsky *et al.* (33) compared the atherogenic effects of refined, bleached, and deodorized (RBD) palm oil with those of randomized RBD palm oil. The RBD palm oil contained 41.2% palmitic acid, of which 2.6% was at the *sn*-2 position. In the randomized palm oil, 13.6% palmitic acid was at the *sn*-2 position. The randomized palm oil was significantly more atherogenic for rabbits than was the RBD palm oil, consistent with other findings of Kritchevsky *et al.* that increasing the amount of palmitic acid at the *sn*-2 position of a fat led to an increased atherogenic effect.

Four synthetic fats whose triglyceride structures were SOS, SSO, POP, and PPO (S = stearic acid, P = palmitic acid, O = oleic acid) were evaluated for their atherogenic potential in rabbits (34). The fats were fed in semisynthetic diets containing 15% fat, of which 58% was the synthetic fat, 24% was sunflower oil, and 18% was high-oleic safflower oil. All of the diets contained 0.05% cholesterol and were fed for 20 wk. The blood lipid levels (total cholesterol, % HDL, and triglycerides) were similar in all four groups. Average atherosclerosis was similar in rabbits fed SOS compared to SSO (2.4 or 28% of stearic acid in the *sn*-2 position, respectively); however, it was much greater in rabbits fed PPO compared to POP (Table 4). The authors noted that these findings confirmed their earlier observations that randomization of fats affects their atherogenicity but not their lipidemic effects. Specifically, fats bearing palmitic acid (but not stearic acid) in the *sn*-2 position have increased atherogenicity compared to fats with palmitic acid in the *sn*-1 or *sn*-3 positions. The mechanism of this effect was suggested to be related to greater absorption of the atherogenic fat.

An important observation in these studies by Kritchevsky *et al*. (28,29,31,32,34) was that feeding diets containing interesterified fats compared with native fats resulted in differences in atherogenicity that would not have been predicted from the blood lipid and lipoprotein levels of the animals. In all of these studies, there were no consistent differences in blood lipid or lipoprotein levels as a result of feeding an interesterified fat compared with the corresponding native fat. On the other hand, the feeding of interesterified fats compared with native fats showed significant differences in the development of atherosclerosis (Table 4). With the exception of peanut oil, the more atherogenic fats were those in which there was a higher level of palmitic acid in the *sn*-2 position compared with the *sn*-1 or *sn*-3 positions. Thus, native lard (with palmitic acid primarily *sn*-2) was more atherogenic than interesterified lard (31), and interesterified cottonseed oil (having more palmitic acid *sn*-2) was more atherogenic than native cottonseed oil (32). The increased atherogenicity of native peanut oil compared with randomized peanut oil (29) may have been more closely related to a higher level of lectin in the native oil than to a change in triglyceride structure.

It has been reported that the presence of a saturated fatty acid in the *sn*-2 position of a triglyceride slows its removal from the bloodstream (35). Thus, the *in vivo* residence of a fat with palmitic acid in the *sn*-2 position would be increased and the probability of its deposition increased. Kritchevsky *et al.* (32) suggested that the combination of enhanced absorption and persistence in the circulation of fats with palmitic acid in the *sn*-2 position adds up to increased availability and exposure to the aorta, even when levels in the blood do not differ.

Nicolosi, Wilson, and Lawton (personal communication) have conducted atherogenicity studies in which hamsters were fed for 6 wk on native or randomized tallow, lard, or peanut oil as part of semipurified diets. Consistent with results of Kritchevsky *et al*., Nicolosi *et al.* found no differences in various blood lipid parameters after feeding the native compared to the randomized oils. However, in contrast to the atherogenicity results of Kritchevsky *et al.* (in which atherogenicity increased when the level of palmitic acid in the *sn*-2 position of the dietary fat increased), Nicolosi *et al.* found no differences in atherogenicity after feeding the native compared to the randomized fats. This difference in atherogenicity results between the studies of Nicolosi *et al.* and Kritchevsky *et al.* may be related to a difference in responsiveness between the species used, hamsters compared to rabbits.

Innis and coworkers have conducted several experiments with piglets to evaluate the effect of dietary triacylglycerol structure on fatty acid absorption and on the composition and distribution of plasma lipoprotein fatty acids. In a fatty acid absorption experiment, Innis *et al.* (36) fed similar concentrations of palmitic acid (27–31%) to piglets, either in the form of sow's milk (55% of palmitic acid in *sn*-2) or in a formula containing interesterified triglycerides (from palm, sunflower, and canola oils; 70% of palmitic acid in *sn*-2). The piglets fed sow's milk or the interesterified fat showed increased incorporation of palmitic acid into plasma triglycerides and cholesterol esters 4 h after feeding compared with piglets fed palm oil (4% of palmitic acid in *sn*-2). These results were consistent with those of Lien *et al*. (20), who reported increased absorption of palmitic acid in rats if the palmitic acid was in the *sn*-2 position.

In a plasma lipoprotein study, Innis and Dyer (37) fed piglets formula containing synthesized triacylglycerols (32% 16:0 in the *sn*-2 position) or palm olein (4.2% 16:0 *sn*-2). These results were compared with those of piglets fed sow's milk (55% 16:0 in the *sn*-2 position). The formulas were made without or with cholesterol added to a level similar to that in human and sow's milk. The piglets were fed from birth until 18 d after birth. Plasma cholesterol and triacylglycerol levels were significantly higher in piglets fed milk than in piglets fed either the palm olein formula (with or without cholesterol) or the synthesized triacylglycerol formula (with or without cholesterol). In considering piglets fed either the palm olein or the synthesized triacylglycerol formula, plasma cholesterol levels were similar for these treatment groups without added cholesterol [1.98 ± 0.11 vs. 1.83 ± 0.17 mmol/L (76.6 vs. 70.8 mg/dL), respectively] or with added cholesterol [2.50 ± 0.06 vs. 2.11 ± 0.09 mmol/L (96.7 vs. 81.6 mg/dL), respectively]. These treatment differences (either with or without cholesterol) were not reported to be significantly different from each other. Also, plasma triacylglycerol levels were similar for the piglets fed either the palm olein or the synthesized triacylglycerol formula (with or without cholesterol).

Feeding sow's milk resulted in higher levels of total 16:0 in chylomicron triacylglycerols compared with feeding the palm olein or synthesized triacylglycerol formula. Also, feeding formula with 16:0 at the triglyceride *sn*-2 position or feeding sow's milk resulted in higher levels of 16:0 in the *sn*-2 position of chylomicron triacylglycerols than when palm olein was fed. This suggested that dietary triacylglycerol *sn*-2 position fatty acids are conserved during digestion, absorption, and reassembly into chylomicron triacylglycerols. The increased 16:0 at the *sn*-2 position of chylomicron triacylglycerols in piglets fed synthesized triacylglycerols was accompanied by lower chylomicron triacylglycerol arachidonic and docosahexaenoic acids than in piglets fed formula with palm olein. The authors suggested that there may be an interaction between the positional distribution of saturated fatty acids on the dietary triacylglycerols and the transport of n-6 and n-3 fatty acids. The nature of such an interaction, however, is unknown.

In a subsequent study, Innis *et al*. (38) investigated whether piglets fed formula with randomized oils (16:0 equally distributed among all positions of the triglycerides) influenced growth or the distribution of fatty acids in plasma and liver lipids compared with piglets fed formula with native oils or sow's milk. After feeding from birth until 18 d after birth, piglets fed formula with palm olein randomized with canola oil (co-randomized) had higher weight gain per liter of formula intake and higher 16:0 in the chylomicron triglyceride *sn*-2 position than piglets fed formula with randomized or native palm olein oil blended with canola oil. Also, piglets fed sow's milk had higher plasma total and HDL-cholesterol levels than piglets fed the formula with native oils, randomized palm olein, or co-randomized palm olein with canola oil. The investigators concluded that the fatty acid distribution of formula triglycerides is an important determinant of pathways of 16:0 absorption, and consequently of plasma lipid fatty acids in formula-fed piglets.

A study by Renaud *et al*. (21) using rats indicated that it is mostly dietary fatty acids in the *sn*-2 position that are able to influence lipemia and platelet reactivity and to be transformed by desaturation and elongation to longer-chain unsaturated fatty acids. Interesterification of lard resulted in significantly lower plasma triglyceride levels. Feeding interesterified lard was associated with reduced palmitic acid in plasma total lipids, and this was consistent with the decrease in palmitic acid in the *sn*-2 position compared with native lard. In addition, dietary levels of specific fatty acids in the *sn*-2 position, namely linoleic, palmitic, and oleic, were directly related to plasma levels of arachidonic (20:4n-6), palmitoleic (16:1n-7), and eicosatrienoic (20:3n-9) acids, respectively.

In summary, a key finding of many animal studies has been that randomization procedures have little or no impact on blood lipid and lipoprotein levels except in very young animals, such as piglets. In spite of this finding, however, randomization that increases the level of palmitic acid in the *sn*-2 position appears to increase the risk of atherosclerosis in susceptible species such as rabbits, whereas procedures that decrease the level of palmitic acid in the *sn*-2 position appear to decrease atherosclerosis risk. The increased risk of atherosclerosis from palmitic acid in the *sn*-2 position may be related to the combination of enhanced absorption and less active removal from the circulation of palmitic acid. This, in turn, could lead to increased exposure of the aortic tissue to this fatty acid and possibly to increased fat deposition. Other fatty acids that have been studied following randomization (e.g., stearic acid) have not shown the same properties with respect to atherosclerosis. Studies with randomized peanut oil, in sharp contrast to studies involving other randomized vegetable oils, have shown that increasing the level of long-chain saturated fatty acids in the *sn*-2 position (or reducing peanut oil's lectin content) appears to decrease the risk of atherosclerosis.

## HUMAN CLINICAL STUDIES INVOLVING INTERESTERIFIED FATS

*Selection of human studies for review*. The focus of this section is to assess from published literature how altering dietary triglyceride structure may affect blood lipids and lipoproteins in humans. The rabbit atherosclerosis studies discussed previously are not directly applicable to humans; however, such studies are

of interest because human studies cannot readily assess the atherogenic potential of a dietary component. Human studies were selected for review if they: (i) compared effects of feeding native (nonrandomized) fats with those of feeding interesterified (randomized) fats or (ii) used synthetic fats in which the positions of fatty acids on the triglycerides differed from those normally found in native fats and oils. Most of the studies utilized conventional food diets. Feeding periods lasted at least 3 wk, which is considered adequate to observe changes in blood lipid and lipoprotein levels. Of interest is whether there is a threshold level of fat in the diet at which there is no effect regardless of the position of the fatty acid on dietary triglycerides. On the other hand, the studies reviewed in this paper had design differences, such as the amount of total fat in the diet or the amount of test fat in the diet, which make comparisons difficult. These design differences are pointed out in the text.

*Human studies involving effects of interesterified fats on blood lipids and lipoproteins.* Four recent human studies have evaluated effects on blood lipids and lipoproteins of feeding native fats compared with interesterified fats. Key results from these studies are summarized in Table 5. Relevant study details are noted below.

Zock *et al.* (39) fed 60 normocholesterolemic male and female subjects (average ages = 29 yr for the men; 32 yr for the women) each of two diets containing 40–41% energy as fat in a crossover design for 3 wk. One diet contained a margarine high in conventional palm oil, and the other a margarine high in an interesterified palm oil. The conventional palm oil had 82% of the palmitic acid in the *sn*-1 and *sn*-3 positions and 18% in the *sn*-2 position. In contrast, the interesterified palm oil (derived by interesterification of palm oil with sunflower oil fatty acids) had 35% of the palmitic acid in the *sn*-1 and *sn*-3 positions and 65% in the *sn*-2 position. Regarding stearic acid, 78% was in the *sn*-1,3 positions and 22% in the *sn*-2 position in the conventional palm oil diet, and 72% was in the *sn*-1,3 positions and 28% in the *sn*-2 position in the modified palm oil diet. Following the modified palm oil diet, considering all subjects, there were nonsignificant ($P > 0.13$) increases of 0.06 mmol/L (2.3 mg/dL), 0.03 mmol/L (1.2 mg/dL), and 0.04 mmol/L (1.5 mg/dL) in total, HDL-, and LDL-cholesterol, respectively, compared with the conventional palm oil diet. The small increases in total and LDL-cholesterol were significant in the men but not in the women. Overall, a large difference in dietary fatty acid configurations had little effect on blood lipid and lipoprotein concentrations. Considering the hypercholesterolemic nature of palmitic acid, these results suggested that the position of palmitic acid on the triglyceride is not significant with respect to its effect on blood lipid and lipoprotein levels.

Nestel *et al.* (40) fed 27 moderately overweight hypercholesterolemic men (average age 49 yr) diets containing one of three special margarines. One margarine was high in linoleic acid and moderate in *trans* fatty acids (TFA); one was high in a palm oil blend; and one contained an interesterified form of the high palm oil blend. The test margarine high in linoleic acid and moderate in TFA and the margarine high in the noninteresterifed palm oil blend had palmitic acid primarily in the *sn*-1 and *sn*-3 positions. The margarine made with the interesterified palm oil blend had palmitic acid roughly evenly distributed among the *sn*-1, *sn*-2, and *sn*-3 positions. The positional distribution of stearic acid was approximately the same in both the noninteresterified and interesterified fat blends (i.e., *sn*-2 = 11–12% of total fatty acids; *sn*-1,3 = 9% of total fatty acids in both fat blends). The fat content of the diets was about 35% energy. Total, LDL-, and HDL-cholesterol values were not different after subjects consumed either the high palm oil blend margarine or the interesterified palm oil blend margarine. These levels, however, were significantly higher ($P < 0.002$) than those seen after the high-linoleic, moderate *trans* blend. Blood triglyceride levels were not affected by any of the three dietary treatments. Overall, the process of interesterification did not influence the plasma lipid and lipoprotein response.

In a further study, Nestel *et al.* (41) compared the effects of feeding a stearic acid-rich, structured fat (Salatrim™; 18:0 = 66% total fatty acids; 18:0 randomly incorporated into the *sn*-1, *sn*-2, and *sn*-3 positions) with those of feeding a palmitic acid-rich fat. The triglycerides of the palmitic acid-rich diet had both palmitic and stearic acids primarily in the *sn*-1 and *sn*-3 positions. The fats were incorporated into margarines, cookies, and muffins and fed to 15 hypercholes-

**TABLE 5**
**Blood Lipid and Lipoprotein Levels in Human Subjects Fed Native vs. Interesterified Fats[a]**

| Study | Feeding period | Dietary fat type | TC (mg/dL) | LDL-C (mg/dL) | HDL-C (mg/dL) | TG (mg/dL) |
|---|---|---|---|---|---|---|
| Zock *et al.* (Ref. 39; 60 normocholesterolemic male and female subjects) | 21 d | Palm oil | 180 | 101 | 61.9 | 85.9 |
| | | Interesterified palm oil | 183 | 103 | 63.0 | 83.2 |
| Nestel *et al.* (Ref. 40; 27 hypercholesterolemic male subjects) | 21 d | Palm oil blend | 245 | 171 | 41.4 | 166 |
| | | Interesterified palm oil blend | 249 | 176 | 40.6 | 165 |
| Nestel *et al.* (Ref. 41; 15 hypercholesterolemic male and female subjects) | 35 d | Palmitic acid-rich margarine | 213 | 143 | 42.2 | 140 |
| | | Stearic acid-rich margarine (structured TG Salatrim) | 209 | 141 | 41.8 | 132 |
| Meijer and Weststrate (Ref. 42; 60 normocholesterolemic male and female subjects) | 21 d | Control fat blend (8% en; largely coconut and palm oils) | 186 (male)<br>205 (female) | 116 (male)<br>128 (female) | 46.4 (male)<br>61.9 (female) | 97.4 (male)<br>88.5 (female) |
| | | Interesterified fat blend (8% en; largely coconut and palm oils) | 189 (male)<br>205 (female) | 124 (male)<br>124 (female) | 50.3 (male)<br>61.9 (female) | 97.4 (male)<br>97.4 (female) |

[a]Data shown from Zock *et al.* (39) and Nestel *et al.* (41) are for combined male and female subjects. Meijer and Weststrate (42) did not report combined data for their male and female subjects. TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol. See Table 1 for other abbreviation.

terolemic men and women (average age = 51 yr) for 5 wk each in random order. A low-fat diet (21% energy as fat) was fed for 2 wk as a baseline "run-in" treatment. The test diets contained 41–42% energy as fat. The fat intake provided by the test fats was *ca.* 25% energy. Total cholesterol levels were not significantly different from each other after feeding the low-fat, high-stearic, or high-palmitic diets, but they were significantly lower ($P < 0.001$) than that measured during the habitual diet period before the study started. HDL-cholesterol and triglyceride levels were not significantly different among the three diet treatments. Overall, a similar increase in the intake of stearic and palmitic acids to ensure a high fat intake led to total- and LDL-cholesterol levels that did not differ significantly from those during the period of a low fat diet. The authors attributed the lack of difference between the palmitic and stearic treatments to the small difference in the palmitic and stearic acid contents of the diets (around 5% total energy for each). Also, the lack of difference in LDL-cholesterol between the palmitic and low-fat diets was consistent with results previously reported by these authors.

Meijer and Weststrate (42) fed 60 healthy male and female subjects (average ages = 34 yr for the men and 37 yr for the women) a blend of commonly consumed vegetable fats. Half of the subjects received a control (untreated) fat blend, and half received an interesterified fat blend. The control and interesterified fat blends had the same fatty acid composition, but differed primarily in the triglyceride distributions of palmitic, stearic, and oleic acids. In the control fat, palmitic and stearic acids were primarily in the *sn*-1 and *sn*-3 positions, and oleic acid was primarily in the *sn*-2 position. In the interesterified fat, these fatty acids were roughly evenly distributed among the *sn*-1, *sn*-2, and *sn*-3 positions. Both fat blends were fed at two energy levels (4 and 8% energy) in margarine. At either energy level, the fat blends were consumed according to a crossover design for two periods of 3 wk. The total dietary fat level was 34% energy. Neither the type of fat blend nor the energy level at which the fat blends were consumed resulted in significant changes in blood lipid levels or in various clinical chemical and hemostasis parameters. The investigators concluded that the inclusion of interesterified vegetable fat blends in the diet of healthy people does not influence fasting blood lipids, blood enzymes, or hemostasis parameters in an adverse way compared with a non-interesterified fat blend with the same fatty acid composition.

While the position of selected fatty acids on dietary triglycerides has been reported not to affect blood lipid levels of human adults, there appears to be an impact in infants. A study by Nelson and Innis (43) evaluated whether the position of 16:0 in human milk and infant formula triglycerides influences the position of fatty acids in postprandial plasma chylomicron triglycerides. These investigators fed full-term infants formula with 25–27% total 16:0 with either 39% of the 16:0 (synthesized triglyceride) or 6% of the 16:0 (standard formula) esterified at the *sn*-2 position of the triglycerides from birth to 120 d of age. Some infants were breast-fed (23% total 16:0, 81% at the *sn*-2 position). Palmitic acid (16:0) is predominantly esterified in the *sn*-2 position of human milk triglycerides but in the *sn*-1 and *sn*-3 positions in the oils used in infant formulas. Infants fed the synthesized triglyceride formula, standard formula, or breast milk had 15.8, 8.3, and 28.0%, respectively, 16:0 in the chylomicron triglyceride *sn*-2 position. These results suggested that 50% or more of the dietary triglyceride *sn*-2 16:0 is conserved through digestion, absorption, and chlylomicron triglyceride synthesis in breast-fed and formula-fed infants. Total plasma cholesterol levels were higher in the breast-fed infants than in both groups of formula-fed infants, and both groups of formula-fed infants had the same total cholesterol levels. Plasma triglyceride levels were the same among all three groups. Infants fed the synthesized triglyceride formula had significantly lower HDL-cholesterol and apolipoprotein (apo) A-I concentrations than infants fed the standard formula or who were breast-fed. On the other hand, the plasma apo B levels of infants fed the synthesized triglyceride formula were significantly higher than those of infants fed the standard formula and were not different from those found in the breast-fed infants.

In summary, consumption of interesterified fats by adult humans appears to have no significant effect on blood lipid and lipoprotein parameters compared with consumption of native fats (Table 5). On the other hand, one study with human infants indicated that feeding a synthesized triglyceride formula (with an increased level of palmitic acid in the *sn*-2 position) led to decreased HDL-cholesterol and apo A-I and increased apo B concentrations compared with feeding a standard formula (with less palmitic acid in the *sn*-2 position). In contrast, breast-fed infants (who consumed milk with a higher level of palmitic acid in the *sn*-2 position compared with the synthesized triglyceride formula) had higher HDL-cholesterol and apo A-1 concentrations than did the infants fed the synthesized triglyceride formula. Also, in view of the limited number of studies that have been conducted in which effects of native and interesterified fats have been compared, it is not possible to determine whether the degree of randomization or the level of fat in the diet has influenced the outcome of the study. In addition, the limited number of available studies does not permit any conclusions to be drawn about whether there is a threshold level of total fat in the diet at which there is no effect, regardless of the position of the fatty acid on the dietary triglycerides.

## HUMAN STUDIES INVOLVING EFFECTS OF SPECIFIC FATTY ACIDS ON BLOOD LIPIDS AND LIPOPROTEINS

Effects of specific fatty acids on blood lipids and lipoproteins have been evaluated using interesterified fat blends but without comparing directly the effects of a native fat with those of the corresponding interesterified fat. In a study to assess the cholesterol-raising potential of specific saturated fatty acids in humans, McGandy *et al*. (44) fed male subjects (aged 41–56 yr) a number of synthesized fats with an excess of a specific saturated fatty acid. The test fats were prepared by interesterifying natural fats with either trilaurin, trimyristin, tripalmitin, or almost fully hydrogenated soybean oil (85% stearic acid) in a ratio of 3:1. (Thus, interesterification was used to enrich the test fats with specific fatty acids, not to study positional effects.) The results obtained with the interesterified fats (in

which the tested saturated fatty acid was randomized among the three positions) conflicted with studies using naturally occurring fat sources. McGandy *et al*. found that the stearic acid interesterified fat showed a similar hypercholesterolemic effect compared with the palmitic or myristic acid fats.

Previous work by Hegsted *et al.* (3), in contrast to that of McGandy *et al*. (44), found that stearic acid fed as cocoa butter (with stearic acid primarily in the *sn*-1 and *sn*-3 positions) was neutral with respect to blood cholesterol raising. Hegsted *et al.*'s study was designed to compare various native fats that are different in degree of saturation and chain length. This study did not include interesterified fats. McGandy *et al*. suggested that the difference in cholesterolemic action of stearic acid in the two studies may have been related to the position of stearic acid on the triglycerides being fed. McGandy *et al.*'s work also contrasted with that of Grande and coworkers (45), who found no differences in either plasma total cholesterol or triglyceride levels in human adults fed native cocoa butter compared with an interesterified fat blend having the same fatty acid composition as cocoa butter but no added cocoa butter *per se*. In the interesterified fat blend, stearic and palmitic acids were randomized among the *sn*-1, *sn*-2, and *sn*-3 positions. This work suggested that the positions of stearic and palmitic acids on the triglycerides did not affect blood lipid levels. [These early (1965 and 1970) studies did not report levels of HDL- and LDL-cholesterol because analytical methods for blood lipoproteins were not well established at that time.]

Positional effects of stearic acid also can be considered by comparing studies conducted by Judd *et al*. (46) and Kris-Etherton *et al*. (47). Judd *et al.* (46) fed 50 normocholesterolemic men (average age = 42 yr) a series of six diets for 5 wk each to study effects of individual fatty acids on blood lipid and lipoprotein levels. Across diets, 8% energy was replaced as follows: (i) carbohydrate (CHO; 1:1 simple to complex); (ii) oleic acid (OL); (iii) TFA; (iv) stearic acid (STE); (v) TFA (4% energy) plus STE (4% energy); or (vi) the saturated fatty acids lauric, myristic, and palmitic (LMP). The CHO diet contained 30% energy as fat, and the other diets about 39% energy as fat. For four of the six diets (CHO, OL, TFA, and LMP), the fats were not modified to rearrange the positions of the fatty acids on the triglycerides. In these fats, STE and palmitic acid were primarily in the *sn*-1 and *sn*-3 positions. The high-stearic fat used in the STE and TFA/STE diets was randomized, so STE in these diets would have been equally distributed among the *sn*-1, *sn*-2, and *sn*-3 positions. Compared with the CHO control diet, the STE diet had no effect on LDL-cholesterol, but it lowered HDL-cholesterol. Also, the STE diet showed a directional (but not significant) increase in plasma triglyceride level compared with the CHO diet. Compared with the LMP diet, the STE diet did not change LDL-cholesterol, but it lowered HDL-cholesterol and raised the plasma triglyceride level. Plasma triglycerides were highest after the STE diet [1.134 mM (100.4 mg/dL)] and lowest after the OL diet [0.878 mM (77.7 mg/dL)]. [This study by Judd *et al*. (46) has been submitted for publication and at present is published only in abstract form.]

The study by Kris-Etherton *et al*. (47) compared the effects on blood lipids and lipoproteins of feeding either one milk chocolate bar or a high-carbohydrate snack per day as part of a National Cholesterol Education Program/American Heart Association (NCEP/AHA) Step 1 diet. Following a 21-d run-in period on the NCEP/AHA Step 1 diet (29% calories as fat), Kris-Etherton *et al.* fed 42 normocholesterolemic men (aged 21–35 yr) the same diet with the addition of either one milk chocolate bar per day (total dietary fat = 34% energy) or a high-carbohydrate snack per day (total dietary fat = 29% energy) for 27 d. Although the intakes of individual saturated fatty acids (except for stearate) were the same for subjects consuming the high-carbohydrate snack compared with the milk chocolate bar, subjects consuming the milk chocolate bar had greater intakes of oleic acid (11.4 energy %) than those consuming the high-carbohydrate snack (9.2 energy %). For subjects consuming the milk chocolate bar, HDL-cholesterol levels were 0.08 mmol/L (3.1 mg/dL) higher ($P < 0.01$), and plasma triglyceride levels were 0.06 mmol/L (5.3 mg/dL) lower ($P < 0.05$) compared with subjects consuming the high-carbohydrate snack. In contrast, Judd *et al.* found that subjects consuming the high-stearate diet had HDL-cholesterol levels that were 0.04 mmol/L (1.5 mg/dL) lower ($P \leq 0.01$) and triglyceride levels that were not significantly different compared with those of subjects consuming the high-carbohydrate diet.

The inconsistency in responses of HDL-cholesterol and triglyceride levels to increased intake of STE in the Judd *et al.* study compared to the Kris-Etherton *et al.* study may be related to differences in triglyceride structure and dietary levels of fat and STE between the two studies. In the STE diet used by Judd *et al.*, STE was randomized (i.e., distributed equally among the *sn*-1, *sn*-2, and *sn*-3 positions), whereas the cocoa butter used in the milk chocolate bars in the study by Kris-Etherton *et al.* was not randomized (STE primarily in the *sn*-1 and *sn*-3 positions). Also, the STE diet used by Judd *et al.* contained higher levels of total fat (39 vs. 34% energy) and of STE (about 38 vs. 17 g/d, based on a caloric intake of around 3000 kcal/d) compared with the milk-chocolate-bar-supplemented diet used by Kris-Etherton *et al.* Furthermore, the stearate and carbohydrate exchange was 8 energy % in the Judd *et al.* study but much lower (2.8 energy %) in the Kris-Etherton *et al.* study. Thus, much more STE would have been available for absorption in the Judd *et al.* study than in the Kris-Etherton *et al.* study. It is possible that the effect of dietary STE on blood lipid and lipoprotein levels depends not only on its level in the diet but also on whether it has been randomized on the dietary triglycerides.

Also in contrast to the study by Judd *et al.*, Nestel *et al.* (41) found no difference in HDL-cholesterol or triglyceride levels after feeding a high-STE diet (STE intake ≈ 43 g/d in a diet containing 2500–2600 kcal/d and randomly incorporated into the *sn*-1, *sn*-2, and *sn*-3 positions) compared with feeding a high-carbohydrate diet (baseline diet; fat = 21% energy). Three recent human studies (6,48,49) have indicated that a high-stearate diet (nonrandomized; i.e., STE, primarily *sn*-1 and *sn*-3) tended to lower or conserve HDL-cholesterol levels without raising triglyceride levels compared with a high-palmitate diet. Bonanome and Grundy (6) fed three liquid for-

mula diets, each containing 40% energy from fat (17% energy from palmitic, STE, or oleic acids), to hypercholesterolemic men for 3 wk each. The STE diet resulted in lower total, LDL-, and HDL-cholesterol compared with the palmitic acid diet but no change in triglyceride level. Snook *et al.* (48) fed premenopausal women each of three conventional food diets containing 40% energy from fat (13% energy from either myristic, palmitic, or stearic acid) for 5 wk. The STE diet resulted in lower total and LDL-cholesterol compared with the palmitic acid diet. HDL-cholesterol and triglyceride levels were the same after all three diets. Tholstrup *et al.* (49) fed three liquid formula diets, each containing 40% energy from fat (15% energy from either myristic plus lauric acids, palmitic acid, or STE) to male subjects for 3 wk each. The STE diet lowered total and LDL-cholesterol compared with both the palmitic and myristic acids plus lauric acid diets. HDL-cholesterol also was lower after the STE diet than after either the palmitic or myristic diet plus lauric acid diet. Triglyceride levels were the same after all three diets.

Zock [personal communication; presented at a special AHA conference (50)] has found that based on a meta analysis, high-stearate diets (having an average stearate content of 3.1% energy, range 0.7–16.5%) slightly decreased the total/HDL-cholesterol ratio when substituted for carbohydrates. The total fat content of the diets averaged 34.4% energy (range 4.5–53%). The high-stearate diets did not increase HDL-cholesterol levels as other saturated fatty acids did compared with carbohydrates. In this analysis, the stearate diets resulted in the same HDL levels as did the carbohydrate diets. Also, stearate diets significantly decreased plasma triglyceride levels compared with carbohydrate diets, and the decrease with stearate diets was to the same extent as that with other saturated fatty acid diets.

Aro *et al.* (51) compared effects of diets high in STE, TFA, or dairy fat for 5 wk on serum lipid and lipoprotein levels in 80 normocholesterolemic men and women (average age = 29 yr). As was the case with the study by Judd *et al.* (46), Aro *et al.* used a high-STE diet in which STE was provided in an interesterified fat blend. Thus STE in this fat was equally distributed among the *sn*-1, *sn*-2, and *sn*-3 positions. On the other hand, unlike the Judd *et al.* study, all three test diets used by Aro *et al.* provided 32–34% energy as fat and no lower-fat, high-carbohydrate diet was used. Thus a high-carbohydrate–high-STE comparison was not possible. Nevertheless, Aro *et al.* found that compared with the baseline dairy fat diet (high in saturated fatty acids), STE and TFA decreased total cholesterol levels similarly (13 and 12%, respectively). The STE and *trans* diets both reduced HDL-cholesterol, with the *trans* diet showing a larger decrease in HDL-cholesterol (17%) than the STE diet (11%). The STE diet decreased serum triglyceride levels by 10% ($P < 0.01$); however, triglyceride levels were unchanged by the *trans* diet. The authors concluded that dietary fats low in both saturated fatty acids and TFA should be favored for optimizing blood lipid and lipoprotein profiles.

Mascioli *et al.* (52) provided 30 healthy adult men and women (average age = 59 yr) baked goods (muffins or cookies) made with either of two fats: butter or an interesterified mixture of butter, medium-chain triglycerides (MCT), and safflower oil. The subjects followed each of two individualized meal plans incorporating either of the two fats in a crossover study design for periods of 5 wk each. The diets consisted of 36% energy as fat. In the butter diet, the levels of lauric, myristic, and palmitic acids in the *sn*-2 position exceeded the levels of these fatty acids in the *sn*-1,3 positions. In contrast, the level of STE in the *sn*-1,3 positions greatly exceeded that in the *sn*-2 position. In the diet containing the interesterified butter/MCT mixture, the saturated fatty acids were equally distributed among the *sn*-1, *sn*-2, and *sn*-3 positions. The investigators found that total-, LDL-, and HDL-cholesterol levels were not significantly different between the two dietary periods. Plasma triglyceride levels were significantly higher after feeding the interesterified butter-MCT mixture than after feeding butter [1.96 mmol/L vs. 1.75 mmol/L (174 vs. 155 mg/dL), $P < 0.05$]. These results were consistent with other cited reports that triglyceride levels increased when subjects consumed MCT. The authors concluded that an interesterified mixture of butter, MCT, and safflower oil compared with butter had no appreciable effect on plasma cholesterol concentrations but was associated with a modest rise in plasma triglycerides.

In addition, studies involving the feeding of high levels of STE as cocoa butter (47,53,54) or as shea butter (sheanut oil) (49,55) have not shown reductions in HDL-cholesterol or increases in triglyceride levels compared with baseline or control diets. In cocoa butter and shea butter, STE is primarily in the *sn*-1 and *sn*-3 positions, whereas in the study by Judd *et al.* (46), it was equally distributed among the *sn*-1, *sn*-2, and *sn*-3 positions. In the study by Judd *et al.*, STE was fed as part of a high-fat diet (39% energy as fat), which was similar to the levels of dietary fat in the studies involving cocoa butter and shea butter (34–40% energy). Thus, the effect of STE on certain blood lipid parameters may have been related to whether the fat had been structurally rearranged. As far as I am aware, human studies comparing effects of native vs. randomized cocoa butter or native vs. randomized shea butter have not been conducted.

In summary, responses among studies of blood lipids and lipoproteins to increased intake of specific fatty acids have been inconsistent. These inconsistencies may be related to differences in triglyceride structure and in dietary levels of the specific fatty acid and of total fat. Among dietary saturated fatty acids, high levels of STE fed in nonrandomized (i.e., native) form have been associated with no change in HDL-cholesterol (although at least one study showed an increase in HDL-cholesterol) and with decreased triglyceride levels compared with a high-carbohydrate diet. Compared with a high-palmitate diet, feeding high levels of stearate in nonrandomized form resulted in lowering or conserving HDL-cholesterol without raising triglycerides. Considering two studies in which STE was fed in randomized (i.e., interesterified) form, one study found the high-stearate diet to lower HDL-cholesterol without changing triglycerides compared to a high-carbohydrate diet, whereas the other study reported no differences in HDL-cholesterol or

triglyceride levels compared with a high-carbohydrate diet. Thus, the effect of dietary STE on levels of blood lipids and lipoproteins appears to depend on both the level of STE in the diet and whether STE has been randomized on the dietary triglycerides.

## IS THERE A RELATIONSHIP BETWEEN THE STEREOSPECIFIC POSITION OF A FATTY ACID AND ITS BIOLOGICAL EFFECTS?

This literature review has attempted to evaluate whether the stereospecific position of a fatty acid can be related to its biological effects. Based on studies conducted by Kritchevsky *et al.* (28,29,31,32,34), only two fatty acids, palmitic acid and, to a lesser degree, STE, have been evaluated in this regard. Based on these studies, it appears that fats with increased levels of palmitic acid in the *sn*-2 position may be more atherogenic to rabbits than those with palmitic acid largely in the *sn*-1 and *sn*-3 positions. For instance, randomized cottonseed oil was found to be more atherogenic to rabbits than native cottonseed oil (32). On the other hand, the stereospecific positioning of palmitic acid appears to have little effect on blood lipid and lipoprotein levels, as indicated by studies that showed no significant differences in blood lipids and lipoproteins after feeding native compared to interesterified fats (28,29,31,32,34). In addition, enhancing the level of STE in the *sn*-2 position has not been found to affect either atherogenic potential or levels of blood lipids and lipoproteins (34). Thus, to date, palmitic acid is the only fatty acid I am aware of with stereospecific positioning related to the atherogenicity of the test fat.

Similar studies on atherogenicity have not been reported with the other recognized cholesterolemic saturated fatty acids, namely, lauric and myristic. Furthermore, different species may vary in their responsiveness to dietary palmitic acid in the *sn*-2 position, considering that rabbits have shown atherosclerotic susceptibility (28,29,31,32,34) but hamsters have not (Nicolosi, R.J., Wilson, T., and Lawton, C., personal communication).

Studies with human adults conducted to date have not shown significant effects on blood lipid parameters of interesterified fats compared with native fats. On the other hand, one study with human infants (43) reported higher levels of HDL-cholesterol in infants fed breast milk (large amount of palmitic acid at the *sn*-2 position) compared with infants fed formulas with much less palmitic acid at the *sn*-2 position. More work is needed in order to establish whether a relationship exists between the stereospecific position of a fatty acid and its biological effects. Also it should be noted that comparisons of individual human studies involving the feeding of specific fatty acids frequently are complicated by many variables in the ways the studies were conducted. Such variables include level of fat (or fatty acid) in the diet, total calorie level of the diet, duration of feeding period, age of subjects, whether subjects were hypercholesterolemic or normocholesterolemic, and whether all foods were provided or only test foods were provided.

## RECOMMENDATIONS FOR FURTHER RESEARCH

Two areas of further research are recommended on effects of dietary fatty acids as related to their position on triglycerides. The first is to answer the fundamental question of whether there is a difference in the metabolic clearance and turnover of various fatty acids at the *sn*-2 compared to *sn*-1 and *sn*-3 positions of triglycerides. The second is to determine the relative hypercholesterolemic and atherogenic effects of fatty acids at the *sn*-2 compared with the *sn*-1 and *sn*-3 positions.

*Metabolic clearance and turnover of fatty acids at* sn-*2 vs.* sn-*1 and* sn-*3 positions.* Human studies could be conducted in which triglycerides with various fatty acids at the *sn*-2 compared with the *sn*-1 and *sn*-3 positions are fed. A single study would focus on a single fatty acid. Parameters such as the uptake of labeled (e.g., with deuterium) fatty acids into chylomicrons and the transport and metabolism of the fatty acids could be followed. Such studies would help to clarify whether the triglyceride position of a specific dietary fatty acid affects its uptake and metabolism.

*Relative hypercholesterolemic and atherogenic effects of fatty acids at the* sn-*2 position compared with the* sn-*1 and* sn-*3 positions.* (i) One study could compare in humans the effects of feeding a synthetic fat in which a cholesterolemic fatty acid (e.g., palmitic acid) or a noncholesterolemic fatty acid (e.g., STE) is completely in the *sn*-1 and *sn*-3 positions (little or none in the *sn*-2 position) with one in which the fatty acid is completely in the *sn*-2 position (little or none in the *sn*-1 and *sn*-3 positions). Human studies conducted to date on effects of native vs. interesterified fats have found no significant differences in various blood lipid and lipoprotein parameters. One possible explanation for this lack of differences may have been insufficient exaggeration of the level of fatty acid in the *sn*-1, *sn*-2, and *sn*-3 positions. Such a study could be conducted at both high and low-to-moderate levels of total dietary fat.

(ii) Another study could evaluate whether the stereospecific structure of triglycerides affects additional parameters (beside blood lipid and lipoprotein levels) that could be related to the promotion of atherosclerosis. Such parameters could include cellular adhesion molecules and postprandial triglyceride clearance and composition.

(iii) A long-term primate feeding study could be conducted to determine whether changing the positions of fatty acids on the trigylcerides (e.g., by interesterification) alters the atherogenicity of the fat. Studies with rabbits fed interesterified fats frequently have found that atherogenicity increased as the level of palmitic acid in the *sn*-2 position increased. Similar long-term studies conducted with primates might be more predictive of human atherosclerosis risk.

(iv) The n-3 fatty acids could be studied to determine if they have different physiological effects if incorporated into randomized fats compared with nonrandomized fats. Nawar (12) has noted that the long-chain polyunsaturated fatty acids characteristic of marine oils are preferentially located at the *sn*-2 position. In contrast, certain commercial preparations of n-3 fatty acids may have these fatty acids randomized on the triglycerides. Randomization might decrease the degree of

absorption of these n-3 fatty acids and thus modify their lipidemic properties.

## SUMMARY AND CONCLUSIONS

Animal studies have shown that altering the stereospecific distribution of certain fatty acids on dietary triglycerides can impact various biological parameters. The changes in specific biological parameters are thought to be related to the enhanced absorption of the fatty acid in the *sn*-2 position of the dietary triglycerides. Specifically, enhancing the level of the saturated fatty acid palmitic acid in the *sn*-2 position of a triglyceride (e.g., by interesterification of the fat to randomize the positions of the fatty acids along the glycerol backbone) has been shown to increase the atherogenic potential in rabbits of the fat without significantly impacting the levels of blood lipids and lipoproteins. In contrast, enhancing the level of STE in the *sn*-2 position has been found not to affect either atherogenic potential or levels of blood lipids and lipoproteins in rabbits. A limited number of human studies have shown no significant effects of interesterified fats on blood lipid parameters. However, it is not known whether modifying the stereospecific structure of dietary triglycerides would affect atherogenicity or other long-term health conditions in humans. It is possible that incorporation of palmitic acid in the *sn*-2 position of milk fat is of benefit to the human infant (as a source of energy for growth and development) but not to human adults. Additional research is needed to determine whether processes like interesterification, which can be used to alter physical parameters of dietary fats (e.g., melting characteristics), may result in favorable or unfavorable long-term effects in humans.

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# Dietary Supplementation with Conjugated Linoleic Acid Increased Its Concentration in Human Peripheral Blood Mononuclear Cells, but Did Not Alter Their Function[1]

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**ABSTRACT:** The purpose of this study was to examine if conjugated linoleic acid (CLA) supplementation of diets would alter fatty acid (FA) composition and function of peripheral blood mononuclear cells (PBMC). Seventeen women, 20–41 yr, participated in a 93-d study conducted at the Metabolic Research Unit. The same diet (19, 30, and 51% energy from protein, fat, and carbohydrate, respectively) was fed to all subjects throughout the study. Seven subjects (control group) supplemented their diet with six daily capsules (1 g each) of placebo oil (sunflower) for 93 d. For the other 10 subjects (CLA group), the supplement was changed to an equivalent amount of Tonalin capsules for the last 63 d of the study. Tonalin provided 3.9 g/d of a mixture of CLA isomers (*trans*-10,*cis*-12, 22.6%; *cis*-11,*trans*-13, 23.6%; *cis*-9,*trans*-11, 17.6%; *trans*-8,*cis*-10, 16.6%; other isomers 19.6%), and 2.1 g/d of other FA. PBMC isolated on study days 30 and 90 were used to assess intracellular cytokines by flow cytometry, secreted cytokines, and eicosanoid by enzyme-linked immonosorbent assay, and FA composition by gas–liquid chromatography. After supplementation, total CLA concentration increased from 0.012 to 0.97% ($P < 0.0001$) in PBMC lipids, but it did not significantly alter the concentration of other FA. CLA supplementation did not alter the *in vitro* secretion of prostaglandin $E_2$, leukotriene $B_4$, interleukin-1β (IL-1β), or tumor necrosis factor α (TNFα) by PBMC simulated with lipopolysaccharide, and the secretion of IL-2 by PBMC stimulated with phytohemagglutinin. Nor did it alter the percentage T cells producing IL-2, interferon γ, and percentage of monocytes producing TNFα. The intracellular concentration of these cytokines was also not altered. None of the variables tested changed in the control group. Our results show that CLA supplementation increased its concentration in PBMC lipids, but did not alter their functions.

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Conjugated linoleic acid (CLA) is a mixture of 18:2 fatty acid isomers that have conjugated double bonds. One of the prominent isoforms of CLA, the 9-*cis* and 11-*trans* (9*c*,11*t*-18:2) isomer, is found naturally in beef and dairy products. This isomer has been commonly named rumenic acid, because it is formed by the microbes in the rumen from linoleic acid (18:2n-6) and *trans*-11-18:1 (1). Several other isomers of CLA are produced industrially during the processing of vegetable oils. The most abundant among these isomers include the 8*t*,10*c*-18:2, 10*t*,12*c*-18:2, and 11*c*,13*t*-18:2. In addition, there are several other minor isomers.

Trace amounts of different CLA isomers have been found in several human tissues, including adipose tissue, blood, milk, and bile (2–7). In animal models, the concentration of CLA in tissue (liver, heart, bone marrow, and spleen) lipids increased when the dietary intake of CLA was increased (8–12). Feeding diets containing CLA to animals also altered the production of prostaglandin $E_2$ ($PGE_2$), leukotriene $B_4$ ($LTB_4$), and cytokines by the splenocytes and peritoneal exudate cell (10,13–17).

The effects of dietary CLA on the fatty acid composition and functions of human or animal peripheral blood mononuclear cells (PBMC) have not been reported. The purpose of this study was to examine the effect of supplementing diets of healthy human adults with CLA on the fatty acid profile of the PBMC, and the *ex vivo* production of eicosanoids and cytokines. We examined the effect of feeding a mixture of CLA isomers on PBMC fatty acid profile, and *ex vivo* production of $PGE_2$, $LTB_4$, interleukin 2 (IL-2), interferon γ (IFN γ), and tumor necrosis factor α (TNF α). These indices were selected because CLA feeding in animal models altered these functions in splenocytes and peritoneal monocytes. Also they are relevant to other changes in immune functions reported in animal models (13–17). Supplementing diets of healthy women with a mixture of CLA isomers at 3.9 g/d for 63 d significantly increased the concentration of several CLA isomers in PBMC lipids, but it did not affect the production of eicosanoids and cytokines studied.

## METHODS AND MATERIALS

*Subjects and study design*. The study protocol was approved by the human use committees of the University of California, Davis, and the U.S. Department of Agriculture (USDA)

[1]Parts of data included here were published as abstracts for the Experimental Biology and AOCS 2001 meetings.

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Abbreviations: CD, cluster designation; CLA, conjugated linoleic acid; CY5, cyanin 5; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GC, gas chromatograph; Hu, human; IFN γ, interferon γ; IL, interleukin; LPS, lipopolysaccharide; $LTB_4$, leukotriene $B_4$; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PE, phycoerythrin; $PGE_2$, prostaglandin $E_2$; RDA, recommended daily allowance; TNFα, tumor necrosis factor α.

(Houston, TX). Healthy women ($n$ = 17) were selected to participate in two cohorts (9 and 8) of a 93-d metabolic research unit study after a physical and clinical evaluation by a physician. They were all nonsmokers and nondrug users and had body weights within 110–120% of the ideal body weight (1983, Metropolitan Life Insurance Co., New York, NY). The age, body weight, and body mass index (mean ± SEM) for the CLA and control groups were 27.0 ± 1.8 and 29.3 ± 2.6 yr, 63.1 ± 2.1 and 63.2 ± 4.3 kg, and 23.6 ± 0.5 and 21.9 ± 1.2, kg/m$^2$, respectively. The body weight of the subjects was maintained within 2% of the initial body weight throughout the study, by adjusting their caloric intake if necessary. All subjects lived at the metabolic suite of the Western Human Nutrition Research Center (San Francisco, CA) for the duration of the study, except when going for daily walks (2 miles, twice daily) or other scheduled outings. Subjects were under supervision when going out of the metabolic unit. They consumed only those foods prepared by the staff of the metabolic unit. For the first 30 d, all study participants were fed a stabilization diet supplemented with six capsules of a placebo (sunflower oil 6 g/d). For the next 63 d, subjects were divided into two groups; seven subjects remained on the placebo supplement for the entire period of the study (control group), while for the remaining 10 subjects, 6 g of Tonalin (providing 3.9 g/d of CLA isomers) replaced the placebo supplement. This CLA source was selected because it has been used in many animal studies, and the purified CLA isomers for human consumption were not available at the time the study was conducted. CLA concentration used in our study provided about 1.5% of the total energy intake and was comparable to the percentage energy from CLA in many animal studies.

The nutrient content of the diets was calculated using *USDA Handbook 8* (18); all known nutrients were at or above the recommended daily allowance (RDA) level, and were not different between the two diets. Diets contained 1 × RDA of vitamin E from natural foods and were supplemented with an additional 100 mg capsule of α-tocopherol (Bronson Pharmaceutical, St. Louis, MO) every 5 d. The proportions of energy from protein, fat, and carbohydrate in both diets were 19, 30, and 51%, respectively. Diets were fed with a 5-d rotating menu, comprised of three meals and a post-dinner snack. Specific menus were designed so that each of the saturated, monounsaturated, and polyunsaturated fats provided 10% of the total energy for both the control and intervention groups. Tonalin, a gift from Pharmanutrient, Inc. (Lake Bluff, IL), was the source of CLA. It was provided as capsules by replacing an equivalent amount of the placebo oil. Both Tonalin and placebo oil capsules were administered to the subjects before each meal (breakfast, lunch, dinner) under the supervision of the kitchen staff. CLA isomers made up 65 wt% of the total fatty acids present in Tonalin, while other fatty acids made up the remainder 35%. The major isomers of CLA present in Tonalin, expressed as percentage of total CLA were: *t*10,*c*12, 22.6%; *c*11,*t*13, 23.6%; *c*9,*t*11, 17.6%; *t*8,*c*10, 16.6%; other isomers 19.6%. Thus, the daily intake of different CLA isomers was: 881 mg of *t*10,*c*12; 920 mg of *c*11,*t*13; 686 mg of *c*9,*t*11; 647 mg of *t*8,*c*10; and 764 mg of other isomers. Placebo capsules were made from sunflower oil containing 72.6% linoleic acid and no detectable CLA. Other details regarding this study can be found in our previously published papers (19–22).

*Laboratory procedures*. Blood samples for PBMC fatty acid analysis were collected on study days 30 and 90, and for cytokine and eicosanoid production on study days 15, 22, 29, 78, 85, and 92. Samples were collected between 0700 and 0800, after a 12-h fast, by antecubital venipuncture into evacuated tubes without anticoagulants (for sera preparation) or containing heparin (all other uses).

*Isolation and culture of PBMC for cytokine and eicosanoid secretion.* PBMC were isolated using Histopaque-1077 as previously reported (23) and cultured with or without lipopolysaccharide (LPS, 1.0 mg/L) in 24-well flat-bottom culture plates ($5 \times 10^5$ PBMC/mL/well). The culture medium used was RPMI-1640, (Gibco, Grand Island, NY) containing 10% autologous serum and L-glutamine (2 mmol/L), penicillin (100 KU/L), streptomycin (100 mg/L), and gentamicin (20 mg/L). The tissue culture media were collected by centrifugation 24 h after stimulation with LPS and stored frozen at −70°C until the cytokine (IL-1β and TNFα) and eicosanoid ($PGE_2$ and $LTB_4$) concentrations were determined. For the stimulation of IL-2 synthesis, PBMC were cultured with PHA (10 mg/L) for 48 h, before collecting the cell culture media. These mitogen concentrations were found to cause maximal stimulation of cytokine production in our previous studies (23–25). Enzyme-linked immunosorbent assay (ELISA) kits for cytokine and eicosanoid assays were purchased from Cayman Chemical Company (Ann Arbor, MI).

*Intracellular cytokines*. The number of T cells producing IL-2 and INFγ was determined with a flow cytometer and reagents provided by Becton Dickinson (San Jose, CA) as previously reported (24). Briefly, the T lymphocytes were activated by mixing 500 µL sodium-heparin blood with 500 µL RPMI-1640, containing 10 µg brefeldin A, 25 ng phorbol 12-myristate 13-acetate (PMA), and 1 µg inomycin. After 4 h incubation at 37°C in 5% $CO_2$, the cells were recovered by centrifugation. The pellet was stained with either anti-human (Hu)-IL-2 PE or anti-Hu-IFNγ phycoerythrin (PE) and incubated for 30 min. The pellet was washed once with phosphate buffered saline (PBS) and resuspended in 1% paraformaldehyde. The number of IL-2 or IFNγ producing cells was analyzed with a flow cytometer by gating on the cluster designation (CD)3+ cells. The fluorescent geometric mean was also determined for these antigens. Isotype controls were run for both the stimulated and unstimulated control samples.

The number of monocytes producing TNFα was determined in a fashion similar to that used for the T cells producing IL-2, except that the cells were stimulated with LPS (1 µg/mL) instead of PMA and inomycin. Surface markers used for gating the monocytes were CD14 fluorescein isothiocyanate (FITC) and CD45 PE-CY5. After permeablization, anti-Hu-TNFα PE was added to the cell pellet. The number of cells expressing TNFα and the fluorescent geometric mean were determined.

In addition to determining the percentage of cells expressing specific cytokines, the amount of PE conjugated antibody bound per cell was determined using Becton Dickinson Phycoerythin Fluorescence Quantitation Kit. A standard curve was constructed plotting the log PE mean fluorescent intensity vs. the log of the number of PE molecules per bead. Assuming a 1:1 ratio of PE to monoclonal antibody, the antibody bound per cell was calculated by substituting the log fluorescent geometric mean of the PE-stained beads with the PE fluorescence expressed by the cells stained with the anti-Hu-TNFα PE antibody. The intracellular concentrations of cytokines are expressed as number of PE molecules/cell.

*PBMC isolation and fatty acid analysis*. The PBMC were isolated using Histopaque-1077 and purified as previously reported (23,24). Briefly, the isolated cells were washed with Dulbecco's PBS, resuspended in PBS, and layered over Histopaque-1077. The tubes were centrifuged to remove contaminating erythrocytes. PBMC were washed with PBS, mixed with LYMPHO-KWIK (One Lambda, Inc., Canoga Park, CA), and incubated for 15 min at 37°C. The PBMC were then overlaid with PBS and centrifuged. The cell pellet was washed with PBS and stored frozen at –20°C until fatty acid analysis. This isolation procedure removed most of contaminating platelets and erythrocytes and yielded cells containing 90–95% mononuclear cells as determined by differential cell counting.

Because of the limited sample size, the PBMC lipids were not extracted with chloroform/methanol, but were lyophilized and then transmethylated. In order to avoid isomerization of CLA isomers (26), the total lipids and CLA standards were transmethylated with 0.2 M sodium methylate (methoxide) for 10 min at 55°C followed by a reaction with 1 N hydrochloric acid in methanol for 15 min at 55°C (27). The fatty acid methyl esters (FAME) were extracted with hexane and purified by thin-layer chromatography as described elsewhere (28) before dilution and injection into the gas chromatograph (GC).

The FAME were analyzed by GC (model 6890; Hewlett-Packard, Palo Alto, CA) with a computer. An SP-2380 column (100 m × 0.25 mm i.d. × 0.2 mm film thickness; Supelco Inc., Bellefonte, PA) was used. The column was heated to 75°C for 4 min and then temperature-programmed at 13°C/min to 175°C, and held there for 27 min, followed by a second temperature program at 4°C/min to 215°C, and finally held there for 31 min. The total run time was 79.69 min. Fatty acids were identified by comparison of their retention times with authentic standards. If a GC peak was not clearly identified, then ion trap mass spectra were compared to mass spectra from NIST or mass spectra prepared in our laboratory (22).

*Data analysis*. The data from the two cohorts were combined for a repeated measure of analysis of variance using the SAS PROC MIXED procedure (29). Day, diet, and the interaction were considered the fixed effects, while cohort, diet × cohort, subjects within diet × cohort, and diet × cohort × day were the random effects. Period 1 (stabilization) vs. period 2 (intervention) × diet contrast was partitioned out of the day × diet effect. Pooled means ± SEM for the three measurements made at the ends of stabilization (days 15, 22, and 29) and intervention (days 78, 85, and 92) periods are shown in the Results section. For consistency with the fatty acid data, cytokines and eicosanoids data are also labeled as day 30 and day 90. Since none of the response variables in the control group changed from day 30 to day 90, data for this group are not shown. Paired *t*-test was used to evaluate the changes in the fatty acid composition data of PBMC. Changes in the parameters examined are considered significant for $P < 0.05$ unless stated otherwise.

## RESULTS

Both diets provided 19, 30, and 51% energy from protein, fat, and carbohydrates, respectively. The average daily caloric intake was approximately 2100 kcal (2117 ± 191, mean ± SD) and was not significantly different between the two groups. Fatty acid composition of the diets is shown in Table 1. Fatty acids other than CLA and linoleic acid were not significantly different between the two diets. The concentration of CLA in the control diet was below the detection limit, while the sum total of all CLA isomers in the CLA diet was 5.28% of the total fatty acids. The linoleic acid content of the control diet was about 5% higher than that in the CLA diet. This was because the placebo supplement contained sunflower oil with 73 wt% linoleic acid.

*Effect of CLA on fatty acid profile of PBMC*. The women in the CLA group received a placebo supplement until study day 30 and a CLA supplement from study day 31 to 93. The fatty acid profiles of the PBMC isolated on study day 30 and

**TABLE 1**
**Fatty Acid Composition (wt%) of Experimental Diets**

| Fatty acid | Diet without supplement[a] | Diet + placebo[a] | Diet + CLA[a] |
|---|---|---|---|
| 12:0 | 0.67 ± 0.20 | 0.70 ± 0.22 | 0.70 ± 0.21 |
| 14:0 | 3.29 ± 0.56 | 3.14 ± 0.62 | 3.11 ± 0.60 |
| 14:1n-7 | 0.18 ± 0.02 | 0.19 ± 0.02 | 0.18 ± 0.02 |
| 14:1n-5 | 0.32 ± 0.02 | 0.32 ± 0.03 | 0.30 ± 0.03 |
| 16:0 | 19.04 ± 0.50 | 18.19 ± 0.61 | 17.99 ± 0.65 |
| 16:1*t* | 0.13 ± 0.01 | | |
| 16:1n-9 | 1.14 ± 0.12 | 1.07 ± 0.11 | 1.06 ± 0.12 |
| 18:1n-7 DMA | 0.28 ± 0.00 | 0.26 ± 0.00 | 0.26 ± 0.00 |
| 18:0 | 8.24 ± 0.50 | 7.91 ± 0.47 | 7.77 ± 0.46 |
| 18:1*t*, all isomers | 5.23 ± 1.14 | 4.96 ± 1.12 | 4.58 ± 0.93 |
| 18:1n-9 | 25.13 ± 1.17 | 24.34 ± 1.20 | 25.23 ± 1.13 |
| 18:1n-7 | 1.24 ± 0.10 | 1.19 ± 0.10 | 1.21 ± 0.10 |
| 18:1n-5 | 0.98 ± 0.26 | 1.08 ± 0.17 | 1.06 ± 0.17 |
| 18:2*tt* | | | 0.25 ± 0.01 |
| 18:2n-6 | 29.85 ± 1.42 | 32.97 ± 1.15 | 27.56 ± 1.15 |
| 18:3n-3 | 1.83 ± 0.15 | 1.74 ± 0.13 | 1.71 ± 0.14 |
| 9*c*,11*t*- and 8*t*,10*c*-18:2 | 0.23 ± 0.00 | | 2.22 ± 0.31 |
| 11*c*,13*t*-18:2 | | | 1.34 ± 0.20 |
| 10*t*,12*c*-18:2 | | | 1.30 ± 0.20 |
| 9*t*,11*t*- and 10*t*,12*t*-18:2 | | | 0.44 ± 0.06 |
| 20:3n-6 | 0.25 ± 0.06 | 0.28 ± 0.07 | 0.30 ± 0.06 |
| 20:4n-6 | 0.19 ± 0.02 | 0.20 ± 0.00 | 0.20 ± 0.02 |
| 24:0 | 0.24 ± 0.00 | 0.25 ± 0.00 | 0.24 ± 0.00 |
| Unidentified | 1.31 ± 0.52 | 1.29 ± 0.59 | 1.14 ± 0.39 |

[a]Data are mean ± SEM, $n = 5$. DMA, dimethyl acetal; CLA, conjugated linoleic acid.

day 90 for this group are shown in Table 2; comparisons between CLA concentrations on study days 30 and 90 are pictorially shown in Figure 1. The concentrations of all other fatty acids, except CLA, remained unchanged between study days 30 and 90, while that of CLA increased significantly ($P <$ 0.0001; Table 2 and Fig. 1). In the PBMC isolated on day 30, there were only trace amounts of CLA, and the sum of all CLA isomers was 0.12 wt% of the total fatty acids. The CLA isomers that were present included CLA1(9*c*,11*t*) + CLA2 (8*t*,10*c*), CLA 3 (11*c*,13*t*), and CLA4 (10*t*,12*c*) at 0.05, 0.03, and 0.04 wt%, respectively. Total concentration of CLA in PBMC on study day 90 increased to 0.97 wt%, which was an eightfold increase over its concentration on study day 30. Concentration of CLA as well as that of the other fatty acids in the PBMC from the control group remained unchanged between study days 30 and 90 (not shown).

*Effect of CLA supplementation on* in vitro *eicosanoid and cytokine secretion*. Concentrations of $PGE_2$ secreted by the LPS-stimulated PBMC from the CLA group on day 30 and day 90 were 22.7 ± 3.2, and 20.5 ± 2.7 (mean ± SEM) ng/million cells, respectively; corresponding values for $LTB_4$ secretion were 6.5 ± 2.4 and 10.3 ± 3.2 pg/million cells. Concentrations of neither of the two eicosanoids were different between day 30 and day 90. Likewise, the concentrations of these eicosanoids did not change from day 30 to day 90 in the control group (not shown). The concentrations of both these eicosanoids in the unstimulated PBMC from both dietary groups were below the detection limit of the ELISA assays used.

The concentrations (ng/mL) of IL-1β secreted by monocytes in response to LPS stimulation for the CLA group on day 30 and day 90 were 2.47 ± 0.47, and 2.98 ± 0.55, respectively; corresponding values for TNFα were 1.45 ± 0.13, and 1.27 ± 0.17, respectively. IL-2 secreted into the medium by T cells stimulated with phytohemagglutinin on days 30 and 90 was 0.28 ± 0.09, and 0.28 ± 0.12, respectively. Without stimulation, the concentrations of these cytokines secreted were not detectable. Thus, mitogen treatment caused a several-fold increase in the concentrations of the cytokines secreted by the monocytes and T cells, when compared to the corresponding values in the unstimulated cultures. However, CLA supplementation did not alter the concentrations of these cytokines secreted after mitogen stimulation. Nor did these cytokine concentrations change from day 30 to 90 in the control group (data not shown).

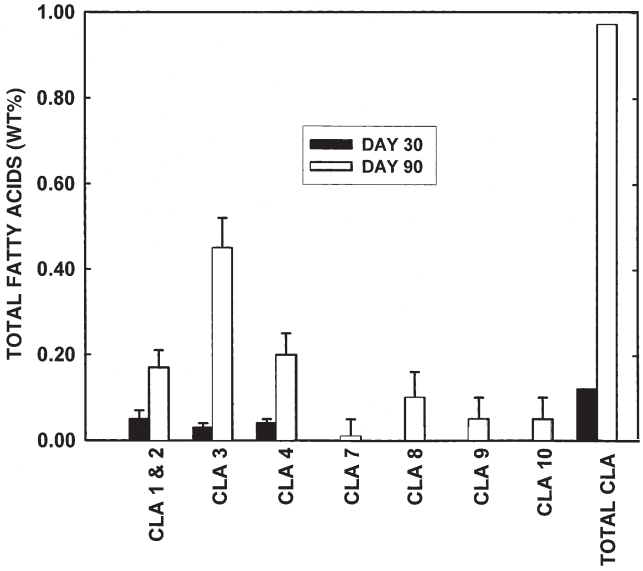


**FIG. 1.** Effect of conjugated linoleic acid (CLA) supplementation on CLA concentration of peripheral blood mononuclear cells. Data shown are the mean ± SEM ($n$ = 10) for study day 30 (end stabilization) and day 90 (end intervention). CLA supplementation significantly ($P < 0.05$) increased the concentration of all the CLA isomers. CLA 1 = 9*c*,11*t*; CLA 2 = 8*t*,10*c*; CLA 3 = 11*c*,13*t*; CLA 4 = 10*t*,12*c*; CLA 7 = 10*c*,12*c*; CLA 8 = 11*c*,13*c*; CLA 9 = 11*t*,13*t* + 8*t*,10*t*; CLA 10 = 9*t*,11*t* + 10*t*,12*t*.

**TABLE 2**
**Effect of CLA Supplementation on Fatty Acid Composition (wt%) of Peripheral Blood Mononuclear Cells[a]**

| Fatty acid | End stabilization (day 30) | End intervention (day 90) |
|---|---|---|
| 16:0 DMA | 2.22 ± 0.17 | 2.38 ± 0.06 |
| 16:0 | 16.35 ± 0.36 | 15.60 ± 0.36 |
| 18:0 DMA | 1.77 ± 0.12 | 2.10 ± 0.09 |
| 18:0 | 19.51 ± 0.33 | 19.23 ± 0.18 |
| 18:1n-9*t* | 1.37 ± 0.05 | 1.53 ± 0.09 |
| 18:1n-9 | 8.37 ± 0.18 | 8.73 ± 0.17 |
| 18:1n-7 | 2.51 ± 0.06 | 2.01 ± 0.07 |
| 9*c*,11*t*- + 8*t*,10*c*-18:2 | 0.05 ± 0.02 | 0.16 ± 0.01* |
| 11*c*,13*t*-18:2 | 0.03 ± 0.01 | 0.44 ± 0.02* |
| 10*t*,12*c*-18:2 | 0.04 ± 0.01 | 0.19 ± 0.01* |
| 11*c*,13*c*-18:2 | 0.00 ± 0.00 | 0.10 ± 0.01* |
| Other CLA | 0.00 ± 0.00 | 0.10 ± 0.01* |
| 18:2n-6 | 11.20 ± 0.23 | 11.12 ± 0.26 |
| 20:2n-6 | 1.12 ± 0.02 | 1.00 ± 0.03 |
| 20:3n-6 | 2.11 ± 0.16 | 1.95 ± 0.13 |
| 20:4n-6 | 19.99 ± 0.47 | 19.50 ± 0.51 |
| 24:1n-9 | 2.11 ± 0.14 | 2.22 ± 0.08 |
| 22:5n-3 | 1.79 ± 0.05 | 1.86 ± 0.07 |
| 22:6n-5 | 2.05 ± 0.14 | 2.00 ± 0.15 |
| Minors and unknowns | 7.41 | 7.78 |

[a]Data are mean ± SEM ($n$ = 10). *Significantly different between two periods ($P < 0.0001$). Minor fatty acids include those with concentration less than 1%, except CLA. For the control group, none of the fatty acids changed between days 30 and 90 (not shown). See Table 1 for abbreviations.

*Effect of CLA supplementation on the number of cells producing cyokines and their intracellular concentrations.* Percentages of T cells expressing IL-2 in the CLA group on days 30 and 90 were 40.3 ± 4.1 and 36.2 ± 4.4, respectively; corresponding values for IFNγ were 34.6 ± 4.2 and 26.5 ± 2.9, respectively. Percentages of monocytes expressing TNFα on day 30 and day 90 were 73.9 ± 3.9, and 78.9 ± 2.9, respectively. None of these percentages was different between day 30 and day 90, indicating that CLA did not alter the expression of these cytokines. Nor did CLA alter the intracellular concentrations of these cytokines, as determined by the PE molecules per cell (data not shown). On study day 30, the percentages of these cells that expressed TNFα, IL-2, and IFNγ were 73.9, 40.3, and 34.6, respectively. The corresponding values on study day 90 were 78.9, 36.2, and 26.5%, respectively. Thus, CLA supplementation did not alter the percentage of cells expressing these three cytokines. Nor did it alter the intracellular concentration of these cytokines as determined by the number of PE mole-

cules/cell, which was used to tag the specific monoclonal antibodies against these cytokines (data not shown). The percentage of cells expressing these cytokines and their intracellular concentrations did not change in the control group either between study day 30 or 90 (data not shown).

## DISCUSSION

CLA supplementation of the diets caused an eightfold increase (0.12 to 0.97 wt%) in its concentration in PBMC lipids. Although the concentrations of several CLA isomers increased, the largest increase occurred for the isomer 11*c*,13*t* (0.03 to 0.44%). This isomer represented 23.6% of all the CLA isomers in the diet but accounted for 45% of the total CLA in PBMC lipids. Since most of the health effects of CLA in animal models have been attributed to the 9*c*,11*t* and 10*t*,12*c* isomers, we do not know if this selective increase in 11*c*,13*t* isomer has any physiologic significance. We also do not know if this is due to increased transport or reduced metabolism of this isomer. Even if the concentration of CLA increased eightfold in PBMC lipids, it did not significantly reduce any of the other fatty acids in PBMC lipids.

In our study, CLA supplementation of the diets did not alter the *in vitro* production of $PGE_2$, $LTB_4$, IL1β, IL-2, and TNFα. Nor did it alter the intracellular concentrations of TNFα, IL-2, and IFNγ, or the number of PBMC positive for these cytokines. The lack of CLA effects on eicosanoid and cytokine production in our study is consistent with the lack of a change in the PBMC arachidonic acid, which is the precursor for these eicosanoids. Our results regarding $PGE_2$ production by the PBMC are consistent with those obtained with mice (14) and rat splenocytes (15), but are at variance with those obtained with rat peritoneal macrophages, where CLA feeding was reported to decrease $PGE_2$ production (10). Our results for $LTB_4$ production are at variance with others, who reported a decrease in its production by splenocytes from rats fed CLA-supplemented diets (15). The lack of an effect on IL-1β and TNFα production in our study is consistent with the results obtained with mice splenocytes (14) and rat peritoneal macrophages (10). CLA supplementation of the diets in our study did not alter the production of IL-2 and IFNγ. Our results regarding IL-2 production are not consistent with the increase reported in mice splenocytes (14,17).

We are not certain of the reasons for the difference in our results in humans with those of others in animal models. We had a small number of participants in our study, but based on our experience with the feeding of other fatty acids under metabolic unit conditions (23–25,30–32), the power to detect effects of CLA was more than adequate. Based on percentage energy from CLA, the amount we used was comparable to that used in animal studies. However, since animals eat several times more food per kg body weight than humans, the amount of CLA fed per day in our study was 5–10-fold less than that used in the animal studies. Considering that the average intake of CLA in the U.S. is only 100–200 mg/d (33), the amount of CLA used in our study was 20–40 times higher than the average daily intake. Amounts higher than those used in our study will also raise safety concerns. CLA supplementation in mice has been reported to cause an enlargement of livers and spleens, increase the circulating level of insulin, and decrease that of leptin (34,35). Most of the animal studies report that the CLA mixture used was an equal mixture of the *c*-9/*t*-11, and *t*-10/*c*-12 isomers, while in our study these two isomers represented only about 40% of the total CLA isomers. We believe the CLA mixture we supplemented was similar to those used in the animal studies; the discrepancy may be due to the analytical methods (27). Differences in species, cells studied, analytical methods, feeding regime, amount and the composition of CLA isomers, and duration of its feeding may have contributed to the disparate results. Inconsistencies exist not only between results from human and animal models but also between those obtained from different animal models.

In summary, our results show that CLA supplementation of human diets increased CLA concentration in PBMC lipids, but did not alter many of their functions investigated. These results, along with those previously published from this study that showed no change in a number of other indices of immune response, body composition, appetite, and blood lipids (19–22), do not indicate any health benefits to humans from feeding a mixture of CLA isomers. Future studies with purified isomers of CLA are needed to determine if there may be any health benefits of the individual isomers.

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# Fatty Acid Content of Plasma Lipids and Erythrocyte Phospholipids Are Altered Following Burn Injury

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**ABSTRACT:** The objective of this study was to examine compositional and quantitative changes in fatty acids of plasma components and red blood cell phospholipids (PL) immediately following and during recovery from burn injury. Subjects ($n$ = 10) with >10% total body surface area burn had blood drawn at specific timepoints (0 to >50 d) following burn injury. Fatty acid composition of red blood cell PL and plasma PL, cholesteryl esters (CE), and triglycerides was determined using gas–liquid chromatography after separating each fraction from extracted lipids by thin-layer chromatography. Total plasma PL and CE in burn patients were lower than in healthy control subjects with reduced 20:4n-6, n-6, and n-3 fatty acids and higher levels of monounsaturated and saturated fatty acids early after burn. CE levels remained half that of healthy control values up to 50 d post-burn. Red blood cell PL had decreased 20:4n-6 content and profiles similar to that of an essential fatty acid deficiency early after burn. These results suggest an impairment in lipoprotein and polyunsaturated fatty acid metabolism in the early post-burn period. Lower levels of 20:4n-6 and n-3 fatty acids in every plasma fraction suggest increased use of these fatty acids for wound healing and immune function following burn injury. Further work is needed to determine the ability of burn patients to utilize essential fatty acids in order to design nutritional intervention that promotes wound healing and immunological functions consistent with recovery in these patients.

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There are profound alterations in fatty acid metabolism following burn injury (1,2). Fatty acid oxidation rates, free fatty acid turnover, and lipolysis are elevated (3,4). In addition, plasma levels of 18:2n-6 (linoleic acid) and 20:4n-6 (arachidonic acid) are reduced (5) while saturated fatty acid (SFA) and oleic acid are increased, suggestive of an essential fatty acid deficiency (2,4). Diet is an important part of therapy for burn patients, and lipids must be included in the diet to provide energy, aid in the absorption of fat-soluble vitamins, and prevent essential fatty acid deficiency. Essential fatty acids are involved in wound healing (6,7) and serve important roles in immune functions (8). Dietary lipids have profound effects on immune functions and inflammatory processes through changing cellular membranes and altering substrate availability for the synthesis of lipid mediators such as prostaglandins (PG) (9–13) that may predispose patients to infection. Preventing infection and reducing inflammatory processes are important goals in the treatment of burn patients. Before diets designed to improve immune recovery in these patients can be formulated, an understanding about the metabolism of specific fatty acids, particularly the essential fatty acids involved in wound healing and immunological functions following burn injury, is needed. The objective of this study is to characterize quantitative and compositional changes in fatty acids in plasma components and red blood cells (RBC) immediately following and during recovery from burn injury.

## EXPERIMENTAL PROCEDURES

*Materials.* H-plates and G-plates were purchased from Analtech (Newark, DE). All solvents were purchased from VWR (Edmonton, Canada). Ficoll Hypaque gradients, bovine serum albumin (BSA) (Fraction V), chemicals for buffers, and all other lipid supplies, including standards, were purchased from Sigma Chemicals (St. Louis, MO).

*Subjects.* The burn study was approved by the University of Alberta Faculty of Medicine Research Ethics Board. The blood sampling on healthy subjects was approved by the Faculty of Agriculture, Forestry, and Home Economics Ethics Review Board. Subjects ($n$ = 10) were recruited from patients admitted to the Firefighter's Burn Treatment Unit at the University of Alberta Hospital (Edmonton, Canada). Patients who had >10% total body surface area (TBSA) burn and who gave informed consent were included in the study. Patients who were under the age of 18, overtly malnourished, had a history of alcohol or drug abuse, were taking immunosuppressive drugs, or had autoimmune disease were excluded. All subjects were previously healthy. Included in the study were 2 females and 8 males. Burns were a result of flame (8:10), hot tar (1:10), and electrical (1:10). Their age range was from 20 to 65 yr of age (mean = 40 ± 4 yr). The range of burn size was 12–90% [mean = 37 ± 5% TBSA]. Length of hospital stay ranged from 13 to 82 d (mean = 38 ± 9 d). All subjects survived their injuries. Subjects were resuscitated in Ringer's lactate (14) using the Parkland formula (15). All patients underwent wound excision and skin grafting of deep second- and third-degree wounds commenc-

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Abbreviations: BSA, bovine serum albumin; CE, cholesteryl ester; MCT, medium-chain triglycerides; MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; RBC, red blood cell; SFA, saturated fatty acid; TBSA, total body surface area; TG, triglyceride.

ing the first week of hospitalization. All subjects received similar wound-care treatment, analgesia, and antibiotic therapy using preestablished protocols.

Energy requirement for each subject was determined using the Harris Benedict equation with a stress factor based on injury severity. Continuous enteral feeding began within 24 h after injury and stopped when feeding complications persisted and 6 h prior to surgery. No patients received total parenteral nutrition. The enteral diet fed provided higher amounts of nitrogen and is especially designed for critical-care patients (Nitro-Pro™, Nutrition Medical, Minneapolis, MN). Nitro-Pro™ provided 5,188 kJ, 60 g protein (caseinates), 160 g carbohydrates (maltodextrin), 80% of the U.S. Department of Agriculture (USDA) recommended intakes for all minerals and water-soluble vitamins as well as 240% of the USDA recommended intake of vitamin C per 1000 mL formula. Pro-Mod™ (Ross Laboratories, Columbus, OH) was supplemented as an extra source of protein when required. The fat content of the diet was composed of half medium-chain triglycerides (MCT) and the other half corn oil for a total of 29% of total energy provided by fat. The fatty acid composition of the enteral diet fed is shown in Table 1. Regular blood draws on surgery days were taken the morning prior to surgery, thus avoiding the potential effects of blood or serum products provided during surgical procedures. A nonfasting blood sample was drawn from healthy volunteer subjects ($n$ = 8) recruited from the University of Alberta who gave informed consent.

*Sample collection.* A nonfasting venous blood sample (10 mL) was collected by the medical staff during the patient's regular blood work on the following timepoints after admission to the burn unit: within the first 12 d [(timepoint (t) 1], between 12 and 19 d (t2), between 20 and 35 d (t3), between 36 and 49 d (t4), and after 50 d (t5). For subjects with smaller burns or those who were discharged prior to 50 d, the final sample was obtained at the subject's first out-patient visit to clinic. When more than one sample was obtained within a time period, the results were combined and the mean reported.

*Serum collection.* Whole blood (3 mL) was layered on top of Ficoll Hypaque gradients and centrifuged as previously described (16). Serum was collected from the top and stored immediately at −70°C for plasma lipid analysis. After gradients were removed, the remaining RBC were washed once with Krebs-Ringer HEPES buffer with BSA (5 g/L). RBC were frozen immediately at −70°C for fatty acid analysis of phospholipids (PL).

*Fatty acid analysis*. A modified Folch method was used to extract lipids from RBC as previously described (17). Serum (500 µL) fatty acids were extracted using chloroform/methanol (2:1, vol/vol) as previously described (18). Individual PL from RBC and plasma were separated on H-plates (19), and serum lipid classes [PL, triglycerides (TG), and cholesteryl esters (CE)] were separated on G-plates as previously described (18). Bands corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) from RBC, and the PC, PE, and PI fractions from serum PL classes were visualized with 8-anilino-1-naphthalene-sulfonic acid and identified under ultraviolet light using appropriate standards. Bands were scraped and directly methylated. Internal standards were added to the TG and CE (20 µg of 15:0) bands, and they were saponified and methylated as previously described . The 17:0 standard was added to the scraped serum PL (10 µg of 17:0) followed by direct methylation.

Fatty acid methyl esters were prepared by methylation using 14% (wt/vol) $BF_3$/methanol reagent and separated by automated gas–liquid chromatography (Vista 6010; Varian Instruments, Georgetown, Canada) on a fused-silica BP20 capillary column (25 m × 0.25 mm internal diameter; Varian Instruments), as previously described (17). Peaks of fatty acid methyl esters were identified by comparisons with standards purchased from Supelco Canada and Sigma Chemical companies. Fatty acid contents of serum lipid classes were calculated using the area peak of the internal standard. Total PL, CE, TG, and their respective contents of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), as well as the essential fatty acids and their elongation/desaturation products, were calculated on both a quantitative and percentage basis. Percentage fatty acids in RBC PL and major PL in serum were determined.

*Statistical analysis.* Data are reported as means ± SEM. Subjects were grouped into groups based on size of burn (large = >35%, $n$ = 5 and small = <35%, $n$ = 5), and when differences existed between these two groups, results are presented. When there were no differences between the two burn injury groups, all subjects were included in the analysis and the overall mean was presented. To determine differences between post-burn timepoints, a repeated measures analysis of variance was used to identify differences in fatty acids in each plasma fraction at the five post-burn time periods described above. The numbers of subjects sampled at each timepoint were as follows: t1, $n$ = 10; t2, $n$ = 10; t3, $n$ = 10; t4, $n$ = 8; t5, $n$ = 8. Subjects with burns >35% TBSA were sampled at each timepoint. When significant differences were identified, differences between time periods were identified using least square means. A Student's $t$-test was used to identify differences between post-burn timepoints and control values. All statistical analyses were conducted using the SAS statistical package (Version 6.12; SAS Institute, Cary, NC).

**TABLE 1**
**Fatty Acid Composition of Nitro-Pro™[a]**

| Fatty acid | g/1000 mL | Fatty acid | g/1000 mL |
|---|---|---|---|
| 8:0 | 14.62 | 18:0 | 0.42 |
| 10:0 | 5.76 | 18:1 | 4.64 |
| 12:0 | 0.21 | 18:2 | 11.58 |
| 14:0 | 0.02 | 18:3 | 0.32 |
| 16:0 | 2.29 | 20:0 | 0.07 |
| 17:0 | 0.02 | 22:0 | 0.02 |

[a]The clinical diet fed was composed of half medium-chain triglycerides and the other half corn oil for a total of 29% of total energy coming from fat. Mean linoleic and linolenic acid consumption per day coming from this diet was 24 ± 1 and 0.7 ± 0.1 g/d, respectively.

## RESULTS

*Dietary intake*. Continuous enteral feeds began within 24 h after injury and were generally well tolerated. Complications included diarrhea (2:10 subjects), emesis (2:10 subjects), and hyponatemia (1:10 subjects). Enteral feeds were stopped when feeding complications persisted and 6 h prior to surgery. Subjects consumed an average of 9,132 kJ/d during the in-patient period when enteral feeding provided the primary source of energy. The average intake of linoleic acid (18:2n-6) during the in-patient period was 24 ± 1 g/d (903 kJ/d, 10% energy), which approximates the intake of healthy, free-living North Americans who are estimated to consume 3–18% energy from linoleic acid (20). Mean linolenic acid (18:3n-3) consumption per day from the enteral feed diet was estimated at 0.7 ± 0.1 g/d. The enteral diet used did not contain long-chain n-3 fatty acids.

*Plasma total PL.* Percentages of 20:4n-6 and total n-6 in plasma PL were significantly lower at t1 compared to t5 (Table 2). Although not different on a relative percentage basis, quantitatively, the n-3 fatty acid content was lower at t1 (14.7 ± 1.9 μg/mL) and t2 (17.5 ± 2.2 μg/mL) than at t5 (25.1 ± 2.9 μg/mL; $P < 0.05$). The total percentage of MUFA in plasma PL was higher at t1 compared to t3, t4, and t5 (Table 2). The mean concentration of 20:3n-9 in plasma PL of the burn patients was 1.7 ± 1% (0.4–4.1%) and 0.1 ± 0.04% (undetectable–0.2%) in healthy subjects. The plasma concentration of 20:3n-9 did not differ significantly between post-burn timepoints. Compared to healthy control subjects, the relative proportion of total n-6 fatty acids was lower and SFA higher at all post-burn timepoints (Table 2). At t1 and t2, plasma PL exhibited lower percentage of 20:4n-6 and higher proportion of MUFA, compared to healthy control subjects. There were no significant differences in total plasma PL concentration between post-burn timepoints; however, at t1 and t2, the total plasma PL concentration was significantly less ($P < 0.03$) than healthy controls (Table 2).

*Major PL fractions*. There was no significant difference in the 20:4n-6 content in the major plasma PL fractions (PC = 6.7 ± 0.9%; PE = 14.8 ± 1.8%; PI = 16.9 ± 1.6%) between post-burn timepoints; however, PE and PI fractions contained less 20:4n-6 than control subjects (PE = 20.2 ± 2.2%; PI = 22.9 ± 1.9%; $n = 6$) at t1 and t2, and t1–t3 post-burn timepoints, respectively (data not illustrated). SFA content of the PI and PE fractions was significantly greater ($P < 0.03$) at t1 and t3 (PI = 47.5 ± 1.3%; PE = 40.9 ± 1.4%) as compared to control subjects (PI = 41.3 ± 2.3%; PE = 34.3 ± 1.5%). At all post-burn timepoints, PC and PE contained less percentage n-3 fatty acids than controls (Table 3).

*CE.* Proportions of 20:4n-6 and total n-6 fatty acids in plasma CE were significantly ($P < 0.04$) lower at t1 and t2 compared to t5 (Table 4). CE isolated at t5 contained significantly ($P < 0.03$) more n-3 fatty acids than any other post-burn timepoint (Table 4). The percentage of SFA was higher ($P < 0.04$) at t1 and t2 compared to t5. The mean concentration of 20:3n-9 in plasma CE was 0.7 ± 0.4% (ranging from undetectable levels to 1%) and that of healthy controls was 0.3 ± 0.1%. On a percentage basis, CE isolated from burn patients at t1, t2, and t3 contained significantly less ($P < 0.03$) n-6 fatty acids than control subjects (Table 4). The percentage SFA was higher than that of control subjects at sample points t1–t4. At every post-burn timepoint there was a lower concentration of CE in plasma and significantly ($P < 0.01$) lower amounts of 20:4n-6 (23.04 ± 4.1 μg/mL), n-6 (208.84 ± 26.4 μg/mL), and n-3 (5.75 ± 0.69 μg/mL) fatty acids compared to control values (data not illustrated).

*TG*. The total percentage n-6 fatty acids in plasma TG was lower at t1 than at t4 and t5 (Table 5). Total n-3 fatty acid content was lower ($P < 0.05$) at t2 compared to t5. The average concentration of 20:3n-9 in plasma TG of the patients was 1.3 ± 0.6% (ranging from undetectable levels to 5%) and did not differ significantly between post-burn timepoints. The level of 20:3n-9 in healthy controls was 0.2 ± 0.02%. Quantitatively, 20:4n-6 content at t1 (4.5 ± 0.9 μg/mL) was less ($P < 0.03$) than that of control subjects (8.5 ± 1.4 μg/mL). The n-3 fatty acid content at the first four timepoints (overall mean = 6.1 ± 0.6 μg/mL) was half that of control subjects (15.5 ± 2.6 μg/mL, data not illustrated, $P < 0.01$).

**TABLE 2**
**Fatty Acid Analysis of Plasma PL Following Burn Injury[a]**

| Post-burn time | 20:4n-6 | Σn-6 | Σn-3 | Σ SFA | Σ MUFA | Total PL (μg/mL)[c] |
|---|---|---|---|---|---|---|
| | | | (% of total fatty acids)[b] | | | |
| t1 | 6.9 ± 0.5[a,*] | 27.8 ± 0.9[a,*] | 3.8 ± 0.3 | 49.0 ± 0.5[a,*] | 18.7 ± 0.7[a,*] | 432.4 ± 39.0* |
| t2 | 7.4 ± 0.5[a,b,*] | 30.1 ± 1.0[a,b,*] | 3.5 ± 0.3 | 49.2 ± 0.5[a,b,*] | 16.6 ± 0.8[a,b,*] | 470.4 ± 46.1* |
| t3 | 8.3 ± 0.7[a,b] | 30.3 ± 1.3[a,b,*] | 3.6 ± 0.4 | 50.5 ± 0.7[a,b,*] | 15.5 ± 1.0[b] | 569.9 ± 61.1 |
| t4 | 8.2 ± 0.8[a,b] | 28.9 ± 1.5[a,b,*] | 3.6 ± 0.4 | 51.7 ± 0.8[b,*] | 15.2 ± 1.2[b] | 515.0 ± 69.1 |
| t5 | 8.7 ± 0.7[b] | 31.5 ± 1.4[b,*] | 3.6 ± 0.4 | 50.7 ± 0.7[b,*] | 14.2 ± 1.0[b] | 551.5 ± 61.5 |
| Reference | 9.1 ± 0.6 | 35.6 ± 1.2 | 3.8 ± 0.3 | 45.6 ± 1.0 | 14.1 ± 0.9 | 684.8 ± 56.1 |

[a]Values are means ± SEM (for $n$ see "Statistical analysis" in the Experimental Procedures section). Means within a column not sharing common superscript roman letters are significantly ($P < 0.05$) different. *$P < 0.05$ vs. reference (healthy control subjects; $n = 8$).
[b]Fatty acid composition of plasma phospholipids (PL) at specific timepoints after burn injury was determined using gas–liquid chromatography and is expressed as percentage of total fatty acids.
[c]Total plasma PL were calculated using 17:0 (10 μg) as the standard and are expressed as μg/mL of plasma. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids.

**TABLE 3**
**The n-6 and n-3 Content in PC, PE, and PI of Plasma Following Burn Injury[a]**

| Post-burn time | PC | | PE | | PI | |
|---|---|---|---|---|---|---|
| | ∑n-6 | ∑n-3 | ∑n-6 | ∑n-3 | ∑n-6 | ∑n-3 |
| | (% of total fatty acids)[b] | | | | | |
| t1 | 31.3 ± 1.7[a,b] | 3.1 ± 0.4* | 31.4 ± 1.7 | 11.1 ± 1.0* | 31.1 ± 1.2[a,*] | 5.6 ± 0.6[a] |
| t2 | 30.2 ± 1.8[a,b] | 3.8 ± 0.4* | 33.9 ± 1.8 | 11.8 ± 1.1* | 35.8 ± 1.1[b] | 3.3 ± 0.6[b] |
| t3 | 27.0 ± 1.9[a] | 2.9 ± 0.5* | 33.5 ± 2.1 | 9.9 ± 1.2* | 35.3 ± 1.4[b] | 3.9 ± 0.7[a,b] |
| t4 | 34.5 ± 2.7[b] | 2.3 ± 0.6* | 31.5 ± 2.6 | 10.5 ± 1.5* | 38.9 ± 2.1[b] | 3.2 ± 1.1[a,b] |
| t5 | 35.4 ± 2.1[b] | 3.3 ± 0.5* | 34.8 ± 2.3 | 9.1 ± 1.3* | 34.9 ± 1.6[a,b] | 5.2 ± 0.9[a,b] |
| Reference | 31.4 ± 5.3 | 6.8 ± 1.2 | 35.7 ± 2.5 | 15.9 ± 1.5 | 35.6 ± 1.8 | 4.8 ± 0.9 |

[a]Values are means ± SEM (for *n* see "Statistical analysis" in the Experimental Procedures section). Means within a column not sharing common superscript roman letters are significantly ($P < 0.05$) different. *$P < 0.05$ compared to reference values (healthy control subjects; $n = 6$).
[b]Fatty acid composition of major plasma PL fractions at specific timepoints post-burn was determined using gas–liquid chromatography and is expressed as percentage of total fatty acids. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol. See Table 2 for other abbreviation.

**TABLE 4**
**Fatty Acid Analysis of CE in Plasma Following Burn Injury[a]**

| Post-burn time | 20:4n-6 | ∑n-6 | ∑n-3 | ∑ SFA | ∑ MUFA | Total (μg/mL)[c] |
|---|---|---|---|---|---|---|
| | (% of total fatty acids)[b] | | | | | |
| t1 | 4.5 ± 0.4[a] | 45.4 ± 1.9a* | 1.3 ± 0.1[a] | 24.8 ± 1.3a* | 28.1 ± 1.2* | 336.4 ± 28.5[a,*] |
| t2 | 4.8 ± 0.4[a] | 48.1 ± 2.0[a,b,*] | 1.3 ± 0.1[a] | 24.1 ± 1.4[a,*] | 27.0 ± 1.3* | 393.5 ± 30.5[a,b,*] |
| t3 | 5.4 ± 0.5[a,b] | 47.5 ± 2.6[a,b,*] | 1.2 ± 0.1[a] | 22.8 ± 1.9[a,b,*] | 27.5 ± 1.7* | 443.1 ± 39.6[b,*] |
| t4 | 6.0 ± 0.7[a,b] | 51.4 ± 3.3[a,b] | 1.1 ± 0.1[a] | 21.6 ± 2.3[a,b,*] | 24.8 ± 2.1 | 414.8 ± 49.9[a,b,*] |
| t5 | 6.7 ± 0.6[b] | 53.0 ± 2.9[b] | 1.7 ± 0.1[b] | 18.6 ± 2.1[b] | 25.9 ± 1.9 | 495.6 ± 44.3[b,*] |
| Reference | 5.3 ± 0.5 | 57.6 ± 2.0 | 1.2 ± 0.1 | 16.6 ± 1.7 | 22.0 ± 1.4 | 818.5 ± 40.9 |

[a]Values are means ± SEM (for *n* see "Statistical analysis" in the Experimental Procedures section). Means within a column not sharing common superscript roman letters are significantly ($P < 0.05$) different. *$P < 0.05$ compared to reference values (healthy control subjects; $n = 8$).
[b]Fatty acid composition of plasma CE at specific timepoints post-burn was determined using gas–liquid chromatography and is expressed as percentage of total fatty acids.
[c]Total plasma CE were calculated using 15:0 (20 μg) as the standard and are expressed as μg/mL of plasma. CE, cholesteryl esters. See Table 2 for other abbreviations.

*Erythrocyte PL.* The fatty acid content of major n-6 and n-3 fatty acids for PC and PE, the major PL of RBC, during the post-burn period are depicted in Tables 6 and 7, respectively, and reference ranges of healthy adults reported in the literature are shown. In the PC (Table 6), PS (data not illustrated), and PI (data not illustrated) PL fractions of RBC, the relative percentage of 20:4n-6 was significantly ($P < 0.03$) lower at t1 than at t5. Every RBC PL fraction exhibited half the levels of n-3 fatty acids of reference values at all post-burn timepoints (data shown for PC and PE only in Tables 6 and 7). The fatty

**TABLE 5**
**TG in Plasma Following Burn Injury[a]**

| Post-burn time | 20:4n-6 | ∑n-6 | ∑n-3 | ∑ SFA | ∑ MUFA | Total (μg/mL)[c] |
|---|---|---|---|---|---|---|
| | (% of total fatty acids)[b] | | | | | |
| t1 | 1.2 ± 0.2 | 15.5 ± 1.0[a] | 1.3 ± 0.1[a,b] | 34.0 ± 1.0 | 45.8 ± 1.5 | 493.6 ± 53.5* |
| t2 | 1.2 ± 0.2 | 17.0 ± 1.1[a,b] | 1.1 ± 0.2[a] | 36.1 ± 1.1* | 44.9 ± 1.6 | 547.2 ± 58.2 |
| t3 | 1.0 ± 0.3 | 17.3 ± 1.4[a,b] | 1.3 ± 0.2[a,b] | 37.1 ± 1.4 | 44.5 ± 2.1 | 515.2 ± 70.0 |
| t4 | 1.4 ± 0.3 | 21.1 ± 1.8[b] | 1.3 ± 0.3[a,b] | 35.8 ± 1.8 | 41.4 ± 2.7 | 468.2 ± 88.3* |
| t5 | 1.3 ± 0.3 | 19.8 ± 1.6[b] | 1.7 ± 0.2[b] | 35.0 ± 1.6 | 44.0 ± 2.4 | 481.7 ± 78.4* |
| Reference | 1.1 ± 0.2 | 18.2 ± 1.4 | 1.7 ± 0.3 | 31.3 ± 1.6 | 44.8 ± 1.9 | 682.7 ± 65.6 |

[a]Values are means ± SEM (for *n* see "Statistical analysis" in the Experimental Procedures section). Means within a column not sharing common superscript roman letters are significantly ($P < 0.05$) different. *$P < 0.05$ compared to reference values (healthy control subjects; $n = 8$).
[b]Fatty acid composition of plasma triglycerides (TG) at specific timepoints post-burn was determined using gas–liquid chromatography and is expressed as percentage of total fatty acids.
[c]Total plasma TG were calculated using 15:0 (20 μg) as the standard and are expressed as μg/mL of plasma. See Table 2 for other abbreviations.

**TABLE 6**
**Fatty Acid Subsets of RBC PC Following Burn Injury[a]**

| Post-burn time | 18:2n-6 | 20:3n-6 | 20:4n-6 | 18:3n-3 | 20:5n-3 | 22:6n-3 | ∑n-6 | ∑n-3 |
|---|---|---|---|---|---|---|---|---|
| | | | | (% of total fatty acids)[b] | | | | |
| t1 | 17.9 ± 0.7[a] | 2.1 ± 0.2[a] | 2.2 ± 0.4[a] | 0.18 ± 0.02 | 0.2 ± 0.1 | 0.5 ± 0.1 | 21.6 ± 1.2[a,b] | 1.0 ± 0.1[a] |
| t2 | 15.1 ± 0.7[b] | 1.4 ± 0.2[b] | 3.2 ± 0.3[a,b] | 0.17 ± 0.02 | 0.2 ± 0.1 | 0.6 ± 0.1 | 24.2 ± 1.0[a] | 1.2 ± 1.2[a,b] |
| t3 | 17.5 ± 0.7[a] | 1.9 ± 0.2[a,b] | 3.2 ± 0.3[a,b] | 0.23 ± 0.02 | 0.2 ± 0.1 | 0.6 ± 0.9 | 23.0 ± 1.1[a,b] | 1.3 ± 0.1[a,b] |
| t4 | 17.5 ± 1.0[a,b] | 1.6 ± 0.3[a,b] | 2.8 ± 0.5[a,b] | 0.13 ± 0.03 | 0.1 ± 0.1 | 0.3 ± 0.1 | 19.8 ± 1.7[b] | 1.1 ± 0.2[a,b] |
| t5 | 16.2 ± 0.9[a,b] | 1.5 ± 0.3[a,b] | 4.0 ± 0.4[b] | 0.21 ± 0.03 | 0.3 ± 0.1 | 0.7 ± 0.1 | 23.9 ± 1.5[a,b] | 1.6 ± 0.2[b] |
| Reference[c,d] | 15.7–25.4 | 2.0–2.6 | 5.8–11.3 | 0–0.3 | 0.4–1.8 | 1.6–3.7 | 29.6–37.0 | 5.0–7.6 |

[a]Values are means ± SEM (for *n* see "Statistical analysis" in the Experimental Procedures section). Means within a column not sharing common superscript roman letters are significantly ($P < 0.05$) different.
[b]Fatty acid composition of red blood cell (RBC) at specific timepoints post-burn was determined using gas–liquid chromatography and is expressed as percentage of total fatty acids.
[c]Innis (47).
[d]Aagren *et al.* (48). See Tables 2 and 3 for other abbreviations.

acid, 20:3n-9 comprised <0.5% each RBC PL fraction, and the proportion of this fatty acid did not change significantly in any RBC fraction during the post-burn period (data not shown).

## DISCUSSION

The results of this study support the hypothesis that essential fatty acid metabolism is altered following burn injury. This study confirms other reports of reduced PL and CE levels following burn injury (5,21–25) and expands existing knowledge by demonstrating that n-6, particularly 20:4n-6, and n-3 fatty acids are reduced in the early post-burn period in plasma and erythrocytes. Reduced amounts of essential fatty acids in plasma suggest decreased availability from the diet or an increase in their utilization by the tissues.

A diet high in linoleic acid (18:2n-6) in healthy subjects would be expected to increase n-6 fatty acids in plasma (20). However, patients in this study exhibited lower n-6 and 20:4n-6 content early after burn injury when 18:2n-6 intake from the enteral diet was high. Despite the return of total plasma PL concentrations to values not different from healthy controls by t3, the content and relative percentage of n-6 and total PUFA in plasma remained low throughout the study. Low levels of 18:2n-6 and 20:4n-6 in total plasma lipid have previously been reported following burn injury (26), and this report confirms that this occurs in each of the lipid fractions that constitute the total plasma lipid pool. Low levels of 20:4n-6 in plasma PL and CE may suggest increased utilization of 20:4n-6 during the early post-burn period. Prostaglandins (PG) derived from 20:4n-6 have been reported to be increased post-burn (27,28) and have been implicated in immunosuppression associated with burn injury (29,30). Fatty acids in plasma have also been reported to occur in immune cell membranes (20) and would potentially affect PG synthesis and wound healing (31).

The n-3 content of each plasma fraction was reduced in the early post-burn period and in some cases persisted even 50 d after the initial injury. Reductions in the content of n-3 fatty acids observed in plasma TG (primarily from chylomicrons and very low density lipoprotein fractions) suggest reduced availability to tissues. The n-3 fatty acids are important in balancing the production of cyclooxygenase products derived from 20:4n-6 (32) and have also been demonstrated to reduce inflammatory responses (33–35). Increased SFA and MUFA were observed in all plasma fractions, which is consistent with increased lipolysis in these patients (36,37).

Mead acid (20:3n-9) is rarely produced, and its presence

**TABLE 7**
**Fatty Acid Subsets of RBC PE Following Burn Injury[a]**

| Post-burn time | 18:2n-6 | 20:3n-6 | 20:4n-6 | 18:3n-3 | 20:5n-3 | 22:6n-3 | ∑n-6 | ∑n-3 |
|---|---|---|---|---|---|---|---|---|
| | | | | (% of total fatty acids)[b] | | | | |
| t1 | 7.1 ± 0.3 | 1.0 ± 0.1[a] | 13.2 ± 1.6[a] | 0.5 ± 0.1 | 0.6 ± 0.1 | 1.8 ± 0.3[a,d] | 26.8 ± 1.9[a] | 4.5 ± 0.7[a] |
| t2 | 7.4 ± 0.4 | 1.2 ± 0.1[a,b] | 15.4 ± 1.3[a,b] | 0.5 ± 0.1 | 0.6 ± 0.1 | 2.5 ± 0.3[a,b] | 28.8 ± 1.6[a,b] | 5.6 ± 0.6[a,b] |
| t3 | 7.0 ± 0.3 | 1.5 ± 0.1[b] | 17.9 ± 1.3[b] | 0.6 ± 0.1 | 0.6 ± 0.1 | 2.7 ± 0.3[b,c,e] | 32.7 ± 1.6[b] | 5.9 ± 0.6[a,b] |
| t4 | 8.0 ± 0.5 | 1.3 ± 0.2[a,b] | 11.9 ± 2.0[a] | 0.4 ± 0.1 | 0.3 ± 0.2 | 1.4 ± 0.4[d] | 24.0 ± 2.5[a] | 4.2 ± 0.9[a] |
| t5 | 7.3 ± 0.4 | 1.4 ± 0.2[b] | 17.8 ± 1.8[b] | 0.5 ± 0.1 | 0.7 ± 0.1 | 3.5 ± 0.3[e] | 29.7 ± 2.2[a,b] | 6.8 ± 0.8[b] |
| Reference[c,d] | 6.6–10.0 | 1.0–2.1 | 23.8–25.8 | 0.2–0.3 | 1.6–2.9 | 7.2–12.6 | 36.8–42.4 | 14.9–20.8 |

[a]Values are means ± SEM (for *n* see "Statistical analysis" in the Experimental Procedures section). Means within a column not sharing common superscript roman letters are significantly ($P < 0.05$) different.
[b]Fatty acid composition of RBC at specific timepoints post-burn was determined using gas–liquid chromatography and is expressed as percentage of total fatty acids.
[c]Innis (47).
[d]Aagren *et al.* (48). See Tables 2, 3, and 6 for other abbreviations.

in plasma is believed to be an indicator of essential fatty acid deficiency. Although there appeared to be higher levels, compared to the healthy subjects, of this fatty acid in the plasma of patients early after burn injury, the levels did not change significantly with recovery from burn injury. This is suggestive of essential fatty acid deficiency, at least in some patients after burn injury. However, elevated MUFA and SFA levels and reduced PUFA levels, particularly 20:4n-6, is a more consistent observation in this patient group. Clearly, metabolism is altered, and the observations reported in this study are likely a combined result of changes in absorption (38), assimilation (22,39–42), oxidation (26), and transport (43) of essential fatty acids that are reported to occur after burn injury.

Quantitative and compositional differences between patients and control subjects in this study suggest impairments in fatty acid assimilation and/or lipoprotein metabolism. It is unknown if the decrease in plasma components hinders utilization of essential fatty acids by tissues or if there is increased tissue utilization causing depletion in the plasma. Lipoproteins differ in CE, TG, and PL constituents (44). Severe reductions in plasma concentrations of CE and PL early after burn observed in this study and supported by others (5,21–25) suggest a change in the relative proportions of the individual lipoprotein fractions or an overall reduction in their synthesis. Unfortunately, the isolation of lipoprotein fractions was not done in the present study. However, concentrations of low density lipoprotein and high density lipoprotein have been reported to be reduced following burn injury (22,25), which would proportionately reduce cholesterol and PL levels. Deficiencies in intestinal lipases (38), lipoprotein carrier proteins (43), carnitine (26), and liver functions (22,39–42) have been reported following burn injury and might contribute to altered lipid metabolism and the compositional and quantitative changes observed in the plasma lipid components in this study.

The fatty acid composition of RBC is frequently used as a means to estimate the composition of dietary fat and abnormalities in essential fatty acid metabolism (45,46). It is likely that a sufficient amount of 18:2n-6 was provided by the diet, as 18:2n-6 levels were relatively constant throughout the study and were similar to control values with the exception of a transient decrease at t2 in the PC fraction. In spite of this, all RBC PL fractions exhibited lower 20:4n-6 levels than reference values throughout the post-burn period (47,48). These observations are inconsistent with Diboune *et al.* (49), who observed increases in 18:2n-6 at 14 d and no change in 20:4n-6 levels when intensive-care patients were provided an enteral diet with similar MCT and linoleic acid content as the enteral diet provided in this study. Despite dietary intake consisting of higher amounts of PUFA than MUFA and SFA (apart from the rapidly oxidized MCT), RBC exhibited higher SFA and MUFA than PUFA early after burn and compared to reference values in their PL. These findings parallel the observations in the various plasma components. Low concentrations of 20:4n-6, 22:6n-3, and 18:2n-6 with high levels of SFA have been reported in total RBC lipids following burn injury (5). In the current study, 22:6n-3 levels remained lower than healthy controls at all post-burn timepoints. If subjects in this study were provided adequate amounts of 18:2n-6, slow conversion to 20:4n-6 is a possible explanation of low 20:4n-6 levels observed in all measured RBC PL fractions and plasma components. Reduced Δ-6-desaturase activity is supported by the observations of low levels of 22:5n-3, 22:6n-3, and 20:3n-6 at t1 compared to t5 (and controls) even though 18:3n-3 content of RBC was similar to healthy controls and exhibited little change throughout the post-burn period. This pathway is reduced by a large essential fatty acid intake (50–52) and with burn injury (53). Several aspects of RBC have been reported to be altered following burn injury (54–57), some of which are known to be affected by PL composition of the membrane (58,59).

This study demonstrates essential fatty acid metabolism to be altered in both plasma and RBC following burn injury and points to deficiencies in key enzymes involved in regulation of lipid metabolism. Every lipid-containing plasma component isolated exhibited lower n-6 and n-3 fatty acid content and higher SFA and MUFA content in early post-burn timepoints compared to later post-burn timepoints and those of control subjects. Decreased essential fatty acids in plasma may be suggestive of the increased use of these lipids in the synthesis of membrane lipids for wound healing and immune functions. Achieving the optimal balance of fatty acids for skin regeneration, immune competence, and inflammatory processes through provision of specific fatty acids through diet is a complex issue. Further work is needed to determine the ability of burn patients to utilize essential fatty acids and to design nutritional intervention that supports optimal wound healing and immunological functions consistent with recovery in these patients.

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# Regulation of Intestinal Apolipoprotein A-I Synthesis by Dietary Phosphatidylcholine in Newborn Swine

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**ABSTRACT:** Phospholipid (PL) from both dietary sources and biliary secretions may be important in the regulation of intestinal apolipoprotein (apo) synthesis. We previously demonstrated the up-regulation of apo A-I secretion by phosphatidylcholine (PC) in a newborn piglet intestinal epithelial cell line. We hypothesized that dietary PC increases small intestinal apo A-I synthesis *in vivo* in the newborn piglet. Two-day-old female swine were fed by gavage for 48 h. Diets consisted of a formula containing 51% of calories as triacylglycerol providing 180 kcal/kg/24 h. The experimental group (+PC, $n = 7$) received 1 g/L added soybean PC, and the control group (–PC, $n = 7$) received no added PC. At the end of the study period, jejunal apo A-I, B, and A-IV synthesis was measured, and apo A-I mRNA levels were quantitated. Jejunal mucosal PL content and serum lipids and apo B and A-I levels were measured. Jejunal apo A-I synthesis was almost twice as high in the +PC group as compared to the –PC group with no difference in apo A-I mRNA levels. Jejunal content of PL was higher in the +PC group than in the –PC group. There were no differences in jejunal apo B and A-IV synthesis or serum levels of lipids and apo-lipoproteins between the two groups. Dietary PC supplementation in newborn swine up-regulated jejunal apo A-I synthesis. Apo A-IV synthesis, which is sensitive to fatty acid flux, was not significantly increased, which suggests a specific effect of PC on apo A-I synthesis. Lumenal PC may be important in the regulation of intestinal apo A-I synthesis in the neonate.

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Phospholipid (PL), specifically phosphatidylcholine (PC), has been shown to be important in intestinal chylomicron assembly and secretion in the adult rat, probably by providing a polar lipid surface coat for the lipoprotein particle (1–3). PC from both the diet and biliary secretions may also be important in the regulation of intestinal lipoprotein synthesis and secretion in the neonatal mammal, which is dependent on a high-fat breast milk diet to support rapid growth and development. In addition to regulating lipoprotein synthesis and secretion, lumenal PC may also be important in the regulation of apolipoprotein (apo) synthesis and secretion. Apo-lipoprotein are peptides that bind to the surface of lipoprotein particles and are crucial for the assembly, secretion, and subsequent metabolism of the particles (4). Apo A-I is the major apo-lipoprotein of plasma high density lipoproteins (HDL), as well as a major protein component of intestinal chylomicrons and very low density lipoproteins (VLDL), and is produced by both liver and intestine in the human, rat, and swine (5–9). Its major metabolic role is that of a cofactor for lecithin:cholesterol acyltransferase, the enzyme responsible for the production of HDL cholesteryl ester by the transfer of a fatty acid from PC to free cholesterol in HDL (10). We previously demonstrated up-regulation of apo A-I secretion by PC *in vitro* in a newborn piglet intestinal epithelial cell line (IPEC-1) (11). The present *in vivo* study was undertaken to determine if dietary supplementation with PC would regulate intestinal apo-lipoprotein synthesis in *vivo* in newborn swine, a model for the human infant.

## EXPERIMENTAL PROCEDURES

*Animals.* Two-day-old female domestic swine were obtained from Tyson Farms (Plummerville, AR). The animals were suckled by the sow during the first 2 d of life and were housed in groups of three to four in heated stalls with straw bedding. The experimental protocol was approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee.

*Diets and feeding.* Animals were bolus-fed by gavage using an 8 French soft plastic feeding tube (Biosearch Medical Products, Somerville, NJ) five times per day for 48 h. Diets consisted of a fat-free formula base (Sowena; Merrick, Madison, WI) blended with lipids to contain 51% of calories as fat, 27% as carbohydrate, and 22% as protein, providing 180 kcal/kg/24 h. Of total lipid calories, 45% were from palm oil, 20% from soybean oil, 20% from coconut oil, and 15% from safflower oil. The experimental group (+PC, $n = 7$) received 1 g/L added refined soybean PC (1% free fatty acid content; ICN Pharmaceuticals, Inc., Costa Mesa, CA), and the control group (–PC, $n = 7$) received no added PC.

*Procurement of samples for determination of intestinal apolipoprotein synthesis.* At the end of the experimental feeding period, 2 h after the final feeding, the animals were anesthetized,

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Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PL, phospholipid; TCA, trichloroacetic acid.

and a 10-cm segment of proximal jejunum was isolated 10 cm distal to the ligament of Treitz by two ligatures. Radiolabeling was performed by instilling 1.0 mCi of L-[4,5-$^3$H]leucine (>120 Ci/mmol; Amersham, Arlington Heights, IL) into the segment. The segment was removed 9 min later and prepared for immunoprecipitation as described next. This labeling time has been shown to be optimal in similar experiments in the adult rat, and we used it previously in the piglet (6,8,9,12–14). A distal adjacent segment was snap frozen in liquid $N_2$ for later RNA extraction.

*Preparation of mucosal cytosolic supernatants for immunoprecipitation.* Radiolabeled intestinal segments were flushed with 50 mL of iced phosphate-buffered saline (PBS) (50 mmol/L phosphate, 100 mmol/L NaCl, pH 7.4)–20 mmol/L leucine, and the mucosa was scraped and homogenized on ice in 1 mL of PBS/1% Triton X-100/2 mmol/L leucine/1 mmol/L phenylmethylsulfonyl fluoride/1 mmol/L benzamidine, pH 7.4, as previously described (8). Aliquots of the homogenate were taken for measurement of trichloroacetic acid (TCA)-precipitable radioactivity. The remainder was pelleted at 105,000 × *g* for 65 min in a 50.3 Ti rotor (Beckman Instruments, Palo Alto, CA), followed by collection of the cytosolic supernatant. Although most intracellular apo-lipoprotein is membrane-associated, this technique was shown previously to result in extraction and solubilization of 84–94% of total recoverable apolipoprotein mass with no discernible effect of the state of lipid flux (12). All procedures were performed at 0–5°C, and the mucosal supernatant samples were stored at –80°C until analysis.

*Apo-lipoprotein immunoprecipitation.* Intestinal cytosolic supernatant fractions were subjected to specific immunoprecipitation of apo B, A-I, and A-IV under conditions of antibody excess as described (9,15). Aliquots of cytosolic supernatants were pre- incubated with washed IgG-SORB$^R$ (The Enzyme Center, Malden, MA) and subsequently reacted with excess rabbit polyclonal anti-swine apo-lipoprotein antiserum for 18 h at 4°C. Following a second addition of IgG-SORB$^R$ and extensive washing, the liberated immunocomplex was applied to either 5.6% (apo A-I, A-IV) or 4% (apo B) sodium dodecyl sulfate-polyacrylamide tube gels. After electrophoresis, gels were sliced into 2-mm slices and solubilized in Solvable$^R$ (DuPont, Boston, MA) at 50°C for 3 h, followed by the addition of Ultima Gold$^R$ scintillation fluid (Packard, Meriden, CT) and incubation overnight at room temperature. Liquid scintillation counting was performed in a Packard Model 2000 liquid scintillation counter (Packard, Downers Grove, IL). Apo-lipoprotein species were identified by comparison to stained co-electrophoresed apo. Apo-lipoprotein synthesis rates were expressed as the percentage of specific immunoprecipitated apoprotein counts as compared to total protein TCA-precipitable counts. Apo-lipoprotein synthesis was thereby expressed as a percentage of total protein synthesis.

*Apolipoprotein A-I mRNA quantitation.* Total RNA was extracted from the frozen jejunal samples by the method of Chomcyznski and Sacchi (16). Intactness of each RNA preparation was verified by agarose gel electrophoresis and visualization of ribosomal RNA subunits. RNA (10 µg) was applied to nitrocellulose filters using a slot blot apparatus (Hoefer, San Francisco, CA). Filters were serially hybridized with a swine apo A-I cDNA (17) and a murine β-actin cDNA (provided by Dr. L. Kedes, Stanford University, CA). Hybridization signals were quantitated using a Bio-Rad Model GS-525 Molecular Imager System and associated software (Bio-Rad, Hercules, CA). Apo A-I mRNA abundance was expressed as apo A-I mRNA/β-actin mRNA signal intensity ratios.

*Phospholipid quantitation.* Proximal jejunal mucosal homogenates and serum samples were subjected to lipid extraction followed by PL quantitation by the method of Bartlett (18).

*Serum lipid and apo-lipoprotein mass determination.* Serum total cholesterol, triacylglycerol, and HDL-cholesterol concentrations were measured using enzymatic assays (Sigma Chemical Co., St. Louis, MO). Serum apo B and A-I concentrations were measured by enzyme-linked immunosorbant assays as previously described (9).

*Statistics.* Student's unpaired *t*-test was used to compare the results from the control and PC-fed animals. Statistical significance was set at a two-tailed *P* value of <0.05.

## RESULTS

Figure 1 shows the PL content of jejunal mucosa from the two groups of animals. The group receiving PC added to their formula had almost twice as much PL in proximal jejunum as compared to the control group.

Jejunal synthesis of apo B, A-I, and A-IV in the two groups of animals is shown in Figure 2. There was no difference in apo B or A-IV synthesis between the two groups. However, jejunal apo A-I synthesis in the PC-supplemented animals was approximately twice that of the control group.

To determine whether the increase in jejunal apo A-I synthesis in the PC-supplemented group was accompanied by a concomitant increase in apo A-I mRNA levels, a slot blot hybridization assay was performed using β-actin as a control

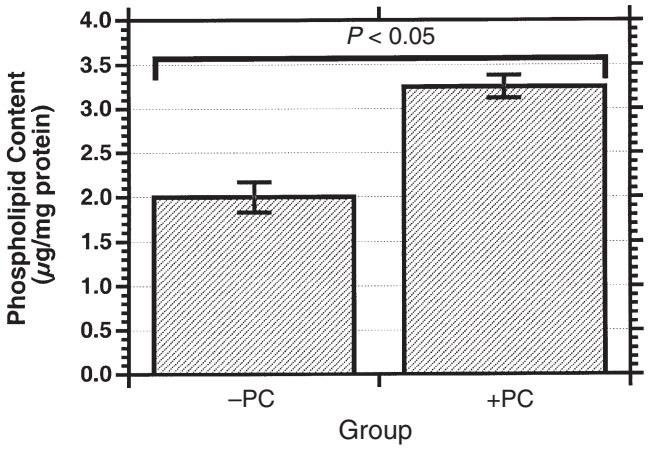


**FIG. 1.** Jejunal phospholipid content expressed as µg phospholipid/mg total mucosal protein in control animals (–PC group, *n* = 7) and animals receiving supplemental dietary phosphatidylcholine (+PC group, *n* =7). Bars represent the mean ± SEM of data from each group. The *P* values were determined using Student's unpaired *t*-test.

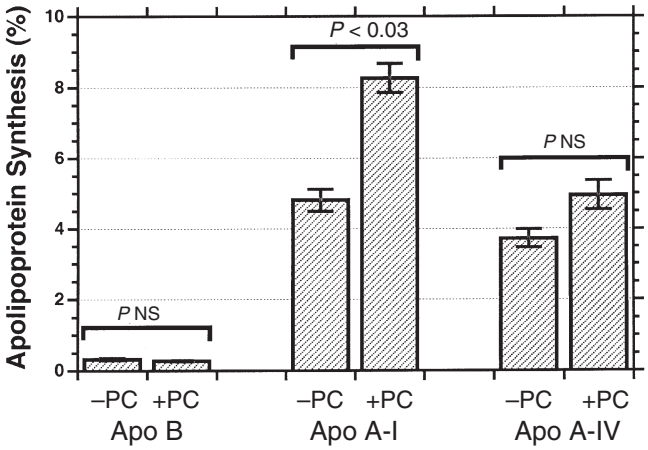


**FIG. 2.** Effect of dietary phosphatidylcholine supplementation on jejunal apolipoprotein (apo) synthesis expressed as a percentage of total protein synthesis in control animals (–PC group, $n = 7$) and animals receiving supplemental dietary phosphatidylcholine (+PC group, $n = 7$). Bars represent the mean ± SEM of data from each group. The $P$ values were determined using Student's unpaired $t$-test. NS, not significant.

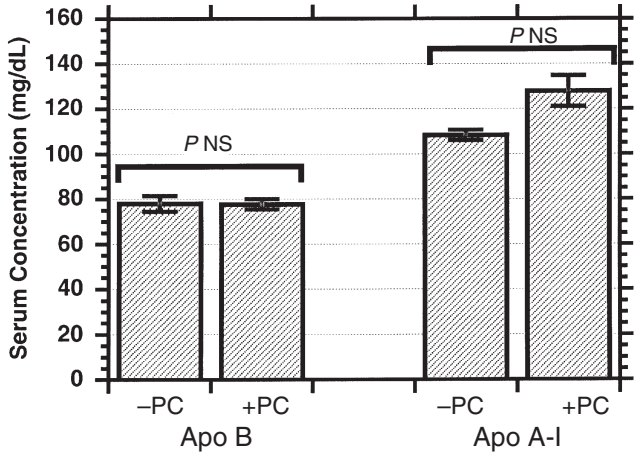


**FIG. 4.** Effect of dietary PC supplementation on serum apo B and A-I concentrations determined by enzyme-linked immunosorbent assays in control animals (–PC group, $n = 7$) and animals receiving supplemental dietary phosphatidylcholine (+PC group, $n = 7$). Bars represent the mean ± SEM of data from each group. The $P$ values were determined using Student's unpaired $t$-test. For abbreviations see Figures 1 and 2.

gene. Results are shown in Figure 3. There were no differences in apo A-I mRNA abundance between the two groups, which suggests that regulation occurs at the translational level.

Apo B and A-I levels were measured in serum samples taken from animals at the time of intestine harvest and euthanasia (Fig. 4). There were no differences in apo B levels. Apo A-I and HDL-cholesterol levels (Fig. 5) were slightly higher in the PC-supplemented group, but the differences did not reach statistical significance. No differences were noted between the two groups in serum total cholesterol, triacylglycerol, or PL (Fig. 5).

## DISCUSSION

In the present study, we report the up-regulation of apo A-I synthesis in newborn swine small intestine by dietary PC supplementation, as compared to a group of animals receiving the identical diet without added PC. This study was prompted by a previous study in a newborn swine intestinal epithelial cell line, IPEC-1, which demonstrated an increase in basolateral apo A-I secretion after incubation with PC in the apical culture medium compartment (11). In these cell culture studies, the regulation of apo A-I secretion appeared to occur at the posttranslational level, as compared to the present *in vivo* study, which demonstrates up-regulation of apo A-I expression at the translational level. The difference in the mechanism of apo A-I regulation (posttranslational vs. translational) between the *in vitro* and *in vivo* studies may be attributable to the timing of the exposure of the intestinal cells to the PC (24 h in

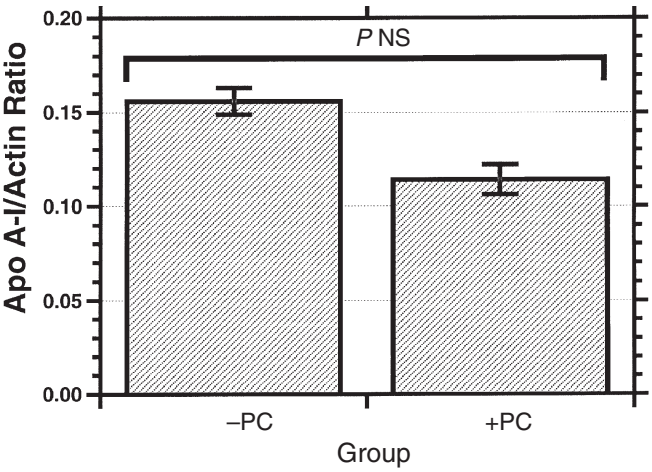


**FIG. 3.** Jejunal apo A-I mRNA abundance expressed as apo A-I to β-actin ratios determined by slot blot hybridization in control animals (–PC group, $n = 7$) and animals receiving supplemental dietary phosphatidylcholine (+PC group, $n = 7$). Bars represent the mean ± SEM of data from each group. The $P$ value was determined using Student's unpaired $t$-test. For abbreviations see Figures 1 and 2.

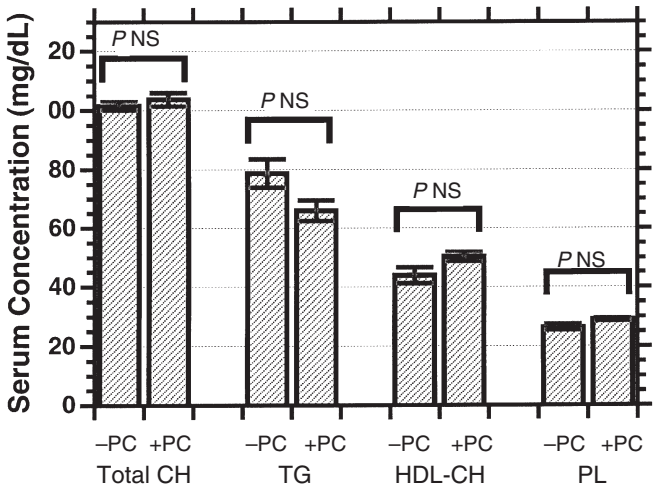


**FIG. 5.** Effect of dietary PC supplementation on serum total cholesterol (CH), triacylglycerol (TG), high density lipoprotein (HDL)-CH, and phospholipid (PL) concentrations in control animals (–PC group, $n = 7$) and animals receiving supplemental dietary PC (+PC group, $n = 7$). Bars represent the mean ± SEM of data from each group. The $P$ values were determined using Student's unpaired $t$-test. For other abbreviations see Figures 1 and 2.

the IPEC-1 cells studies and 48 h in the present piglet feeding study) or intrinsic differences in the model systems. Interestingly, the regulation of apo A-I secretion in IPEC-1 cells appeared to occur through a novel mechanism, which did not require the hydrolysis of PC to lysophosphatidylcholine or cellular uptake of either species, as verified using radiolabeled PC (11). Although developmental deficiency of phospholipase $A_2$, leading to the presence of significant amounts of intact lumenal PC, might exist in newborn swine in the present study, most of the lumenal PC was most likely digested to lysophosphatidylcholine and taken up by the enterocytes. Although this issue was not directly addressed in the present *in vivo* study, we did measure intestinal mucosal PL mass in jejunum of both experimental groups of animals at the end of the study and found a more than 1.5-fold increase in mucosal PL content in the group receiving PC supplementation. This increased mass of PL may have been present within the enterocyte and/or as a component of the cell membrane. This increased PL content may represent both exogenously and endogenously derived PL. Interestingly, in our previous studies in IPEC-1 cells, we observed that apical incubation with intact PC resulted in a striking up-regulation of cellular PL synthesis without uptake of the PC molecule (11). Although the mucosa was rinsed thoroughly at the time of harvest, it is also possible that at least a portion of the additional PL represents intact PC adherent to the brush border membrane.

Another issue is whether the increase in jejunal apo A-I synthesis with PC supplementation might be due to absorption of the fatty acid component of the PC molecule. We previously showed that 18-carbon unsaturated fatty acids can acutely upregulate apo A-I synthesis in newborn piglet jejunum (9). Soybean PC contains predominantly unsaturated fatty acids (18:1, 22%; 18:2, 54%; 18:3, 8%) (19). The added PC contributes approximately 2% to the total lipid mass in the experimental formula and represents an additional 1.8 kcal/kg/d to the total daily caloric intake (180 kcal/kg/d in the –PC group and 181.8 kcal/kg/d in the +PC group). If accounted for by differences in fatty acid content alone, it is doubtful that such a small difference in total fat caloric intake would make such a large difference in jejunal apo A-I synthesis. Furthermore, we previously showed that apo A-IV expression is a sensitive marker for intestinal epithelial fatty acid flux in newborn swine jejunum with a sevenfold increase in synthesis with a high-triacylglycerol diet containing predominantly polyunsaturated fatty acids, as compared to a very low-triacylglycerol diet (13). In a similar study with the same dietary composition and amount, jejunal apo A-I synthesis increased twofold in the animals receiving the high-triacylglycerol diet, as compared to the group receiving the low-triacylglycerol diet (9). In the present study, PC supplementation did not result in a significant increase in apo A-IV synthesis in the face of an almost twofold increase in apo A-I synthesis. This finding suggests that the increase in apo A-I synthesis is a specific effect of the PC molecule itself, rather than the esterified fatty acids.

Lumenal PC results in more efficient processing of absorbed fatty acids and monoglycerides that are reesterified by the monoglyceride pathway, resulting in greater transport of dietary lipid into lymph as chylomicrons (20–23). One possible mechanism for this effect is that the supplemental PC may improve the efficiency of fatty acid absorption by making more PL available to serve as a polar surface coat for nascent chylomicrons. Biliary PC is preferentially used, as compared to dietary PC, for this purpose (24). However, in the adult rat, lumenal PC seems to be inefficiently incorporated into chylomicrons, and enterocyte *de novo* PC synthesis cannot fully compensate for the lack of adequate PC for chylomicron assembly when the absorbed lipid load is high (3). Ultimately, adequate PC is not available to support maximal chylomicron triacylglycerol output unless there is dietary supplementation of PC (3). Therefore, in the present study, the efficiency of dietary lipid absorption may have been improved by the supplemental dietary PC, resulting in increased lipid absorption and resultant up-regulation of apo A-I synthesis. However, if this effect were of sufficient magnitude to increase apo A-I synthesis, it would also have been expected to result in significantly increased apo A-IV synthesis. Again, these findings suggest a specific effect of the supplemental PC on apo A-I synthesis.

Proximal intestinal synthesis of apo A-I increased almost twofold with PC supplementation. Also, serum levels of apo A-I and the lipoprotein associated with apo A-I, HDL, were slightly higher in the +PC group, but the differences did not reach statistical significance. This may be due to the fact that the serum levels of apo A-I are the net result of both synthesis and clearance from the plasma compartment. Therefore, the increased entry of apo A-I into the plasma compartment may be accompanied by increased clearance, resulting in no net significant change in the serum concentration.

Supplementation of a high-fat formula with soybean PC at a concentration of 1 g/L over a 48-h period in newborn swine resulted in an approximately twofold increase in jejunal apo A-I synthesis without a significant increase in apo A-I mRNA levels. The cellular mechanism of regulation of apo A-I translation by lumenal PC in the newborn enterocyte is currently under investigation in our laboratory. This effect of supplemental dietary PC may be beneficial to the newborn mammal.

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# Studies of Phospholipid Metabolism, Proliferation, and Secretion of Stably Transfected Insulinoma Cells That Overexpress Group VIA Phospholipase $A_2$

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**ABSTRACT:** A cytosolic 84 kDa Group VIA phospholipase $A_2$ ($iPLA_2\beta$) that does not require $Ca^{2+}$ for catalysis was cloned from Chinese hamster ovary (CHO) cells, murine P388D1 cells, pancreatic islet β-cells, and other sources. Proposed $iPLA_2\beta$ functions include participation in phosphatidylcholine (PC) homeostasis by degrading excess PC generated in CHO cells that overexpress CTP:phosphocholine cytidylyltransferase (CT), which catalyzes the rate-limiting step in PC biosynthesis; participation in biosynthesis of arachidonate-containing PC species in P388D1 cells by generating lysophosphatidylcholine (LPC) acceptors for arachidonate incorporation; and participation in signaling events in insulin secretion from islet β-cells. To further examine $iPLA_2\beta$ functions in β-cells, we prepared stably transfected INS-1 insulinoma cell lines that overexpress $iPLA_2\beta$ activity eightfold compared to parental INS-1 cells or to INS-1 cells transfected with an empty retroviral vector that did not contain $iPLA_2\beta$ cDNA. The $iPLA_2\beta$-overexpressing cells exhibit a twofold increase in CT activity compared to parental cells but little change in rates of [$^3$H]choline incorporation into or disappearance from PC. Electrospray ionization (ESI) tandem mass spectrometric measurements indicate that $iPLA_2\beta$-overexpressing cells have 1.5-fold higher LPC levels than parental INS-1 cells but do not exhibit increased rates of [$^3$H]arachidonate incorporation into phospholipids, and incorporation is unaffected by a bromoenol lactone (BEL) suicide substrate inhibitor of $iPLA_2\beta$. The rate of appearance of arachidonate-containing phosphatidylethanolamine species visualized by ESI mass spectrometry is also similar in $iPLA_2\beta$-overexpressing and parental INS-1 cells incubated with supplemental arachidonic acid, and this process is unaffected by BEL. Compared to parental INS-1 cells, $iPLA_2\beta$-overexpressing cells proliferate more rapidly and exhibit amplified insulin secretory responses to a protein kinase C-activating phorbol ester, glucose, and a cAMP analog. These findings suggest that $iPLA_2\beta$ plays a signaling role in β-cells that differs from housekeeping functions in PC biosynthesis and degradation in P388D1 and CHO cells.

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Phospholipases $A_2$ ($PLA_2$) catalyze hydrolysis of the *sn*-2 fatty acid substituent from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid (1–7). $PLA_2$ are a diverse group of enzymes, and the first well-characterized members have low molecular weights (*ca.* 14 kDa), require millimolar [$Ca^{2+}$] for catalytic activity, and function as extracellular secreted enzymes designated $sPLA_2$ (3,6). The first $PLA_2$ to be cloned that is active at $Ca^{2+}$ concentrations that can be achieved in the cytoplasm of living cells is an 85-kDa protein classified as a Group IV $PLA_2$ and designated $cPLA_2$ (3,5). This enzyme is induced to associate with its substrates in membranes by rises in cytosolic $Ca^{2+}$ concentrations in the submicromolar range, is regulated by phosphorylation, and prefers substrates with *sn*-2 arachidonoyl residues (5).

A second cytosolic $PLA_2$ has been cloned (8–10) that does not require $Ca^{2+}$ for catalysis; it is classified as a Group VIA $PLA_2$ and has been designated $iPLA_2$ (3,4). The $iPLA_2$ enzymes cloned from hamster (8), mouse (9), and rat (10) cells represent species homologs, and all are 84 kDa proteins containing 752 amino acid residues with highly homologous sequences. Each contains a GXSXG lipase consensus motif and eight stretches of a repeating motif homologous to a repetitive motif in the integral membrane protein-binding domain of ankyrin (8–10). Each of these $iPLA_2$ enzymes is susceptible to inhibition (8–10) by a bromoenol lactone (BEL) suicide substrate (11,12) that is not an effective inhibitor of $sPLA_2$ or $cPLA_2$ enzymes at comparable concentrations (4,11–14). It has been proposed that this enzyme now be designated $iPLA_2\beta$ to distinguish it from a membrane-associated, $Ca^{2+}$-independent $PLA_2$ that contains a peroxisomal targeting sequence and is designated $iPLA_2\gamma$ (15,16).

Proposed functions for $iPLA_2\beta$ include housekeeping roles in phospholipid metabolism. The first such role involves generation of lysophospholipid acceptors for incorporation of arachidonic acid into phosphatidylcholine (PC) of murine P388D1 macrophage-like cells (4,17,18). This proposal (4)

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Abbreviations. BEL, bromoenol lactone suicide substrate; BSA, bovine serum albumin; CAD, collisionally activated dissociation; CHO, Chinese hamster ovary; $cPLA_2$, Group IV phospholipase $A_2$; CT,CTP:phosphocholine cytidylyltransferase; dBcAMP, dibutyryl cyclic AMP; dpm, disintegration per minute; ESI, electrospray ionization; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; $iPLA_2\beta$, Group VIA phospholipase $A_2$; KRB, Krebs-Ringer bicarbonate buffer; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NP-HPLC, normal-phase high-performance liquid chromatography; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; $PLA_2$, phospholipase $A_2$; PMA, phorbol myristate acetate; $sPLA_2$, secretory phospholipase $A_2$; TLC, thin-layer chromatography.

derives from experiments involving inhibition of $iPLA_2$ activity in P388D1 cells with BEL (17) or with an antisense oligonucleotide (18). Inhibition of P388D1 cell $iPLA_2$ activity suppresses incorporation of [$^3$H]arachidonic acid into PC and reduces [$^3$H]lysophosphatidylcholine (LPC) levels in [$^3$H]choline-labeled cells (17,18). Arachidonate incorporation (17,18) reflects a deacylation/reacylation cycle (19,20) of phospholipid remodeling rather than *de novo* synthesis (21), and the level of LPC acceptors is thought to limit the rate of [$^3$H]arachidonic acid incorporation into P388D1 cell PC (17,18).

Recently, a second proposed housekeeping function for $iPLA_2\beta$ in PC homeostasis has been proposed from studies of cells that overexpress CTP:phosphocholine cytidylyltransferase (CT) (22,23), which catalyzes the rate-limiting step in PC biosynthesis *via* the Kennedy pathway. Cells that overexpress CT after transfection with CT cDNA in viral vectors exhibit increased rates of PC biosynthesis and degradation and little net change in PC accumulation (22,23). This suggests that PC degradative mechanisms are upregulated in response to CT overexpression in order to prevent excess PC accumulation and to maintain PC homeostasis. The increased PC degradation observed in CT-overexpressing cells is prevented by BEL, and immunoreactive $iPLA_2\beta$ protein and activity increase in such cells, suggesting that $iPLA_2\beta$ is upregulated in response to CT overexpression (22,23). If general, this could represent an important role for $iPLA_2\beta$ in cell biology because PC biosynthesis is involved in regulation of the cell cycle and apoptosis (24–27).

This model predicts that overexpression of $iPLA_2\beta$ might cause upregulation of CT as a compensatory response to maintain cellular PC homeostasis. To test this possibility, we have prepared INS-1 insulinoma cells that overexpress $iPLA_2\beta$ after stable transfection with a retroviral vector containing $iPLA_2\beta$ cDNA. We have measured CT activity in such cells and in parental INS-1 cells, and we have compared rates of [$^3$H]choline incorporation into and disappearance from PC in these cell lines. We have also measured LPC levels in the cells and compared rates of [$^3$H]arachidonic acid incorporation into PC and effects of BEL on this process. Because the glycerolipid composition of pancreatic islet β-cells may affect insulin secretion (28–31) and because signaling functions of $iPLA_2\beta$ have been proposed for some cells (32–47), including β-cells (48–59), we have also examined rates of cellular proliferation and insulin secretion in response to stimulation with secretagogues in $iPLA_2\beta$-overexpressing and parental INS-1 cells.

## EXPERIMENTAL PROCEDURES

*Materials.* Enhanced chemilumiescence detection reagents and 1-palmitoyl-2-[$^{14}$C]linoleoyl-*sn*-glycero-3-phosphocholine (55 mCi/mmol) were purchased from Amersham (Piscataway, NJ). PC standards were obtained from Avanti Polar Lipids (Birmingham, AL), and arachidonic acid was from Nu-Chek-Prep (Elysian, MN). BEL $iPLA_2$ suicide substrate (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)- 2H-pyran-2-one was purchased from Cayman Chemical (Ann Arbor, MI). Tissue culture media (CMRL-1066, RPMI, and minimal essential minimum), penicillin, streptomycin, Hank's balanced salt solution, and L-glutamine were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT) and Pentex bovine serum albumin (BSA; fatty acid free, fraction V) from ICN Biomedical (Aurora, OH). ATP, ampicillin, and kanamycin were obtained from Sigma Chemical (St. Louis, MO). Krebs-Ringer bicarbonate buffer (KRB) contained 25 mM HEPES (pH 7.4), 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, and 1 mM $MgCl_2$.

*Cell culture.* INS-1 insulinoma cells provided by Dr. Christopher Newgard (University of Texas, Dallas, TX) were cultured as described (60) in RPMI 1640 medium containing 11 mM glucose, 10% fetal calf serum, 10 mM HEPES buffer, 2 mM glutamine, 1 mM sodium pyruvate, 50 mM β-mercaptoethanol, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. RetroPack PT 67 cells (Clontech, Palo Alto, CA) were maintained in Dulbecco's modified Eagle's medium (4.5 mg/mL glucose) containing 10% FBS, 4 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin.

*Preparation of recombinant retrovirus containing the cDNA encoding the rat pancreatic islet $iPLA_2\beta$.* A retroviral system (61,62) was used to stably transfect INS-1 cells with $iPLA_2\beta$ cDNA and achieve overexpression. To construct the retroviral vector, full-length rat pancreatic islet $iPLA_2\beta$ cDNA, was excised from pBK-CMV-$iPLA_2\beta$ vector and subcloned into the retroviral vector pMSCVneo at the recognition sites for restriction endonucleases EcoR I and Xho I. The construct containing the $iPLA_2\beta$ cDNA (pMSCVneo-$iPLA_2\beta$) was transfected into Clontech RetroPack PT 67 packaging cells with a GenePORTER transfection system according to the manufacturer's instructions (Gene Therapy Systems, San Diego, CA). Upon transfection of packaging cells, pMSCVneo integrated into the genome and expressed a transcript containing viral packaging signal, a neomycin resistance gene that confers resistance to the selection agent G418, and $iPLA_2\beta$ cDNA. This transcript is recognized by viral proteins in packaging cells. Introduction of pMSCVneo-$iPLA_2\beta$ into PT 67 cells resulted in production of high-titer, replication-incompetent, infectious virus particles that were released into the culture medium, collected, and used to infect INS-1 cells.

*Infection of INS-1 cells with recombinant retroviral particles and selection of stably transfected cells that overexpress $iPLA_2\beta$.* INS-1 cells were plated on 100-mm petri dishes at a density of 3–5 × $10^5$ per plate 12–18 h before infection. Freshly collected, retrovirus-containing medium was passed through a 0.45-µm filter and added to INS-1 cell monolayers. Polybrene (final concentration 4 µg/mL) was added to culture medium, and medium was replaced after 24 h of incubation. To select stably transfected cells that expressed high levels of $iPLA_2\beta$, retrovirally infected cells were cultured with G418 (0.4 mg/mL) for 1–2 wk. After G418-resistant colonies be-

came apparent, cell culture was continued for several days. Individual colonies were transferred to a 48-well plate for expansion of clonal cells.

*Assay of INS-1 cell iPLA$_2$ activity.* Seeded INS-1 were washed with phosphate-buffered saline (PBS) and detached by trituration. Cells were collected by centrifugation and disrupted by sonication (Vibra Cell High Intensity Processor, (Sonics & Materials, Inc., Danbury, CT) five 1-s pulses, amplitude 12%) in homogenization buffer (250 mM sucrose, 40 mM Tris-HCI, pH 7.1, 4°C). Homogenates were centrifuged (15,000 × *g*, 45 min, 4°C) to yield a cytosolic supernatant. Protein content was measured with Coomassie reagent (Pierce, Rockford, IL) against BSA standard. $Ca^{2+}$-independent PLA$_2$ activity in aliquots of cytosol (25 μg protein) was assayed by ethanolic injection (5 μL) of 1-palmitoyl-2-[$^{14}$C]linoleoyl-*sn*-glycero-3-phosphocholine (final concentration 5 μM) in assay buffer (40 μM Tris, pH 7.5, 5 mM EGTA, total volume 200 μL). Assay mixtures were incubated (5 min, 37°C, with shaking) and reactions terminated by adding butanol (0.1 mL) and vortexing. After centrifugation (2,000 × *g*, 5 min), products in the butanol layer were analyzed by silica gel G thin-layer chromatography (TLC) in petroleum ether/ethyl ether/acetic acid (80:20:1). The TLC plate region containing free fatty acid was identified with iodine vapor and scraped into a scintillation vial. Released [$^{14}$C]fatty acid was measured by liquid scintillation spectrometry, and PLA$_2$ specific activity was calculated from the disintegrations per minute (dpm) of released fatty acid and protein content as described (48).

*Assay of INS-1 cell CT activity.* CT activity was measured in whole cell lysates prepared from parental and transfected INS-1 cells. Cell homogenates were prepared in 10 mM HEPES buffer, pH 7.4, containing 0.34 M sucrose, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethyl sulfonyl fluoride (assay homogenization buffer). Cells were sonicated on ice and then centrifuged (1000 × *g*, 20 min) to remove unbroken cells. CT activity was measured essentially as described (63). The assay depends on conversion of [*methyl*-$^{14}$C] phosphorylcholine to [$^{14}$C]CDP-choline. Substrate and product were separated by TLC on silica plates with methanol/0.5% sodium chloride/ammonium hydroxide (50:50:1) as mobile phase. The phosphorylcholine and CDP-choline bands were identified based on comigration with standards, and the radiolabel in each band was measured by liquid scintillation spectrometry.

*Rate of incorporation of [methyl-$^3$H]choline into PC*. INS-1 cells were plated at 2 × 10$^6$ cells per well in six-well plates and cultured overnight. The following day, cells were incubated with 0.5 μCi/mL [*methyl*-$^3$H]choline. After various incubation periods, lipids were extracted by the method of Bligh and Dyer (64). The organic layer of the extraction, which contained the lipids, was analyzed by TLC on silica plates with chloroform/methanol/ammonium hydroxide (65:25:5) as the mobile phase. PC was identified based on comigration with standards, and associated radiolabel was measured by liquid scintillation spectrometry. Raw data were corrected for extraction and counting efficiencies, normalized to cell protein content, and expressed as $^3$H dpm/μg protein.

*Rate of loss of [$^3$H]pc from cells prelabeled with [methyl-$^3$H]choline.* INS-1 cells were plated at 2.5 × 10$^6$ cells per well in six-well plates and metabolically labeled by incubation with 1.5 μCi/mL [*methyl*-$^3$H]choline for 60 h. Medium was then removed, and cells were washed twice with fresh medium containing 100 mM unlabeled choline. Cells were then incubated in that medium for various periods, and lipids were extracted by the method of Bligh and Dyer (64) and analyzed by TLC to isolate PC as described above. The [$^3$H]PC content was determined by liquid scintillation spectrometry, normalized to cell protein content, and expressed as a fraction of the [$^3$H] dpm/μg protein value observed in the time zero sample obtained immediately after completion of prelabeling with [$^3$H]choline.

*Measurement of INS-1 cell LPC mass and molecular species.* To determine INS-1 cell LPC mass, cells (10$^6$ per condition) were washed twice with and then resuspended and incubated (30 min, 37°C) in KRB medium containing 5.5 mM glucose and 0.1% BSA. Cells were then placed in fresh medium and incubated (30 min, 37°C). Cells were then washed twice with PBS and extracted by the method of Bligh and Dyer (64) with the modifications that 150 mM LiCl in water was aqueous phase and that the chloroform phase contained 17:0-LPC internal standard (300 pmol per condition). Concentrated extracts were analyzed by silica gel G TLC on heat-activated (30 min, 80°C) plates with chloroform/methanol/ammonium hydroxide (65:30:0.8) to separate LPC ($R_f$ 0.38), lysophosphatidylethanolamine ($R_f$ 0.46), and PC ($R_f$ 0.60). LPC was extracted by the method of Bligh and Dyer (64) with the modification that 150 mM LiCl in water was aqueous phase and analyzed by electroscopy ionization tandem mass spectroscopy (ESI/MS/MS).

*Incubation of INS-1 cells with radiolabeled arachidonic acid.* INS-1 cells were washed three times in KRB medium containing 5.5 mM glucose and 0.1% BSA, resuspended in that medium, and preincubated for 30 min at 37°C. The cells (2 × 10$^5$ per condition) were then placed in fresh KRB medium that contained 5.5 mM glucose, 0.1% BSA, and 2.5 mM $CaCl_2$ and incubated (30 min, 37°C) after addition of vehicle only (control) or BEL (10 μM). After the preincubation and loading steps described above, fatty acid incorporation experiments were initiated by adding [$^3$H]arachidonic acid (final concentration 0.5 μCi/mL, 5 nM) to the medium, and incubation was performed for 10–60 min at 37°C. INS-1 cells were then washed three times in KRB medium containing 5.5 mM glucose and 0.1% BSA to remove unincorporated [$^3$H]arachidonate. Cellular lipids were then extracted by the method of Bligh and Dyer (64) and analyzed by TLC or high-performance liquid chromatography (HPLC). The lipid phosphorus content of Bligh-Dyer extracts was measured as described (29).

*Chromatographic analyses of phospholipids*. Incorporation of [$^3$H]arachidonate or unlabeled arachidonate into INS-1 cell phospholipids was determined after TLC (15,16) or HPLC (50,51,57). TLC was performed on silica gel G plates

with hexane/diethyl ether/acetic acid (70:30:1). Phospholipids remain at the origin and are resolved from diglycerides ($R_f$ 0.21–0.24), free fatty acids ($R_f$ 0.58), and triglycerides. Phospholipid head-group classes were separated by normal phase (NP)-HPLC (46,47,53) analyses on a silicic acid column (LiChrosphere Si-100, 10 μm, 250 × 4.5 mm; Alltech, Deerfield, IL) with the solvent system hexane/2-propanol/(25 mM potassium phosphate, pH 7.0)/ethanol/acetic acid (367:490:62:100:0.6) at a flow of 0.5 mL/min for 60 min and then 1.0 mL/min. Retention times of standard phospholipids were: phosphatidylethanolamine (PE), 11 min; phosphatidic acid (PA), 20 min; phosphatidylinositol (PI), 27 min; phosphatidylserine (PS), 38 min; and PC, 102 min. Molecular species of PE were analyzed by ESI mass spectrometry (MS).

*Incubation of INS-1 cells with arachidonic acid to induce phospholipid remodeling.* INS-1 were cells cultured in RPMI medium supplemented with penicillin, streptomycin, fungizone, and gentamicin at a concentration of 0.1% (wt/vol) each. Cells ($1.2 \times 10^6$ per condition) were treated (30 min, 37°C) with vehicle only or with BEL (10 μM). Medium was then removed and replaced with fresh medium containing no supplements other than those described above or containing arachidonic acid (final concentration 70 μM), and cells were cultured at 37°C for various periods. After 0, 4, 8, or 18 h, cells were washed twice with PBS, suspended in homogenization buffer, and disrupted by sonication. One aliquot of homogenate was used to measure protein content, and the remainder was extracted by the method of Bligh and Dyer (64). The extract was concentrated and analyzed by NP-HPLC to separate phospholipid head-group classes.

*ESI/MS analyses of INS-1 cell lipids.* LPC and PC species were analyzed as $Li^+$ adducts by positive ion ESI/MS/MS (57,65), and PE species were analyzed as $[M - H]^-$ ions by negative ion ESI/MS (66) on a Finnigan (San Jose, CA) TSQ-7000 triple-stage quadrupole mass spectrometer with an ESI source controlled by Finnigan ICIS software. Phospholipids were dissolved in methanol/chloroform (9:1, vol/vol) containing LiOH (2 nmol/μL), infused (1 μL/min) with a Harvard syringe pump, and analyzed under described instrumental conditions (57,65,66). For tandem MS, precursor ions selected in the first quadrupole were accelerated (32–36 eV collision energy) into a chamber containing argon (2.3–2.5 mtorr) to induce collisionally activated dissociation (CAD) and product ions analyzed in the final quadrupole. Identities of PE species in the total ion current profile were determined from their tandem spectra (66). To quantitate LPC species, constant neutral loss scanning was performed to monitor LPC $[M + Li]^+$ ions that undergo loss of 59 (trimethylamine) upon CAD (57). Quantitation was achieved by comparing the intensity of the ion for 17:0-LPC internal standard (*m/z* 516) to intensities of ions for the endogenous species 16:0-LPC (*m/z* 502) and 18:0-LPC (*m/z* 530) (57). Standard curve experiments in which a constant amount of 17:0-LPC and varied amounts of 16:0-LPC and 18:0-LPC were added to a series of tubes and analyzed as $Li^+$ adducts by ESI/MS/MS indicated that this method is linear over a wide range that included levels in INS-1 cells.

*Determination of INS-1 cell proliferation rate.* INS-1 cells were seeded into six-well plates at a density of $0.3 \times 10^6$ cells/well and cultured at 37°C in the incubation medium described above. After various intervals, cells from one well were detached with trypsin/EGTA solution, and the number of cells in the suspension was determined with a hemocytometer.

*Determination of insulin secretion by INS-1 insulinoma cells.* Culture medium from INS-1 cells seeded in 24-well plates was removed, and the cells were washed twice in KRB medium and incubated (1 h, 37°C, under an atmosphere of 95% air/5% $CO_2$) in KRB medium (1 mL). Medium was then removed and replaced with KRB medium containing glucose (0-18 mM) with or without phorbolmyristate acetate (PMA) (1 μM) or dibutyryl cAMP (dBcAMP) (1 mM). After addition of final incubation medium, cells were incubated (2 h, 37°C) under the atmosphere described above, and medium was then removed for measurement of insulin by radioimmunoassay (67). Cells were then detached from the plate, and their acid-ethanol extractable insulin was determined by radioimmunoassay (68). Secreted insulin was expressed as a fraction of total cellular insulin content (69).

*Statistical analyses.* Student's *t*-test was used to compare two groups, and multiple groups were compared by one-way analysis of variance with *post-hoc* Newman-Keul's analyses.

## RESULTS

*Comparison of CT activities in control INS-1 cells and in INS-1 cells that overexpress iPLA$_2$β after stable retroviral transfection.* Transfection of INS-1 insulinoma cells with a retroviral construct containing the rat iPLA$_2$β cDNA followed by selection of G418-resistant cells resulted in the isolation of a stably transfected clone that expressed eightfold more iPLA$_2$β activity than parental INS-1 cells (Fig. 1, left set of bars). The iPLA$_2$β-overexpressing (iPLA$_2$-X) cell line also exhibited a twofold increase in specific activity of CT compared to parental INS-1 cells (Fig. 1, right set of bars). The magnitude of the increase in CT activity is thus substantially less than the increase in iPLA$_2$β activity in the transfected cells, but both effects are statistically significant. We also prepared an INS-1 cell line that was stably transfected with an empty retroviral vector that did not contain iPLA$_2$β cDNA, and we prepared a second iPLA$_2$β-overexpressing cell line by methods used to prepare the first such line. Properties of the second iPLA$_2$β-overexpressing line were similar to those of the first line, and properties of the line stably transfected with empty retroviral vector were similar to those of nontransfected parental cells (not shown).

*Comparison of rates of [$^3$H]choline incorporation into and loss from PC in control INS-1 cells and in INS-1 cells that overexpress iPLA$_2$β.* In stably transfected CHO cells that overexpress CT, rates of [$^3$H]choline incorporation into PC are increased, and, in cells in which the PC pool is prelabeled with [$^3$H]choline, rates of disappearance of [$^3$H]choline from PC are also higher in CT-overexpressing cells than in parental

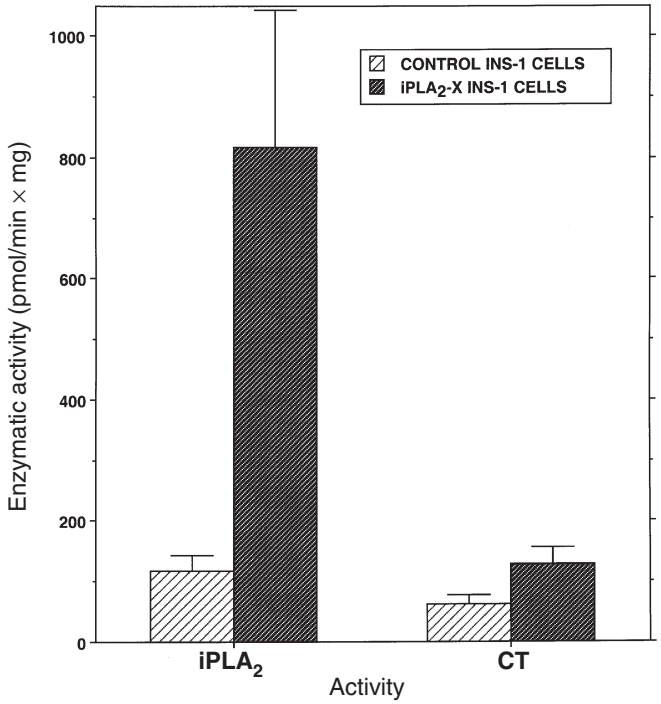


**FIG. 1.** Overexpression of iPLA$_2$β in INS-1 insulinoma cells stably transfected with iPLA$_2$β cDNA in a retroviral vector and effects on expression of CTP:phosphocholine cytidylyltransferase (CT) activity. A clonal INS-1 cell line was isolated after stable transfection with a retroviral vector containing iPLA$_2$β cDNA, and levels of iPLA$_2$β activity (left set of bars) and CT activity (right set of bars) were compared to those of parental INS-1 cells. PLA$_2$ activity was measured in the absence of [$Ca^{2+}$] and presence of EGTA and 1 mM ATP. Light bars reflect activity from parental cells (control INS-1 cells), and dark bars bars reflect activity from the clonal INS-1 cell line transfected with iPLA$_2$ cDNA in the retroviral construct (iPLA$_2$-X INS-1 cells). Mean specific activity values are displayed, and standard errors of the mean are indicated ($n$ = 6). The differences in specific activity between control and iPLA$_2$-X INS-cells were statistically significant ($P < 0.05$) both for iPLA$_2$ and for CT. Abbreviations: iPLA$_2$β, Group IV A phospholipase A$_2$; PLA$_2$, phospholipase A$_2$.

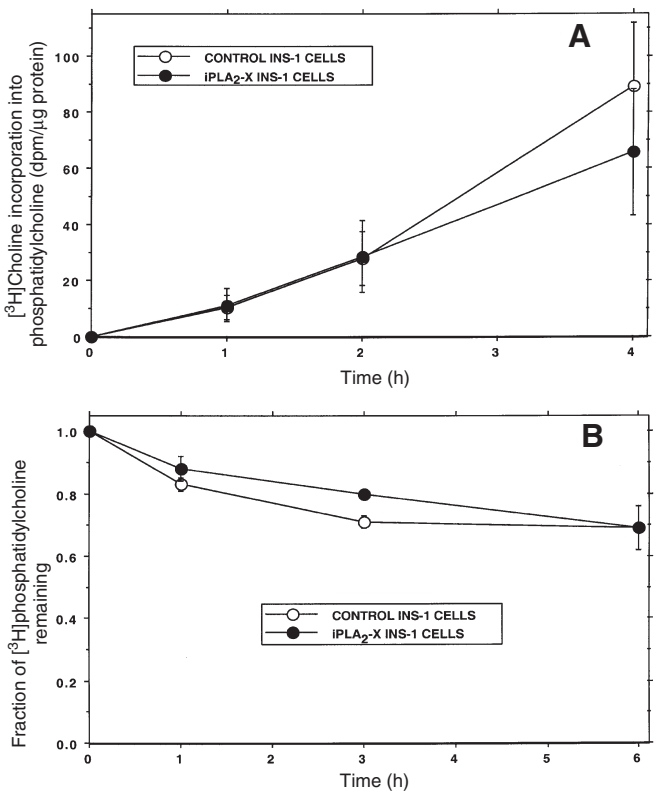


**FIG 2.** Rate of [$^3$H]choline incorporation into and disappearance from phosphatidylcholine (PA) in control and iPLA$_2$β-overexpressing INS-1 cells. The rates of [$^3$H]choline incorporation into PC (panel A) of control (open symbols) and iPLA$_2$β-overexpressing (closed symbols) were measured and expressed as [$^3$H] dpm per μg of cell protein. In panel B, control (open symbols) and iPLA$_2$β-overexpressing INS-1 cells were prelabeled with [$^3$H]choline for 60 h and then incubated in medium containing unlabeled choline for 1 to 6 h. At the end of the incubation, lipids were extracted, and PC was isolated by thin-layer chromatography. The [$^3$H] content was determined by liquid scintillation spectrometry, normalized to cell protein content, and expressed as a fraction of the time zero value. In both panels, mean values are displayed and standard errors of the mean are indicated ($n$ = 4). For abbreviations see Figure 1.

CHO cells upon incubation in medium with unlabeled choline (23). In similar experiments with INS-1 cells, iPLA$_2$β-overexpressing cells did not exhibit an increased rate of [$^3$H]choline incorporation into PC compared to parental cells (Fig. 2A) and did not exhibit accelerated [$^3$H]PC loss after prelabeling with [$^3$H]choline and subsequent incubation in medium with unlabeled choline (Fig. 2B). Effects of iPLA$_2$β overexpression in INS-1 cells thus do not mimic effects of CT overexpression in CHO cells with respect to rates of [$^3$H]choline incorporation into and disappearance from PC, even though the activities of iPLA$_2$β and CT increase together.

*Comparison of LPC levels in control INS-1 cells and in INS-1 cells that overexpress iPLA$_2$β.* Overexpression of iPLA$_2$β in INS-1 cells caused a modest increase in LPC levels (11.53 ± 0.45 nmol/μmol lipid phosphorus) compared to parental cells (7.97 ± 0.92 nmol/μmol lipid phosphorus), as measured by ESI/MS/MS (Fig. 3). The compound 17:0-LPC, which is not an endogenous constituent of INS-1 cells, was added as an internal standard to cell lipid extracts, and LPC species were isolated by TLC and analyzed as $Li^+$ adducts by ESI/MS/MS scans for constant neutral loss of 59 (loss of trimethylamine), which yields a much superior signal-to-noise ratio compared to the ESI/MS total ion current (57). The contents of endogenous 16:0-LPC (*m/z* 502), 18:0-LPC (*m/z* 530), and 18:1-LPC (*m/z* 528) were quantitated relative to the 17:0-LPC internal standard (*m/z* 516) by reference to a standard curve. The 1.5-fold increase in LPC content observed in iPLA$_2$β-overexpressing INS-1 cells compared to parental cells is substantially smaller than the eightfold increase in iPLA$_2$β activity.

*Comparison of rates of [$^3$H]arachidonate incorporation into PC in control INS-1 cells and in INS-1 cells that overexpress iPLA$_2$β.* It has been proposed that cellular content of LPC generated by iPLA$_2$β limits the rate of incorporation of arachidonic acid into PC during phospholipid remodeling and that iPLA$_2$β plays a housekeeping role in the biosynthesis of arachidonate-containing PC species (17,18). Because such a role of iPLA$_2$β might be readily apparent in cells that overexpress

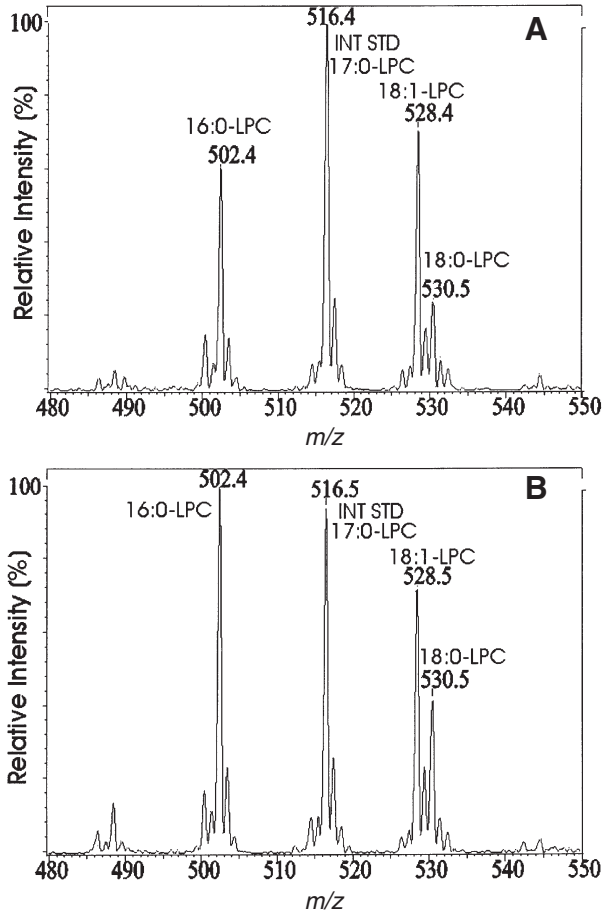


**FIG. 3.** Quantitation of lysophosphatidylcholine (LPC) molecular species by electroscopy ionization tandem mass spectrometry (ESI/MS/MS) in control and iPLA$_2$β-overexpressing INS-1 cells. After incubations described in the Experimental Procedures section, lipids were extracted from control (panel A) and iPLA$_2$β-overexpressing (panel B) INS-1 cells and mixed with 17:0-LPC internal standard. LPC species were isolated by thin-layer chromatography (TLC) and analyzed as $Li^+$ adducts by ESI/MS/MS scans for constant neutral loss of trimethylamine. Internal standard is represented by the ion at *m/z* 516, and endogenous LPC species are represented by ions at *m/z* 502 (16:0-LPC), 528 (18:1-LPC), and 530 (18:0-LPC). Quantities of endogenous species were determined from peak height ratios relative to the internal standard by interpolation from a standard curve. For abbreviations see Figure 1.

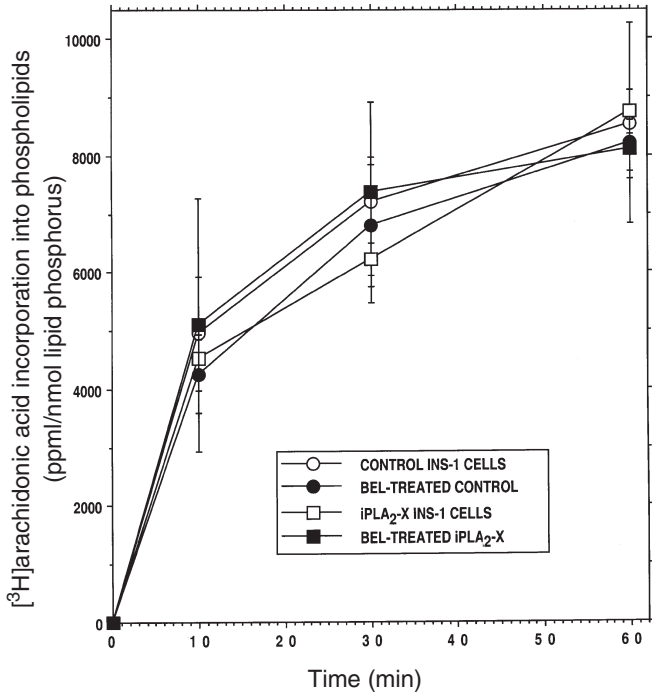


**FIG. 4.** Rates of [$^3$H]arachidonic acid incorporation into phospholipids of control and iPLA$_2$β-overexpressing INS-1 cells and effects of the iPLA$_2$β inhibitor bromoenol lactone (BEL). Control (circles) and iPLA$_2$β-overexpressing (squares) INS-1 cells were pretreated with 10 μM BEL (closed symbols) or BEL-free vehicle (open symbols), washed, and incubated in medium containing [$^3$H]arachidonic acid for various periods. At the end of the incubation, lipids were extracted and analyzed by TLC to isolate phospholipids from fatty acids and neutral lipids. The $^3$H content of the phospholipid band was determined and expressed as a ratio to the lipid phosphorus content of the lipid extract. Mean values are displayed, and standard errors of the mean are indicated ($n$ = 4). For abbreviations see Figures 1 and 3.

the enzyme, we examined rates of [$^3$H]arachidonic acid incorporation into phospholipids of iPLA$_2$β-overexpressing and parental INS-1 cells before and after treatment with the iPLA$_2$β inhibitor BEL (Fig. 4). Neither overexpression of iPLA$_2$β nor its inhibition with BEL significantly affected rates of incorporation of [$^3$H]arachidonic acid into INS-1 cell phospholipids (Fig. 4), arguing against a general role for iPLA$_2$β in biosynthesis of arachidonate-containing PC species. These radiochemical data are consistent with ESI/MS measurements of rates of appearance of arachidonate-containing PC species in INS-1 cells incubated with supplemental arachidonic acid (57).

*Comparison of rates of appearance of arachidonate-containing ethanolamine phospholipid molecular species during culture with arachidonic acid in control INS-1 cells and in INS-1 cells that overexpress iPLA$_2$β.* After initial incorporation of arachidonic acid into PC, it is subsequently transferred in part to PE species in islets and INS-1 cells (57) and in other cells by the action of a CoA-independent transacylase (70,71). With INS-1 cells that are incubated with supplemental arachidonic acid, this process is reflected by a time-dependent increase in the contribution of arachidonate-containing species to the ESI/MS total ion current for PE lipids (Fig. 5). When grown in culture medium without supplemental arachidonic acid, PE lipids from iPLA$_2$β-overexpressing (Fig. 5A) and parental INS-1 cells exhibit virtually identical ESI/MS profiles that include ions at *m/z* 700 (16:0p/18:1-PE), 714 (16:1/18:1-PE), 716 (16:0/18:1-PE), 722 (16:0p/20:4-GPE), 742 (18:1/18:1-PE), 744 (18:0/18:1-PE), 750 (18:0p/20:4-PE), 766 (18:0/20:4-PE), and 790 (18:0/22:6-PE). The designation "p" denotes a plasmalogen, which is identified by acid lability of the species represented by the corresponding ion in the ESI/MS spectrum (66). Identities of the *sn*-1 and *sn*-2 substituents were determined by CAD and tandem MS (66).

When either iPLA$_2$β-overexpressing or parental INS-1 cells are incubated with supplemental arachidonic acid, the contribution of the arachidonate-containing species 18:0/20:4-PE (*m/z* 766) rises progressively with time, and it becomes the predominant component of the mixture by 18 h (Figs. 5B,C). The chain-elongation product 18:0/22:4-PE also becomes a

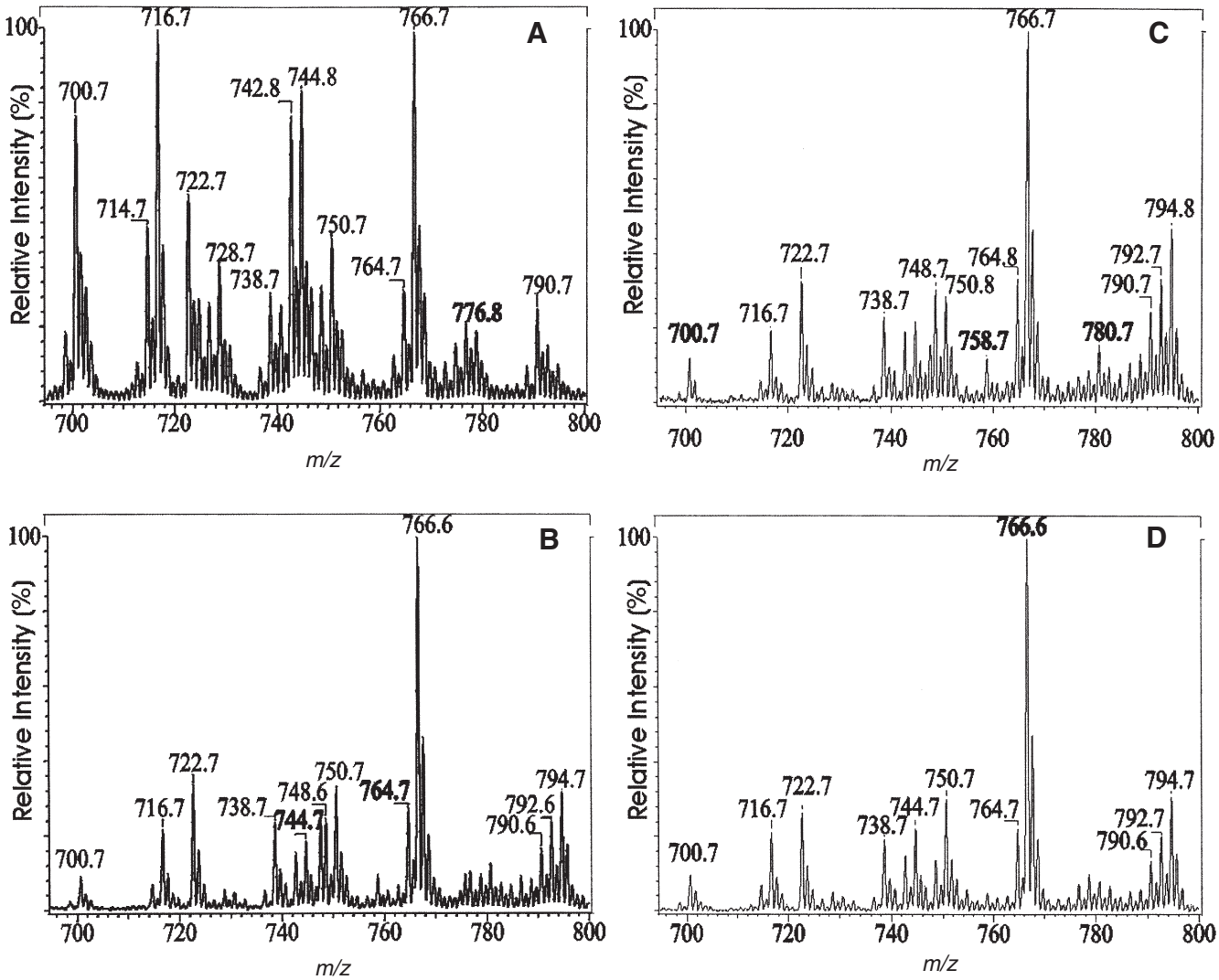


**FIG. 5.** Electrospray ionization mass spectrometry (ESI/MS) analyses of changes in phosphatidylethanolamine (PE) molecular species in control and iPLA$_2$β-overexpressing INS-1 cells incubated with supplemental arachidonic acid and effects of the iPLA$_2$β inhibitor BEL. Control (panel C) and iPLA$_2$β-overexpressing (panels A, B, and D) INS-1 cells were treated with 10 μM BEL (panel D) or with BEL-free vehicle (panels A, B, and C) and then incubated with supplemental arachidonic acid for various periods. At the end of the incubations, lipids were extracted and analyzed by normal-phase high-performance liquid chromatography to isolate PE species, which were analyzed by ESI/MS as $[M - H]^-$ ions. Identities of species represented by ions in the ESI/MS total ion current were determined by collisionally activated dissociation and tandem MS. Tracings displayed are representative of four separate experiments in which time points at 0, 2, 6, and 18 h were examined in quintuplicate. For abbreviations see Figures 1 and 4.

prominent component of the mixture, and the relative abundances of 18:1-containing species (e.g., *m/z* 700, 716, and 742, 744) decline, resulting in a simplified appearance of the spectrum. Neither the rate nor the extent of these effects differed between iPLA$_2$β-overexpressing and parental INS-1 cells (Figs. 5,C), and treatment of the cells with the iPLA$_2$β inhibitor BEL also failed to affect this process (Fig. 5D). The data in Figures 4 and 5 thus indicate that neither overexpression of iPLA$_2$β nor its inhibition with BEL affects the initial incorporation of arachidonate into phospholipids or its subsequent transfer among phospholipid classes in INS-1 cells.

*Comparison of rates of proliferation for control INS-1 cells and for INS-1 cells that overexpress iPLA$_2$β.* During the course of experiments with iPLA$_2$β-overexpressing INS-1 cells, it became apparent that their proliferation rate exceeded that of the parental cell line (Fig. 6). Between 70 and 270 h after application to culture plates, cell numbers for iPLA$_2$β-overexpressing cells increased 2.3-fold more rapidly than that for parental cells, and this property persisted on serial passage in culture.

*Comparison of insulin secretory responses of control INS-1 cells and of INS-1 cells that overexpress iPLA$_2$β.* Enhanced rates of proliferation of insulinoma cells are often associated with reduced insulin secretion (72), but iPLA$_2$β-overexpressing INS-1 cells exhibited greater insulin secretory responses to the protein kinase C-activating phorbol ester PMA and to the cAMP analog (dBcAMP) than did parental INS-1 cells (Fig. 7). The effect of PMA to promote insulin secretion was largely independent of medium glucose concentration (Fig. 7A), but insulin secretory responses to dBcAMP increased

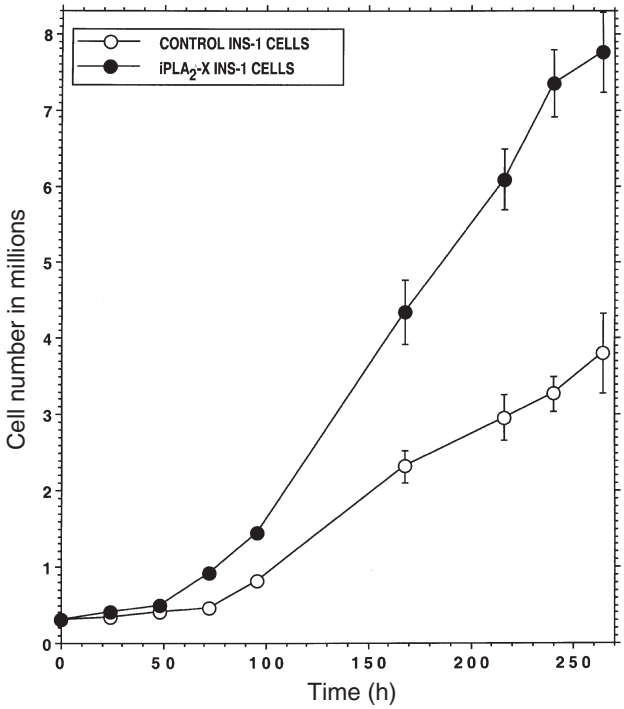


**FIG. 6.** Rates of proliferation of control and iPLA$_2$β-overexpressing INS-1 cells. INS-1 cells were seeded into six-well plates at a density of 0.3 × $10^6$ cells/well and cultured at 37°C. After various intervals, cells from one well were detached with trypsin/EGTA solution, and the number of cells in the suspension was determined with a hemocytometer. Mean values for cell number are displayed, and standard errors of the mean are indicated ($n = 6$). Values for iPLA$_2$β-overexpressing INS-1 cells were significantly higher ($P < 0.05$) than those for control INS-1 cells at all time points after 50 h. For abbreviations see Figure 1.

progressively with the glucose concentration over the range 0 to 11 mM (Fig. 7B). The iPLA$_2$β-overexpressing cells also exhibited greater insulin secretory responses to glucose alone than did the parental cells.

## DISCUSSION

Many potential functions for Group VIA PLA$_2$ (iPLA$_2$β) have been proposed (17,18,22,23,32–59) since it was first cloned (8–10). Among these are two housekeeping roles in PC biosynthesis. Cells that overexpress the rate-limiting enzyme in the Kennedy pathway of PC biosynthesis, CT, after transfection with CT cDNA exhibit increased rates of both PC biosynthesis and degradation with little net change in PC accumulation (22,23). It has previously been reported that CHO cells that overexpress CT activity by seven- to tenfold, compared to parental cells, after stable transfection with CT cDNA in a viral vector exhibit about a two- to threefold increase in iPLA$_2$β activity (23). This suggests that upregulation of PC degradative mechanisms occurs in response to CT overexpression to prevent excess accumulation of PC. Because CT overexpression in CHO cells is associated with increased iPLA$_2$β activity and immunoreactive protein and because the iPLA$_2$β inhibitor BEL retards PC degradation in CT-overexpressing cells, it has been proposed that iPLA$_2$β limits cellular PC accumulation and that its expression is coordinately regulated with that of CT (22,23). We find that overexpressing iPLA$_2$β in INS-1 insulinoma cells stably transfected with iPLA$_2$β cDNA in a retroviral vector is associated with a modest increase in CT activity, but we do not observe the increases in rates of [$^3$H]choline incorporation into or disappearance from PC that occur in CHO cells that overexpress CT. Our findings indicate that, while iPLA$_2$β and CT activity might be coordinately regulated in the same direction, the magnitudes of increases in the two activities are not proportional. Effects of increased expression of iPLA$_2$β and CT may thus depend on the context of the cell-type in which overexpression occurs.

Overexpressing iPLA$_2$β in INS-1 cells is associated with a modest increase in LPC levels, and another housekeeping function proposed for iPLA$_2$β is to generate LPC acceptors for arachidonate incorporation into PC during phospholipid remodeling in murine P388D1 cells (17,18). Although such a role for iPLA$_2$β cannot be demonstrated in pancreatic islets or parental INS-1 cells (57), it is considered possible that this function of iPLA$_2$β may be more readily demonstrable in cells that overexpress the enzyme. We find that neither iPLA$_2$β overexpression nor its inhibition with BEL affects the rate of [$^3$H]arachidonic acid incorporation into INS-1 cell phospholipids, and this is consistent with ESI/MS measurements of the rate of appearance of arachidonate-containing PC species in parental INS-1 cells incubated with supplemental arachidonic acid (57). We also find that neither iPLA$_2$β-overexpression nor its inhibition with BEL affects transfer of arachidonate into PE, as visualized by ESI/MS, in INS-1 cells incubated with supplemental arachidonic acid, and this is consistent with radiochemical studies in pancreatic islets and parental INS-1 cells (57). These findings indicate that iPLA$_2$β is not required for initial incorporation of arachidonic acid into phospholipids or its subsequent transfer among phospholipid head-group classes in β-cells.

It is possible that iPLA$_2$β plays different roles in distinct cell-types and that the function of the enzyme depends on stimulation condition or maturation state. Species of mRNA encoding at least four distinct products of the human iPLA$_2$β gene have been observed, and they are differentially represented among distinct human cell-types (35,39,73,74). The active form of iPLA$_2$β is an oligomer of interacting subunits that associate *via* their ankyrin-repeat domains (8), and distinct iPLA$_2$β gene products form hetero-oligomers with different properties (35). iPLA$_2$β is also subject to proteolytic processing that can increase its catalytic activity (43), and its amino acid sequence contains consensus sites for several posttranslational modifications (74). These findings suggest that iPLA$_2$β gene products might participate in complex regulatory networks that could affect several cell biological properties.

We find that iPLA$_2$β-overexpressing INS-1 cells proliferate more rapidly than parental cells, as reflected by the rate of increase in cell number (Fig. 6). Corresponding increases in the rate of [$^3$H]thymidine incorporation into DNA are observed (Bohrer, A., and Turk, J., unpublished observations),

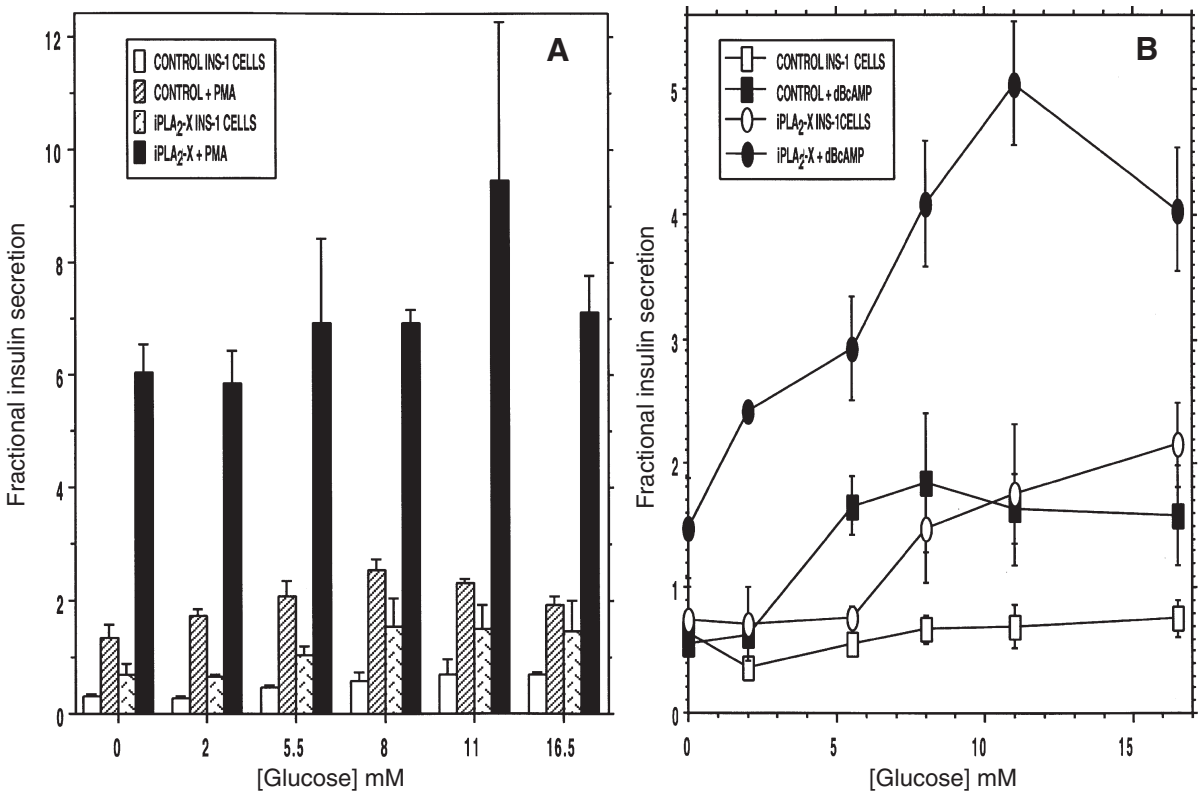


**FIG. 7.** Effects of the protein kinase C-activating phorbol myristate acetate (PMA) ester, glucose, and dibutyryl-cyclic AMP (dBcAMP) on insulin secretion from iPLA$_2$β-overexpressing and control INS-1 cells. Insulin secretion was measured after a 2-h incubation of INS-1 cells in medium containing glucose at concentrations between 0 and 16.5 mM without or with 1 μM PMA (panel A) or without or with 1 mM dBcAMP (panel B). Insulin secreted into the medium was normalized to cell insulin content to yield fractional secretion values. Mean values are displayed, and standard errors of the mean are indicated ($n = 4$). Secretion values for iPLA$_2$β-overexpressing cells were significantly higher ($P < 0.05$) than those for control cells in each comparison in panel A, for each comparison of cells treated with dBcAMP in panel B, and for each comparison at a glucose concentration higher than 5.5 mM in panel B. For other abbreviations see Figure 1.

and iPLA$_2$β inhibition has been reported to reduce [$^3$H]thymidine incorporation and rates of proliferation in lymphocyte lines (75). The bases for these phenomena have not yet been established, but the amino acid sequence of iPLA$_2$β contains a bipartite nuclear localization signal (74). It is thus possible that iPLA$_2$β might affect nuclear events involved in cell division. A favored substrate of iPLA$_2$β is PA, which it converts to lysophosphatidic acid (LPA) (8). LPA is a potent mitogen (75,77), and enhanced proliferation may occur if cellular LPA levels rise as a consequence of iPLA$_2$β overexpression. We are now attempting to quantify INS-1 cell LPA content, but this is technically difficult because of the low LPA levels in cells. Interconversions of LPA, an inverted cone-shaped lipid, and arachidonate-containing species of PA, which are cone-shaped lipids, have been demonstrated to be involved in membrane fission and fusion events in neurosecretion (78,79). Both brain and islets express high levels of iPLA$_2$β, and it is the vastly predominant brain cytosolic PLA$_2$ (80–82). Neurons in brain and β-cells in islets are both electrically active secretory cells that exhibit many biochemical similarities (83), and iPLA$_2$β may play similar signaling roles in secretory events in these cells.

Enhanced rates of proliferation of insulinoma cell lines are often associated with reduced insulin secretory responses (72), but iPLA$_2$β-overexpressing INS-1 cells both proliferate more rapidly and exhibit more robust insulin secretory responses to secretagogues than do parental INS-1 cells. Glucose also stimulates both β-cell proliferation and insulin secretion (84), and common signaling mechanisms, possibly including iPLA$_2$β, may be involved in these two responses. Both phorbol esters and cAMP-elevating agents are known to potentiate insulin secretory responses to glucose in insulinoma cell lines (60,85) and in purified β-cells prepared from dispersed cells from native islets by fluorescence-activated cell sorting (86,87). Products of iPLA$_2$β action may be involved in the interactions of these signaling pathways.

We have not examined the possibilities that enhanced insulin secretory responses to cAMP analogs and phorbol esters in iPLA$_2$β-overexpressing cells reflect increased expression of protein kinase A, protein kinase C, or critical target molecules for these enzymes. We do not observe changes in expression of the Group IVA PLA$_2$ (cPLA$_2$) in iPLA$_2$β-overexpressing INS-1 cells at the level of mRNA or immunoreactive protein (Ma, Z., and Turk, J., unpublished observations), but we cannot exclude compensatory changes in expression of other PLA$_2$ enzymes.

The observation that overexpressing iPLA$_2$β in β-cells enhances their proliferation and amplifies their insulin secretory

responses could prove useful in β-cell engineering. Recently, use of modified immunosuppressive regimens has permitted successful transplantation of human islets in seven consecutive patients with insulin-dependent diabetes mellitus, and each patient remained normoglycemic a year after transplantation without exogenous insulin (88,89). Widespread application of this therapy is precluded by limited availability of donor organs, and β-cell lines that are engineered to proliferate readily in culture but to retain regulated insulin secretion could represent an alternative source of cells for transplantation (72,89). Beta cells with improved secretory responses and other properties have been engineered by introducing genes in viral vectors and by clonal selection strategies (60,90–94). Identifying additional genes whose products affect β-cell proliferation and secretion may permit further progress, and our findings suggest that iPLA$_2$β gene products may be useful in constructing engineered β-cell lines.

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# Metabolism of an Oxysterol, 7-Ketocholesterol, by Sterol 27-Hydroxylase in HepG2 Cells

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**ABSTRACT:** 7-Ketocholesterol (7K) is a quantitatively important oxysterol in both atherosclerotic lesions and macrophage foam cells. We reported recently that radiolabeled 7K delivered to rodents in a modified lipoprotein or chylomicron remnant-like emulsion, both cleared predominantly by the liver, was rapidly excreted into the intestine as water-soluble products, presumably bile acids. Herein, we aimed to elucidate the early or initial reactions in 7K metabolism. The hypothesis was tested that sterol 27-hydroxylase, a mitochondrial cytochrome P450 and the first enzyme of the acidic bile acid pathway, is responsible for the initial metabolism of 7K by HepG2 cells, a human hepatoblastoma cell-line. The 27-hydroxylated product of 7K (27OH-7K) was shown to be the initial, lipid-soluble product of 7K metabolism. It was produced in mitochondrial incubations and whole cells and was readily released into the media from cells. Intact cells generated metabolites of 7K that had undergone conversion from lipid-soluble precursors to water-soluble products rapidly and extensively. Their production was ablated with cyclosporin A, a sterol 27-hydroxylase inhibitor. Furthermore, we demonstrated the effectiveness of two novel selective inhibitors of this enzyme, GW273297X and GI268267X. These inhibitors also ablated the production of water-soluble products by cells; and the inhibitor of choice, GW273297X, decreased the production of 27OH-7K in mitochondrial preparations. This is the first study to demonstrate that sterol 27-hydroxylase plays an important role in the metabolism of oxysterols such as 7K in liver cells.

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Cholesterol oxidation products (oxysterols) have been implicated in atherosclerosis since they were first discovered in atherosclerotic lesions some 50 yr ago. 7-Ketocholesterol (7K; cholest-5-en-3β-ol-7-one) is the most abundant nonenzymatically formed oxysterol detected in human atherosclerotic plaque and in human foam cells isolated from lesions (1,2). As with other oxysterols, 7K possesses a broad spectrum of potent biological effects *in vitro* (reviewed in Refs. 2–4), of which many are potentially pro-atherogenic. Despite the implications that 7K plays a role in atherogenesis, its source remains equivocal (4). It may be derived endogenously by free radical oxidation of cholesterol (5,6) and, indeed, is a major oxysterol in low-density lipoprotein (LDL), which is oxidized *in vitro* (7,8). Alternatively, it has been widely suggested that 7K may be derived exogenously from the diet (9), as it is the major oxysterol present in processed cholesterol-rich foods (10).

Of the estimated 300–500 mg of dietary cholesterol ingested daily by adult humans consuming a mixed Western diet, it has been estimated that as much as 1% of the cholesterol is oxidized (11). The most commonly detected oxysterols are the major cholesterol autoxidation products 7K, 7α-hydroxycholesterol (cholest-5-en-3β,7α-diol), 7β-hydroxycholesterol (7βOH, cholest-5-en-3β,7β-diol), cholesterol α-epoxide, and β-epoxide in foods that include eggs and egg products, meat and meat products, tallow, and dairy products (10,12–15). One example of a carefully executed measurement of 7K in three types of cheeses reported that 7K was consistently the most common oxysterol present, comprising 70% of that detected (10).

Studies in humans (16,17) and animals (18,19) have demonstrated that dietary oxysterols can indeed be absorbed by the intestine and then transported in chylomicrons. However, estimates of the extent to which oxysterols are absorbed vary greatly ranging from as little as 6% to 93% (20). There are also discrepancies for individual oxysterols between species. One study in humans reported that 7K was apparently absorbed preferentially even though it was not the most concentrated oxysterol in the test meal (17), whereas in rats, 7βOH was found to be absorbed to the greatest extent while 7K was least absorbed (19). On balance, however, it appears that oxysterols may not be absorbed as well as cholesterol (2).

Previous investigations showed that 7K can be metabolized *in vivo* to a host of water-soluble, bile acid-like products in rats (21,22), guinea pigs (21), and rabbits (23), although their identities were largely unknown. Recently, using a rat model, we confirmed these results and extended them using a novel mode of administration. In a dual-label approach, $^{14}$C-7K and $^{3}$H-cholesterol contained as steryl esters within a

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Abbreviations: BTSFA plus 1% TMCS, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane; CsA, cyclosporin A; DMEM, Dulbecco's modified Eagle's medium; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; $IC_{50}$, concentration level at which an enzyme's activity is inhibited by 50%; 7K, 7-ketocholesterol (cholest-5-en-3β-ol-7-one); LDL, low-density lipoprotein; 7βOH, 7β-hydroxycholesterol (cholest-5-en-3β, 7β-diol); 19OH, 19-hydroxycholesterol (cholest-5-en-3β-19-diol); 27OH, 27-hydroxycholesterol [(25R)-cholest-5-en-3β, 26-diol]; 27OH-7K, 27-hydroxy-7-ketocholesterol [(25R)-cholest-5-en-3β, 26-diol-7-one]; PBS, Dulbecco's phosphate buffered saline; TMS, trimethylsilyl; TMSOH trimethylsilylhydroxide; UV, ultraviolet.

modified lipoprotein vehicle were injected intravenously, thereby allowing us to compare the behavior of these two sterols directly. In that study, 7K was cleared from the liver and excreted into the intestine as water-soluble products (presumably bile acids) much more rapidly than cholesterol delivered simultaneously (24). We have reproduced these findings in a mouse study (25), in which 7K and cholesterol were delivered simultaneously in a chylomicron remnant-like emulsion. This vehicle models delivery of dietary oxysterols without the complications of intestinal absorption; once absorbed from the diet, oxysterols, including 7K, are incorporated into chylomicrons (17–19) and after lipolysis, their remnants are rapidly cleared by the liver (26–28). In that study, we showed that 24 h after injection, a substantial portion of the injected 7K-derived radioactivity had been excreted in the feces as water-soluble metabolites.

Conversion to bile acids is the major pathway for the excretion of cholesterol. Currently, there are two known pathways of bile acid biosynthesis: the "classical" and the "alternative" pathways (29–31). Since 7K and cholesterol have identical structures but for the addition of one oxygen functional group at C-7, it is conceivable that they may be catabolized by common elements of these pathways. However, owing to the C-7 hydroxyl group, it is unlikely that cholesterol 7α-hydroxylase, the rate-limiting enzyme of the classical pathway, will be able to act upon 7K. Furthermore, cholesterol 7α-hydroxylase is known to have very high substrate specificity and to be competitively inhibited by 7K (32–34). We hypothesized therefore that the initial step in the metabolism of 7K to bile acids (water-soluble products) is catalyzed by sterol 27-hydroxylase, an enzyme unimpeded by any functional groups on 7K, and the initial enzyme of the alternative pathway. We wished to test this hypothesis in a human cell-line that is known to express the two key enzymes of bile acid biosynthesis (cholesterol 7α-hydroxylase and sterol 27-hydroxylase) (35). Therefore, we employed the hepatoblastoma cell-line HepG2. These cells are known to synthesize the primary human bile acids (35–39) albeit in a pattern distinct from that of adult human liver (35–37,39). A cell-line was chosen over primary cultures since they are more readily available and maintained. Moreover, primary cultures of hepatocytes can still display substantial loss of liver-specific function (40).

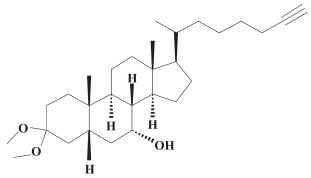
Herein we provide evidence, using the HepG2 human hepatoblastoma cell-line, that 7K is indeed metabolized by sterol 27-hydroxylase. This metabolism can be inhibited with a published inhibitor of sterol 27-hydroxylase, cyclosporin A (CsA) (41–44) and with two novel, more potent inhibitors that we report here for the first time.

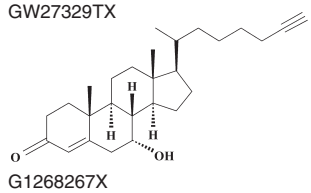
## EXPERIMENTAL PROCEDURES

*Materials.* All solvents were of high-performance liquid chromatography (HPLC) grade (EM Scientific, Kilsyth, Australia; Mallinckrodt, Mulgrave North, Australia; ICN Biomedical, Seven Hills, Australia). General reagents were of analytical reagent (AR) grade or higher and were supplied by Sigma-Aldrich (Castle Hill, Australia) or BDH (Kilsyth, Australia). The two compounds GW273297X and GI268267X (Scheme 1) were a generous gift of GlaxoSmithKline. All other reagents were of the highest grade commercially available and were obtained from the supplier indicated: 27-hydroxycholesterol [27OH; (25*R*)-cholest-5-en-3β,26-diol] (Steraloids, Wilton, NH); fetal bovine serum, L-glutamine, Dulbecco's modified Eagle's medium (DMEM) (Trace Biosciences, Castle Hill, Australia); bicinchoninic acid protein assay kit, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BTSFA plus 1% TMCS) (Pierce, Sydney, Australia); complete protease inhibitor cocktail (Boehringer Mannheim, Castle Hill, Australia); $^{3}$H-7K (American Radiolabeled Chemicals, St. Louis, MO); $^{3}$H-cholesterol (Amersham Pharmacia Biotech, Castle Hill, Australia); liquid scintillation reagents (Canberra-Packard, Mt. Waverly, Australia); and benzoyl chloride, thin-layer chromatography plates (silica gel, 250 μm layer thickness, 5–17 μm particle size, 6 nm pore size), isocitrate, hydroxypropyl-β-cyclodextrin, CsA, Dulbecco's phosphate buffered saline (PBS), Triton X-100, 19-hydroxycholesterol (19OH, cholest-5-en-3β,19-diol), penicillin G, and streptomycin (Sigma-Aldrich).

*Synthesis of 27-hydroxy-7-ketocholesterol (27OH-7K).* 27OH-7K [(25*R*)-cholest-5-en-3β,26-diol-7-one] was synthesized by a modified version of the method described by Kan *et al.* (45), which was based on the methods of Chicoye *et al.* (46) and Parish *et al.* (47). In this method 27OH was used as the starting material rather than cholesterol. Briefly, to 5 mg 27OH, 30 μL benzoyl chloride was added to benzoylate and protect the hydroxyl groups, followed by 300 μL dry pyridine. The preparation was incubated at 60°C for 3 h and then underwent washing and extraction followed by oxidation and isolation as described previously (24). The product was purified by thin-layer chromatography with hexane/ethyl acetate (50:50, vol/vol) as the mobile phase and extracted from the silica with methanol/chloroform (20:80, vol/vol) followed by evaporation under vacuum.

*Characterization of 27OH-7K by gas chromatography–mass spectrometry (GC–MS) and ultraviolet (UV) spectroscopy.* 27OH-7K synthesized from 27OH and $^{14}$C-27OH-7K




**SCHEME 1**

biosynthesized by mitochondria (see below) were analyzed by GC–MS using a Hewlett-Packard (Agilent Technologies, Forest Hill, Australia) 5890 Series II gas chromatograph fitted with a BPX5 column (30 m, 0.25 μm film thickness; SGE, Ringwood, Australia) and a Hewlett-Packard 5989A mass spectrometer. The injector, GC–MS interface, and MS source were all set to 300°C. The temperature program was set at 180°C for 1 min, increased at a rate of 20°C per min to 250°C and 5°C per min to 300°C, and held at 300°C until the stop time at 25 min. The samples were prepared for GC–MS by conversion to the trimethylsilyl (TMS) ether derivatives using BTSFA plus 1% TMCS according to the manufacturer's instructions. A mixed oxysterol standard (5 nmol/μL per oxysterol) was injected (1.5 μL) at a split ratio of 12:1. Each of the 27OH-7K samples was injected in 2-μL volumes with the purge valve closed for 1 min. Normal-phase HPLC was conducted as described previously (8). Briefly, the system comprised a mobile phase of composition hexane/isopropanol/acetonitrile (94.1: 5.6: 0.3, by vol) run at 1.0 mL/min using a Shimadzu (South Rydalmere, Australia) LC-10AT pump, Shimadzu SIL-10A integrated sample cooler and auto-injector, and an Ultramex 3 silica column (3 μm, 150 × 4.6 mm, Phenomenex, Thornleigh, Australia) and 3-μm silica guard column (25 × 4.6 mm). Prior to use, the mobile phase was degassed under vacuum with sonication and continuously sparged with helium during analyses. UV spectra were collected using a Shimadzu SPD-M10AVP diode array detector for both the synthesized 27OH-7K and the $^{14}$C-labeled 27OH-7K produced by HepG2 mitochondria. A mixed oxysterol standard (100 nmol per oxysterol) was injected. Samples were evaporated under vacuum and redissolved in isopropanol/heptane (5:95, vol/vol) for normal-phase HPLC unless otherwise noted.

*Cell culture.* HepG2 cells were cultured in 75 cm$^2$ flasks in DMEM supplemented with 10% (wt/vol) heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 50 U/mL penicillin G, and 50 U/mL streptomycin. Experiments were conducted with confluent cells. The viability of HepG2 cells was determined after 24 h incubation by assay of the release of intracellular lactate dehydrogenase as described previously (48).

*Assay for metabolism of 7K.* $^{14}$C-7K was synthesized as described previously and added to mitochondria as a saturated hydroxypropyl-β-cyclodextrin solution (45% wt/vol, 286 mmol/L) to test the sterol 27-hydroxylase activity toward 7K. Mitochondria were isolated from HepG2 cells according to the method of Winegar and colleagues (44). To a portion of mitochondrial suspension (500 μL), reaction mixture (100 μL) containing isocitrate (5 mmol/L) and isocitric dehydrogenase (1 U) was added and made up to volume (1.0 mL final) with potassium phosphate buffer (100 mmol/L, pH 7.7). At the appropriate time, the reaction was stopped by the addition of methanol (1.3 mL) and extracted by the addition of chloroform (2.7 mL). Samples were then evaporated under vacuum and prepared for either GC–MS or HPLC analysis.

$^3$H-7K in ethanol was added to the media of cell cultures. CsA was used at a final concentration of 10 μmol/L (41–44). GW273297X and GI268267X (Scheme 1) were used at final concentrations of 1.0 and 7.5 μmol/L, respectively. All three inhibitors were added in ethanol, and the final concentration of ethanol, including radiolabeled sterol, did not exceed 0.2% (by vol). At the appropriate time, medium was removed for lipid extraction and analysis of metabolic products in both the aqueous and nonpolar phases. The cells were washed three times in PBS at 37°C and extracted into 0.1% (by vol) Triton X-100 containing complete protease inhibitor cocktail or into 0.2 mol/L sodium hydroxide prior to lipid extraction and HPLC analysis. Mitochondrial and cellular protein concentrations were determined by the bicinchoninic acid method according to the manufacturer's instructions.

*7K metabolism determined by liquid scintillation and lipid analyses of media and cells by normal-phase HPLC.* To test the hypothesis that 7K is metabolized to bile acids or similar products, we determined the level of water-soluble radioactivity as a surrogate measure of bile acids. 7K and its initial metabolic product 27OH-7K (as demonstrated herein) are both chloroform-soluble, whereas bile acids partition into the aqueous phase when subjected to extraction by the method of Folch *et al.* (49) at neutral pH. Media and cells were extracted, and the two phases evaporated under vacuum to ensure removal of residual chloroform. Water-soluble samples were redissolved in water alone or 50% (by vol) methanol, scintillation fluid was added (Ultima Gold or Hionic Fluor, respectively) and samples assayed for radioactivity by liquid scintillation counting. Normal-phase HPLC was conducted as described previously (8) using the parameters indicated above. The system for radiometric detection comprised a Rheodyne manual injector (Rohnert Park, CA), LKB (Amersham Pharmacia Biotech) Bromma 2150 pump, LKB 2151 Variable Wavelength Monitor at 210 nm in series with a Canberra-Packard Series A-100 Radiomatic Flo-one Beta Radio-Chromatography Detector using Ultima-Flo M scintillant (scintillant to effluent solvent ratio of 2).

## RESULTS

*Synthesis and characterization of 27OH-7K.* The putative 27OH-7K was well resolved from other common oxysterols under the conditions employed for GC (Fig. 1A) and normal-phase HPLC (not shown). The other sterols (cholesterol, 7α-hydroxycholesterol, 7βOH, 7K, 19OH, and 27OH) analyzed by GC eluted at least 4 min earlier than 27OH-7K. This synthesized standard co-chromatographed on GC with a peak isolated from HepG2 mitochondria that had been incubated with $^{14}$C-7K (Fig. 1B). MS confirmed the structure of the product synthesized by oxidation of 27OH to be the 27OH-7K. The molecular ion (*m/z* 560) and three significant ions (*m/z* 545, 470, 129) (Fig. 1C) confirmed the finding of one previous publication . Other significant ions were found at *m/z* 455, 365, 269, 187, 174, 161, and 75. The fragment ions at *m/z* 545 (M – 15), 470 (M – 90), 455 (M – 15-90), and 365 (M – 15 – 2 × 90) were attributed to loss of methyl and TMS hydroxide (TMSOH) groups, and the fragment ion at *m/z* 269

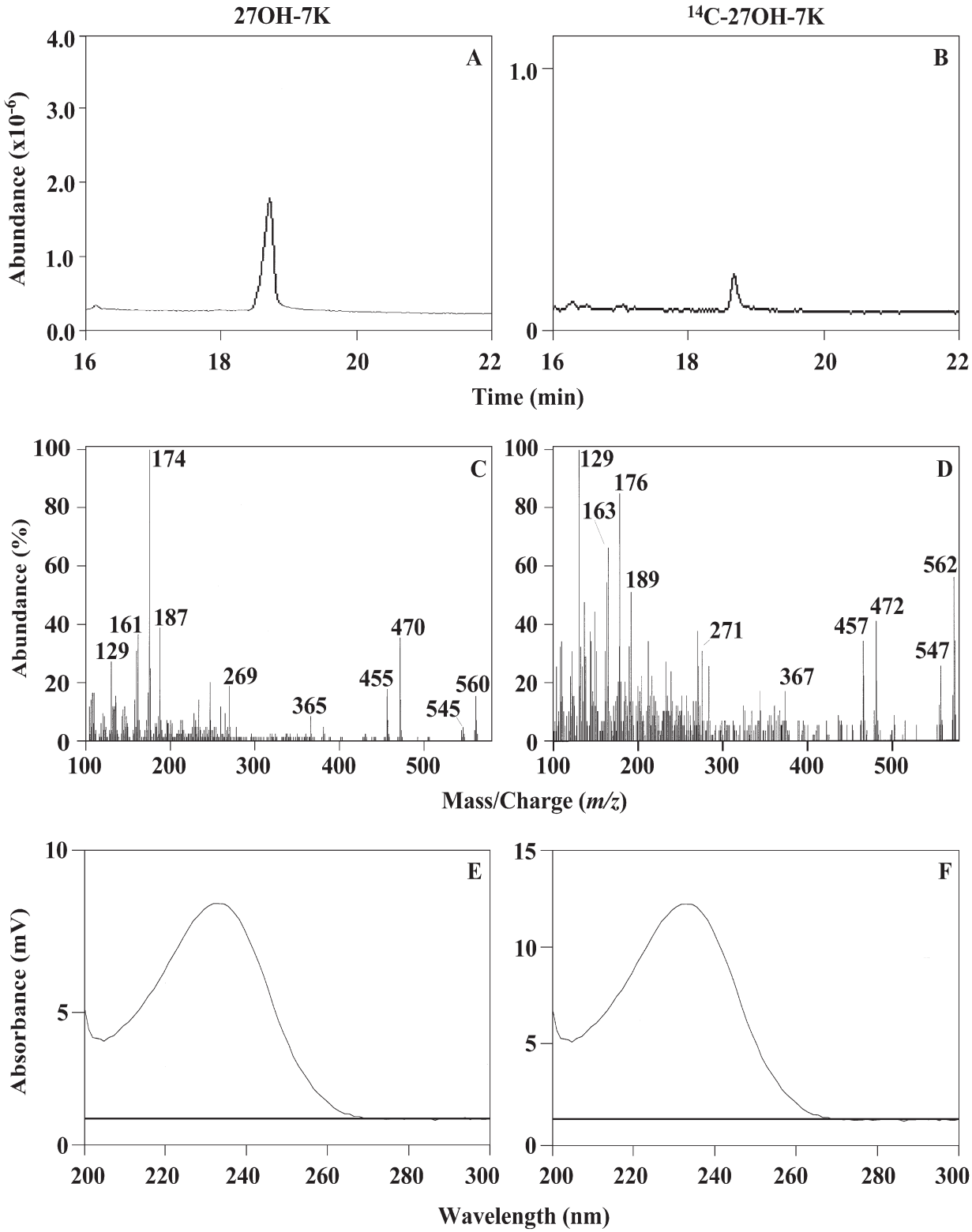



**FIG. 1.** Characterization of 27OH-7K synthesized from 27OH and $^{14}$C-27OH-7K biosynthesized by mitochondria from $^{14}$C-7K. Standard 27OH-7K was well-separated from other oxysterols when analyzed by gas chromatography–mass spectrometry (GC–MS) (panel A). $^{14}$C-27OH-7K generated by mitochondria had an identical retention time to synthetic 27OH-7K on both GC (panel B) and high-performance liquid chromatography (HPLC) (HPLC shown in Fig. 2). The MS data confirm the structure of standard 27OH-7K (panel C). The molecular ion was at *m/z* 560. $^{14}$C-27OH-7K had a similar mass spectrum (panel D) to the synthetic 27OH-7K, but the molecular ion and fragment ions above *m/z* 129 had mass M + 2 due to the $^{14}$C-label at C-4. Sterols were analyzed as their trimethylsilyl derivatives for GC–MS. The ultraviolet (UV) spectra of synthetic 27OH-7K (panel E) and $^{14}$C-27OH-7K (panel F) were indistinguishable and were collected using normal-phase HPLC employing a diode array detector. Methods are as described in the Experimental Procedures section. Abbreviations: 27OH, 27-hydroxycholesterol; 7K, 7-ketocholesterol; 27OH-7K, 27-hydroxy-7-ketocholesterol.

was attributed to the loss of the side chain as the TMS ether and loss of the second TMSOH. The peak generated by mitochondria had a comparable mass spectrum (Fig. 1D) to the standard 27OH-7K and had fragment ions corresponding to M + 2 compared with the synthesized 27OH-7K, for all ions of interest above *m/z* 129. The increase in fragment mass was attributed to the $^{14}C$ label at C-4. In addition, the synthesized standard (Fig. 1E) had an identical UV spectrum with the $^{14}C$-labeled compound (Fig. 1F). These data confirm the identity of the synthesized standard 27OH-7K and indicate that 7K can act as a substrate for sterol 27-hydroxylase in HepG2 mitochondria.

*Mitochondrial and cellular metabolism of 7K.* HepG2 cells were cultured to investigate the metabolism of 7K by human liver cells. After 24 h of incubation, no difference was found in the viability, as determined by lactate dehydrogenase release into the media, of control cells (72 ± 4%, mean ± standard deviation) vs. vehicle-treated cells (76 ± 4%, ethanol 0.2%, by vol). Sterol 27-hydroxylase, the enzyme of interest, is a mitochondrial cytochrome P450 enzyme (29,51) that is located on the inner membrane (52). For this reason we isolated mitochondria from HepG2 cells as a partially purified source of sterol 27-hydroxylase. The activity of this enzyme on cholesterol is such that it can initially introduce a hydroxyl group on the terminal carbon atom to produce 27OH. Thus, we determined the extent of metabolism of cholesterol and 7K by mitochondria through the measurement of 27OH and 27OH-7K, respectively. The primary pathway for excretion of cholesterol is *via* conversion to bile acids that are water-soluble. Therefore, extensive metabolism of the two sterols by intact cells was determined by the measurement of water-soluble radioactivity, where products of 7K were assumed also to be bile acid-like compounds or 7-oxo analogs of cholestenoic acid. The mitochondria, cells, and media were each subjected to lipid extraction and analyzed by normal-phase HPLC with radiometric detection. In a similar manner to GC, the standard 27OH-7K (Fig. 2A) co-chromatographed on normal-phase HPLC with $^{14}C$-27OH-7K produced by mitochondria (Fig. 2B). In addition to being detected in HepG2 mitochondria, 27OH-7K was also detected in whole HepG2 cells (Fig. 2C), and media (Fig. 2D). The relative levels were lower, however, when compared with the isolated mitochondria, a semipurified source of sterol 27-hydroxylase (*c.f.* Fig. 2B). Apart from 27OH-7K, two other chloroform-extractable products were identified by co-elution with authentic standards. These were 7K esters, detected in cells (Fig. 2C), and 7βOH, detected in cells and media (Figs. 2C,D). Both 7K esters (53) and 7βOH (21,22,50,54–58) have been documented previously as products of 7K. A number of other chloroform-extractable products were detected in both cells and media but remain unidentified. Two of these products made sizeable contributions to the products secreted into the media (Figs. 2D, 3B). The results of the radiometric characterization of $^{3}H$-7K metabolites are presented in Figure 3. The coefficient of variation for at least three independent experiments was typically less than 10% (e.g., Fig. 4B). Water-soluble products were formed early and extensively (Fig. 4A), comprising 19% of added radioactivity at 8 h, but the other products were negligible at 1 h (Fig. 3B), even in comparison with 27OH-7K which was readily detected. Thus, 27OH-7K, was the first lipid-soluble product detected in the system.

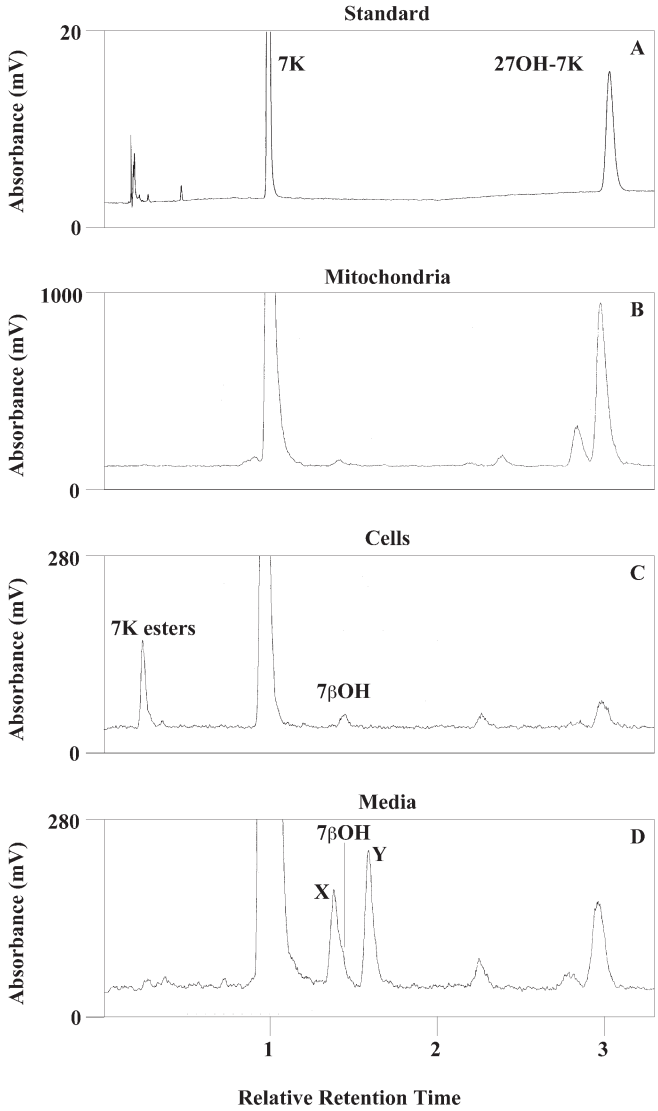



**FIG. 2.** Analysis of 7K metabolites generated by HepG2 mitochondria or intact HepG2 cells. Mixed unlabeled oxysterol standards, including synthesized 27OH-7K, were analyzed using UV detection at 234 nm (panel A) and 210 nm (not shown). Mitochondria were isolated from HepG2 cells and incubated with $^{14}C$-7K (delivered in β-cyclodextrin) in reaction mixture for 8 h then extracted, and the lipid fraction was subjected to normal-phase HPLC analysis with UV detection (not shown) and radiometric detection (panel B). Intact HepG2 cells were incubated with $^{3}H$-7K (delivered in ethanol) for 8 h, and the lipid fractions of the cells (panel C) and media (panel D) were subjected to analysis with radiometric detection. 7K esters and 7β-hydroxycholesterol (7βOH) were detected in cells by co-elution with authentic standards (not shown). Two unidentified chloroform-soluble compounds (peaks X and Y) were detected in the media sample. 7βOH appears as a shoulder on Peak X. Methods are as described in the Experimental Procedures section. Cells and media chromatograms are scaled to show metabolites generated and excreted by intact cells. For abbreviations see Figure 1.

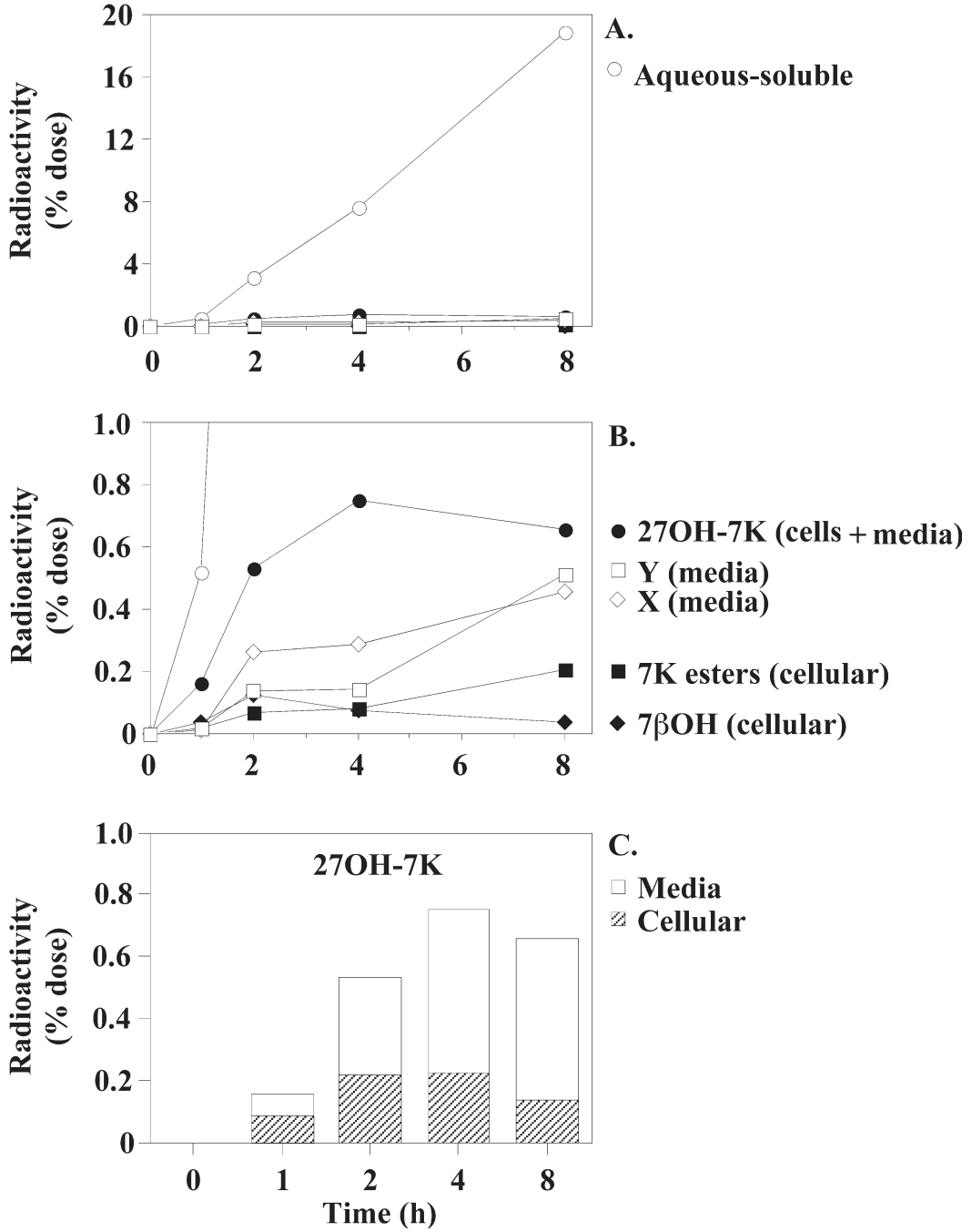



**FIG. 3.** Metabolism of 7K by HepG2 cells. $^{3}$H-7K was incubated with HepG2 cells for periods of up to 8 h. At the appropriate time, cells and media were subjected to lipid extraction. The lipid portions were analyzed by normal-phase HPLC with radiometric detection to detect 7K metabolites, and the aqueous portion (○) was assayed for radioactivity by liquid scintillation counting (panel A). Panel B is an enlargement of the data presented in panel A and shows that 27OH-7K (●, total in cells plus media) was the first lipid-soluble product detected. 7β-Hydroxycholesterol (◆) and 7K esters (■) were detected in the cellular extract. Two unidentified products (X and Y, ◇ and □, respectively) were detected in the media extracts. Panel C indicates the proportions of 27OH-7K detected in the media (open) and in cells (hatched). Data for water-soluble metabolites are representative of three or more experiments with a coefficient of variation <10%. Data for other metabolites in the system were based on a single determination from a pooled sample to achieve sufficient sensitivity for detection by radiometric HPLC. For abbreviations see Figure 1.

Although 27OH-7K is chloroform-extractable (quantitatively recovered, data not shown), it is a relatively polar sterol. It is therefore not surprising that in cell incubations, it was detected predominantly in the media. There was an increasing proportion of this product in the media with increased duration of incubation (Fig. 3C). By 8 h, ~80% of total 27OH-7K was found in the media, indicative of efficient excretion.

*Inhibition of sterol 27-hydroxylase with GW273297X and GI268267X.* In agreement with previous studies (41–44),

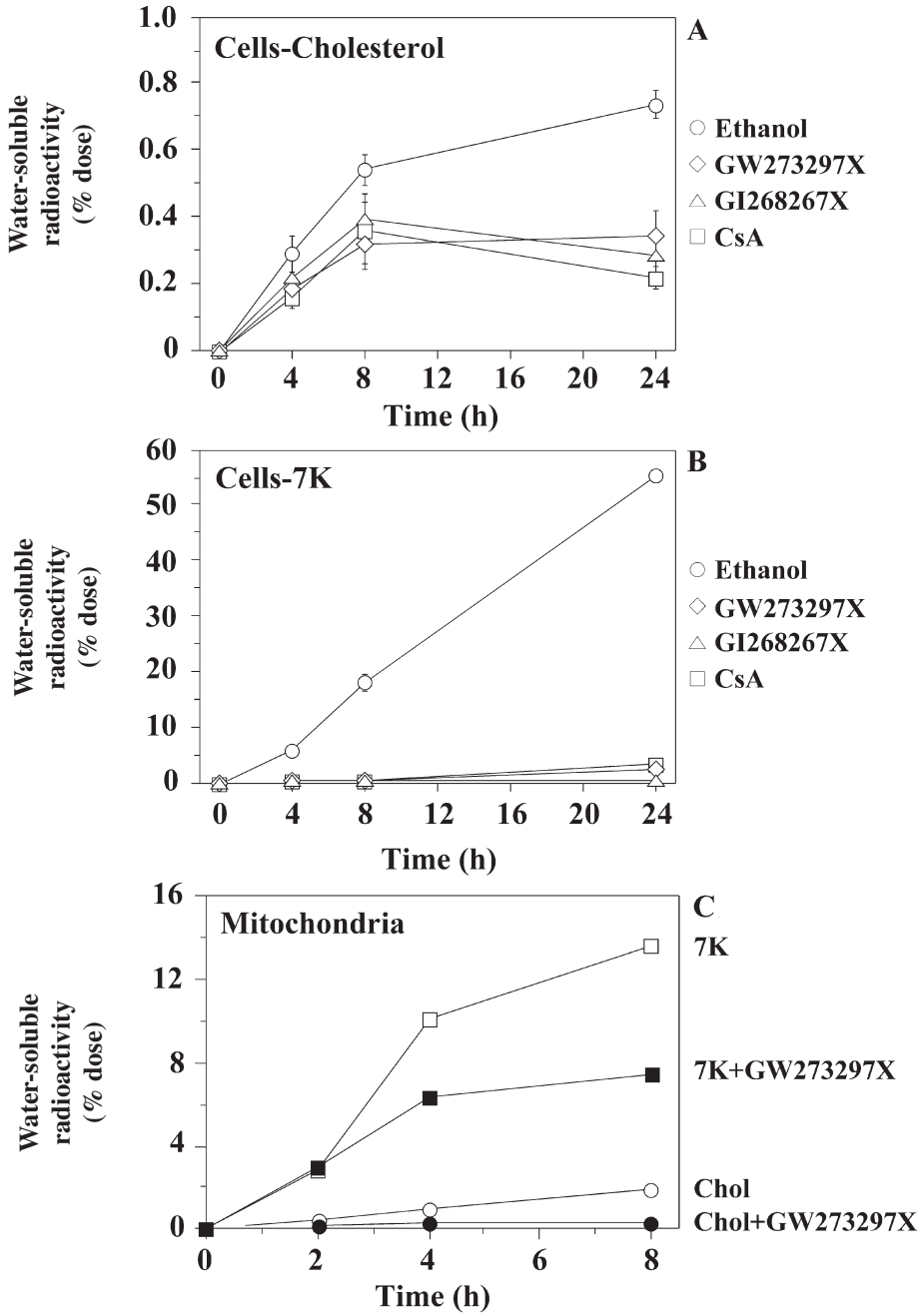



**FIG. 4.** Metabolism of 7K and cholesterol (Chol) by intact HepG2 cells and isolated mitochondria in the presence of inhibitors of sterol 27-hydroxylase. HepG2 cells were incubated for up to 24 h with $^{3}$H-cholesterol (panel A) or $^{3}$H-7K (panel B) in the absence (○, ethanol only, 0.2%, by vol, final concentration) or presence of cyclosporin A (CsA) (□, 10 μmol/L), GW273297X (◇, 1 μmol/L), or GI268267X (△, 7.5 μmol/L). Panel C: Because sterol 27-hydroxylase is a mitochondrial cytochrome P450, metabolism of $^{14}$C-7K (□,■) to 27OH-7K or $^{14}$C-cholesterol (○,●) to 27OH (by mitochondria isolated from HepG2 cells) was tested in the absence (open symbols) or presence (filled symbols) of GW273297X (1 μmol/L). Methods are as described in the Experimental Procedures. Panels A and B show data that are expressed as mean ± standard deviation ($n$ = 3).

sterol 27-hydroxylase activity directed toward cholesterol was inhibited with CsA (Fig. 4A). Inhibition of sterol 27-hydroxylase activity by GW273297X, GI268267X, and CsA was assayed using isolated mitochondria treated with $^{14}$C-cholesterol, and the 50% inhibition values ($IC_{50}$) were determined to be 40, 300, and 874 nmol/L, respectively (Winegar, D.A., unpublished data). GW273297X and GI268267X were used experimentally at final concentrations of 1.0 and 7.5 μmol/L, respectively, while CsA was used at 10 μmol/L as published previously (41–44). At concentrations up to 10 μmol/L, GW273297X and GI268267X were found not to inhibit cholesterol 7α-hydroxylase activity and thus were deemed specific for sterol 27-hydroxylase (Winegar, D.A., unpublished data).

*Inhibition of cellular and mitochondrial metabolism of 7K with CsA, GW273297X, or GI268267X.* The metabolism of cholesterol (Fig. 4A) to water-soluble products was inhibited by all three compounds tested (GW273297X, GI268267X, and CsA), although its absolute extent of metabolism was much less than that of 7K (Fig. 4B). The metabolism of 7K by HepG2 cells was also ablated by incubation with 10 µmol/L CsA (Fig. 4B). To further test whether 7K is metabolized by sterol 27-hydroxylase, we incubated HepG2 cells with two more potent, novel inhibitors of this enzyme, GW273297X and GI268267X. Incubation of HepG2 cells with 7K in the presence of these inhibitors also dramatically blocked the production of water-soluble metabolites over 24 h (Fig. 4B). Based on this result and the lower $IC_{50}$, later experiments were conducted using GW273297X as the inhibitor of choice. No difference was found in the viability (lactate dehydrogenase release) after 24 h of cells treated with 1 µmol/L GW273297X (76 ± 2%) vs. control or vehicle-treated (ethanol 0.2%, by vol) cells (72 ± 4%, 76 ± 4%, respectively, mean ± standard deviation).

Mitochondria were isolated from HepG2 cells to provide a partially purified source of the enzyme and were then incubated with $^{14}$C-7K or $^{14}$C-cholesterol solubilized in a cyclodextrin solution in the presence or absence of inhibitor. As already shown in Figure 2B, 27OH-7K was detected in mitochondrial incubations. GW273297X virtually abolished the production of 27-hydroxycholesterol and reduced the production of 27OH-7K by ~50% (Fig. 4C). Furthermore, we were able to show that mitochondria were able to produce low levels of water-soluble products in addition to their production of 27OH-7K (data not shown). GW273297X did not affect the net cellular uptake of 7K, although the overall proportion metabolized to other chloroform-extractable plus aqueous-soluble products was substantially reduced (Fig. 5).

*Metabolism of 7K vs. cholesterol by sterol 27-hydroxylase.* For the same amount of radiolabeled sterol added, cholesterol was metabolized to a far lesser extent than 7K by both intact HepG2 cells (Figs. 4A and 4B, respectively) and the mitochondrial fraction (Fig. 4C). After addition of equivalent amounts of $^{14}$C-7K or $^{14}$C-cholesterol to HepG2 mitochondria, the resultant 27OH had a specific activity that was one-tenth that of 27OH-7K, reflecting the contribution from endogenous cholesterol in the mitochondria. However, allowing for the specific activities, twice as much 27OH-7K was produced as 27OH on a mass basis (5.4 and 2.5 nmol/mg mitochondrial protein per 8 h, respectively), indicating a greater rate of action of sterol 27-hydroxylase on 7K compared to cholesterol.

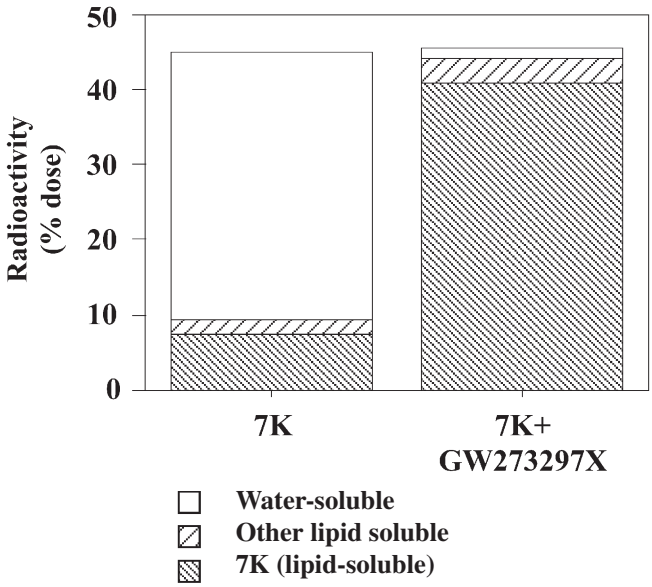



**FIG. 5.** Effect of GW273297X on the metabolic fate of 7K in intact HepG2 cells. HepG2 cells were incubated for 24 h with $^{3}$H-7K in the absence or presence of GW273297X (1 µmol/L). Cells and media were subjected to lipid extraction. The total water-soluble radioactivity (open) was measured by liquid scintillation counting while 7K (fine hatch) and other lipid-soluble radioactivity (coarse hatch) were determined by normal phase high-performance liquid chromatography with radiometric detection.

## DISCUSSION

We recently showed that metabolism of 7K, a quantitatively important oxysterol in the human diet and atherosclerotic lesions, occurred at a greater rate and to a greater extent than that of cholesterol delivered simultaneously to rats or mice. Therefore, our initial experiments were conducted to determine whether human cells were capable of similar metabolic processes. For these experiments we used the human HepG2 hepatoblastoma cell-line and clearly demonstrated that human liver cells are able to extensively metabolize 7K to water-soluble products and that this occurs at a greater rate than for cholesterol.

*7K serves as a substrate for sterol 27-hydroxylase.* The primary objective of this study was to test the hypothesis that 7K is at least initially metabolized by the defining enzyme of the alternative bile acid pathway, sterol 27-hydroxylase. Since sterol 27-hydroxylase is a mitochondrial cytochrome P450 enzyme, we isolated mitochondria from HepG2 cells to determine if 7K can serve as a substrate for this enzyme. The product, 27OH-7K, was identified in mitochondria incubated with 7K by comparison with a synthetic standard whose identity was confirmed by GC–MS and HPLC–UV spectroscopy. It was also detected in whole cell preparations, particularly in the media, along with other metabolic products that had been rapidly generated from 7K. 27OH-7K, however, was the first lipid-soluble product generated. This observation is consistent with the contention that sterol 27-hydroxylase catalyzes the initial catabolism of 7K since, intuitively, conversion of a lipids to a water-soluble product requiring a number of enzymatic steps would initially result in the generation of a more polar but lipid-soluble product. Furthermore, it highlights the speed by which 7K is acted upon by this metabolic pathway. Given the substrate availability, the fact that this product's generation reaches a plateau after 4 h further suggests that it is an intermediate product in the generation of water-soluble

products of 7K metabolism. It is likely also that this is the case for the unidentified products X and Y detected in the media of cells treated with 7K. In accordance with our results, two previous reports detected the 27-hydroxylated product when human fibroblasts (43) or human monocyte-derived macrophages (57) were treated with 7K. These data support the contention that the sterol 27-hydroxylase pathway is a mechanism designed to rid cells of excess sterols including this potentially harmful oxysterol.

*Inhibition of metabolism of 7K with CsA and two novel sterol 27-hydroxylase inhibitors, GI268267X and GW273297X.* Having established that 7K can be acted upon by sterol 27-hydroxylase in HepG2 cells, we further tested the hypothesis that 7K is metabolized by this pathway by using a previously published inhibitor of sterol 27-hydroxylase activity, CsA (41–44). However, CsA is better known for its effects as a potent immunosuppressant drug. Therefore, we also tested two novel inhibitors of sterol 27-hydroxylase, GW273297X and GI268267X, which are effective at lower concentrations than CsA ($IC_{50}$: 40 and 300 nmol/L vs. 874 nmol/L, respectively). These inhibitors, at lower concentrations, abolished the production of water-soluble products of 7K to the same extent as CsA. GW273297X also greatly diminished the production of 27OH-7K in mitochondria incubated with 7K.

The lower level of inhibition of 27OH-7K generation (Fig. 4C), compared with 27OH, may reflect the fact that 7K is able to move into and out of membranes at a greater rate than cholesterol (59). That is, simultaneous addition of inhibitor and sterol, as in this experiment, allowed a certain amount of 7K to be acted upon prior to the entry of the inhibitor into the mitochondrion. Mitochondria were able to produce small amounts of water-soluble products themselves. Presumably this is 3β-hydroxy-7-oxo-5-cholestenoic acid, the 7-oxo analog of cholestenoic acid that has been reported to be produced from 7K by fibroblasts in one previous publication (50). Since mitochondria can metabolize 7K to 27OH-7K (and even perhaps to the water-soluble carboxylic acid), and since inhibition of sterol 27-hydroxylase results in a substantial reduction of metabolism of 7K by both intact HepG2 cells and the mitochondrial fraction, the sterol 27-hydroxylase pathway is clearly an important mechanism for the metabolism of 7K.

In addition to 7K, a variety of other oxysterols have been detected in cholesterol-rich foodstuffs, predominantly those with their oxygen functional groups also present on the B-ring of the sterol (2). We speculate that sterol 27-hydroxylase may also be involved in the metabolism of other dietary oxysterols.

*Implications for dietary oxysterols.* Some oxysterols, including 7K, are absorbed from the diet and appear in triacylglycerol-rich lipoproteins in both rats (18,19) and humans (17). Thus far, however, investigations have been unable to demonstrate directly the accumulation in the artery wall of 7K derived from the diet or from intravenous administration (61,62). In light of the finding from this study that a human hepatocyte cell-line can efficiently metabolize 7K, it will be of great interest to see whether future studies can determine to what extent (if at all) dietary oxysterols contribute to the oxysterols found in atherosclerotic lesions.

This is the first study to identify the human mitochondrial sterol 27-hydroxylase as the enzyme responsible for the metabolism of 7K in liver cells. The initial 27-hydroxylated product of 7K was detected in both mitochondrial preparations and whole cell cultures. Indeed, this product was the first lipid-soluble metabolite produced by cells. The production of 27OH-7K was greatly attenuated by pharmacological inhibition in mitochondrial preparations and intact cultured cells. Sterol 27-hydroxylase also appears to be responsible for the further conversion of 27OH-7K to more polar compounds in a manner analogous to that proposed for cholesterol *via* 27OH because we have demonstrated an inhibition of metabolism of 7K to water soluble products by the human hepatic cell line HepG2 using three different inhibitors of sterol 27-hydroxylase. Moreover, we recently showed that macrophages from a patient with cerebrotendinous xanthomatosis, lacking functional sterol 27-hydroxylase, did not metabolize 7K to water-soluble products in contrast to control macrophages (57,58). Sterol 27-hydroxylase-dependent metabolism of 7K appears to operate in the whole human since we recently showed greatly elevated plasma concentrations of 7K in this same patient with cerebrotendinous xanthomatosis (58). In the absence of hepatic tissue from patients suffering from this rare condition, it would be of interest to repeat these studies in primary human hepatocytes employing the new inhibitors of sterol 27-hydroxylase introduced in this report. Given the continuing interest in oxysterols as physiological regulators of cholesterol homeostasis (63–65), it is noteworthy that Cali and Russell (51),who first cloned the human gene of sterol 27-hydroxylase, stated that "it is conceivable that the enzyme may play an equally important role in inactivating oxysterols." This study has for the first time identified the human mitochondrial sterol 27-hydroxylase as the enzyme responsible for the metabolism of an oxysterol (other than 27OH) in human liver cells and therefore strongly supports such a contention.

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# Incorporation of α-Linolenic Acid and Linoleic Acid into Human Respiratory Epithelial Cell Lines

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**ABSTRACT:** Animal and human studies designed to examine the effects of α-linolenic acid (ALA) and linoleic acid (LA) supplementation on the fatty acid composition of plasma and tissues have demonstrated a marked difference in incorporation into phospholipids of these 18-carbon precursors of the long-chain polyunsaturates. Whereas tissue phospholipid levels are linearly related to dietary ALA and LA, the levels of tissue LA can be 10-fold higher than tissue ALA even when dietary levels are equivalent. There is some dispute whether this disparity is due to ALA being more rapidly metabolized to its products or substantially oxidized by the liver, or whether LA but not ALA is readily incorporated into cellular phospholipids. We examined the level of incorporation of polyunsaturated fatty acids into human respiratory epithelial cell lines (A549, 16HBE) by determining the dose-dependent incorporation of ALA and LA as free fatty acid (5–150 μg FFA/mL). Cell membrane phospholipid ALA and LA were both increased up to ~20–30% total fatty acids, with a concomitant decrease predominantly in monounsaturated membrane fatty acids, before significant toxicity was observed (50 μg/mL). Our data support the concept that rather than any inherent inability by human cells to incorporate ALA into membrane phospholipids, the lack of ALA content in human and animal tissues *in vivo* is due to the rapid metabolism or oxidation of this fatty acid in the liver.

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Numerous animal and human studies examined the effects of α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) supplementation on the fatty acid composition of plasma and tissues. A common feature of these studies is the marked difference in the levels of these 18-carbon precursors of the long-chain polyunsaturates (LCPUFA) incorporated into plasma and tissue phospholipids. Whereas tissue phospholipid levels are linearly related to dietary ALA and LA, the levels of tissue LA can be 10-fold higher than tissue ALA even when dietary levels are equivalent (1–3). In humans, LA appears in high levels (15–30% total fats) in all plasma fractions and competes for incorporation with all long-chain (20- and 22-carbon) LCPUFA into cell membranes (4). In contrast, even when consumed in the same quantities, ALA appears in plasma fractions in only trace amounts (<3% total fats) (2), and there are no reports of ALA being incorporated in amounts comparable to LA into cell membrane phospholipids. A number of hypotheses have been proposed to explain this disparity. ALA may be more rapidly metabolized to its products or substantially oxidized by the liver (5,6).

LA is readily converted to arachidonic acid (AA) and incorporated into tissues in most rodent systems (7,8). Although ALA can be converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), dietary supplementation studies have shown little direct association between high intakes of ALA and increased tissue DHA or EPA (2,9). However, dietary ALA has been shown to decrease AA levels dramatically in lung phospholipids independent of increases in cellular ALA or its metabolites (10).

Patients receiving high n-6/n-3 ratio diets have been shown to have significantly decreased lung function, whereas 50% of those on low n-6/n-3 diets experienced improved lung function (11). As epithelial cells have been shown to play a key role in the regulation of response to respiratory disease it is important to determine the capacity for change within the composition of cell membrane fatty acids of these cells and to what extent these changes may affect the expression of the lungs' natural defense mechanisms. As a first step toward this aim, the level of incorporation of the PUFA ALA and LA in human respiratory epithelial cell lines *in vitro* was examined.

## EXPERIMENTAL PROCEDURES

*Cell lines.* Two human transformed respiratory epithelial cell lines were used: alveolar (A549) and bronchial (16HBE). Both cell lines were maintained in continuous culture prior to each experiment. Culture media for the A549 cells (Dulbecco's modified Eagle's medium; DMEM) and 16HBE cells (RPMI 1640) contained 2 mM L-glutamine, 50 U/mL penicillin, 37.5 U/mL streptomycin, and 5 and 10% heat-inactivated fetal bovine serum (FBS), respectively. The fatty acid content of these base media was consistent regardless of the percentage of FBS added. This fatty acid content is summarized in Table 1.

*Free fatty acid (FFA) incorporation.* Cells were seeded into six-well plates at a density of $4 \times 10^5$ cells/mL. Triplicate wells were supplemented with a range of concentrations from 5–150 μg/mL of ALA or LA FFA (Nu-Chek-Prep Inc., Elysian, MN)

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; FFA, free fatty acid; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acid; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid.

**TABLE 1**
**Total Lipid[a] Composition of Control Media, Compared with Medium Supplemented with 50 μg/mL ALA or LA as FFA[b] (% total fatty acids, *n* = 2)**

| | Control | +ALA | +LA |
|---|---|---|---|
| Total SATS | 44.9 | 12.0 | 11.4 |
| Total MONOS | 24.4 | 6.7 | 6.7 |
| n-7 | 5.7 | 1.6 | 1.5 |
| n-9 | 18.5 | 4.8 | 5.0 |
| 18:2n-6 | 3.6 | 1.3 | 75.3 |
| 20:3n-6 | 2.5 | 0.7 | 0.7 |
| 20:4n-6 | 8.9 | 2.4 | 2.2 |
| 22:5n-6 | 1.1 | 0.3 | 0.3 |
| Total n-6 | 19.9 | 5.6 | 79.3 |
| 18:3n-3 | 0.2 | 73.3 | 0.2 |
| 20:5n-3 | 0.5 | 0.1 | 0.1 |
| 22:5n-3 | 3.0 | 0.8 | 0.8 |
| 22:6n-3 | 4.6 | 1.0 | 1.0 |
| Total n-3 | 8.6 | 75.2 | 2.1 |

[a]The total lipid contribution of fetal bovine serum to the media in a 10% solution equals 17 μg/mL of which approximately 98% is phospholipid.
[b]ALA, α-linolenic acid; LA, linoleic acid; FFA, free fatty acid; total SATS, total saturated acids; total MONOS, total monounsaturated acids = sum of all monounsaturated fatty acids of chain length 11-24, as assessed by gas chromatography.

in 100% ethanol and incubated at 37°C in a 5% $CO_2$ humidified incubator until confluent (24–36 h). The amount of ethanol necessary to obtain these concentration ranges did not exceed 0.5% (vol/vol). Samples (6 mL) of each medium were reserved prior to and after each experiment for fatty acid analyses. At confluence all medium was removed from the wells, and triplicates were pooled and spun ($680 \times g$, 5 min) to pellet nonadherent cells. Triplicates of adherent cells were harvested with 0.25% trypsin/0.04% EDTA in phosphate-buffered saline (PBS), pooled, and added to the nonadherent cells before washing in PBS ($680 \times g$, 5 min). Total cells were then resuspended in 1.5 mL PBS for fatty acid extraction.

*Fatty acid analyses.* Cellular lipids were extracted with propanol/chloroform as described by Broekhuyse (12), and lipids in the culture media were extracted with methanol/chloroform as described by Bligh and Dyer (13). The phospholipid fraction of the cellular lipid extracts and the FFA, phospholipid, triglyceride, and cholesterol ester fractions of medium lipid extracts were separated by thin-layer chromatography using 3:1 petroleum spirit/acetone and 90:15:1 petroleum spirit/acetone/glacial acetic acid (+0.005% butylated hydroxyanisole), respectively. Lipids were transesterified in 1% $H_2SO_4$ in methanol for 3 h at 70°C as described previously (14). Fatty acid methyl esters were separated and quantified using a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA) and identified based on the retention time of authentic lipid standards (Nu-Chek-Prep Inc.).

*Cell viability.* The viability of cells post-FFA incubation was ascertained by the Cell Titer 96® $AQ_{ueous}$ One Solution Cell Proliferation Assay (Promega Corp., Madison, WI). Triplicate wells of a 96-well plate were seeded with $4 \times 10^4$ cells in 100 μL of each experimental FFA concentration in parallel to the FFA incubation experiment. At the time of cell harvesting from the six-well plates, an MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was added to each of the experimental wells and controls in the 96-well plate. The absorbance of colored formazan product produced through bioreduction by metabolically active cells was then read on an automatic plate reader at 490 nm. The viability index was determined by calculating the difference between the experimental wells and controls (no FFA).

*Statistical analysis*. Fatty acid data are presented as mean percentage ± SEM of the total fatty acids measured. The significance of fatty acid composition differences and cell viability index scores between ALA and LA supplementation concentrations was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* analysis using SPSS for Windows (SPSS Inc., Chicago, IL).

## RESULTS

*Incorporation of ALA and LA into respiratory epithelial cells.* Cell membrane phospholipid ALA and LA were increased dose dependently in both A549 and 16HBE cell lines up to a maximal level of incorporation (Fig. 1A–D). LA is readily incorporated into both cell lines at low FFA concentrations, reaching 50% of maximum at 5–10 μg/mL and maximal incorporation at 20–30 μg/mL (Figs. 1B,D). The incorporation of ALA reached 50% at 10–20 μg/mL with maximal incorporation being attained at ~30 μg/mL (Figs. 1A,C). From gas chromatograms, 88–107% of all added fatty acid could be accounted for as incorporated phospholipid or in the media as FFA. As ALA and LA FFA were recovered from the media at similar levels postincubation, the results indicate a slightly higher substrate requirement for equivalent incorporation of ALA than LA in both cell lines.

*Changes in cell membrane PUFA in response to increased ALA and LA incorporation.* The greatest change in membrane composition resulting from incubation with high levels (50 μg/mL) of ALA or LA was a large incorporation of these PUFA into both cell lines, offset by a decrease in monounsaturated fatty acid levels (Table 2). Decreases in the n-7 and n-9 fatty acids as well as a small decrease in DHA were also observed with incorporation of both PUFA in both cell lines. LA incorporation also caused a decrease in the membrane level of EPA and a resultant overall decrease in total n-3 fatty acids in both cell types. However, ALA incorporation resulted in an overall decrease in total n-6 fatty acids in only the A549 cell line.

At low levels of substrate (5 and 20 μg/mL), A549 cells were able to increase the cell membrane phospholipid content of the 20-carbon PUFA EPA and AA, presumably by endogenous synthesis, although this was not measured directly (Figs. 2A,B). The incorporation of EPA and AA by these cells decreased dose dependently with further increases in substrate ALA or LA.

16HBE cells were also capable of increasing cell membrane EPA in the presence of ALA, which was maintained at a constant level at increasing ALA doses (Fig. 2C). However, there was no change in cell membrane phospholipid AA con-

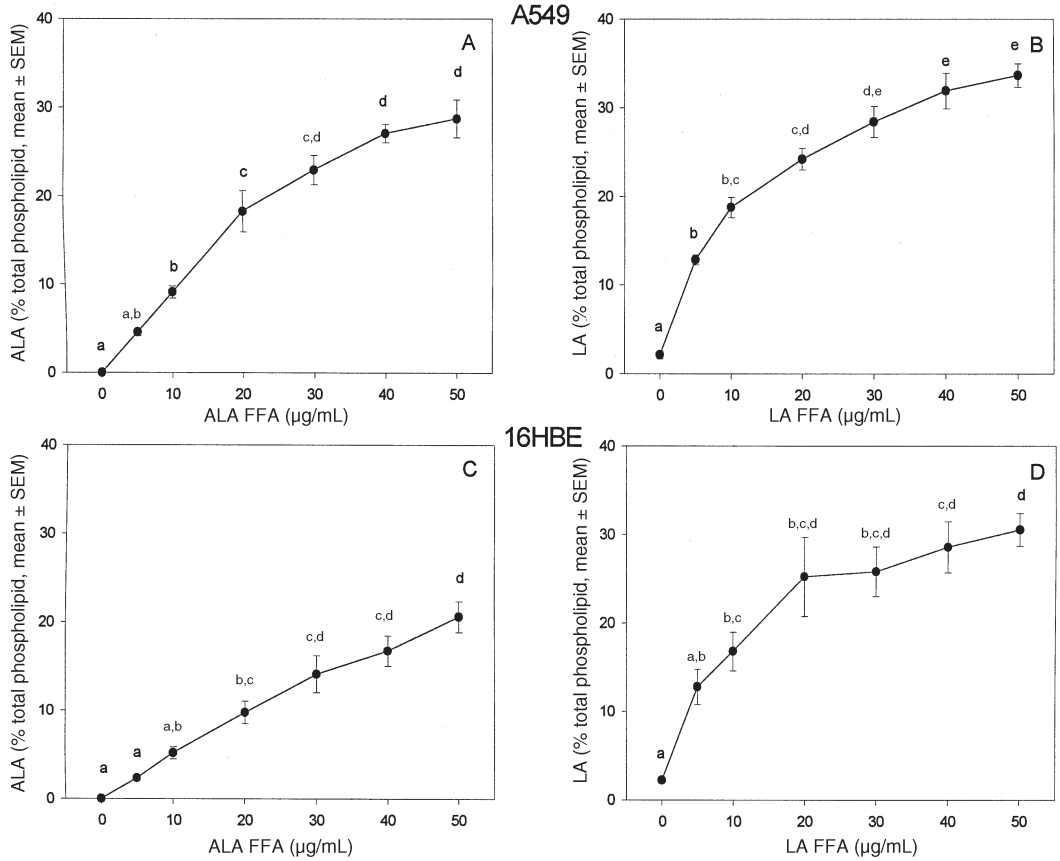


**FIG. 1.** Cell membrane phospholipid α-linolenic acid (ALA) (A,C) and linoleic acid (LA) (B,D) from respiratory epithelial cells A549 (A,B) and 16HBE (C,D) incubated with a range of concentrations of ALA or LA as free fatty acids (FFA). Results from three or four experiments are expressed as a relative percentage of the total fatty acids (mean ± SEM). Points with different superscripts indicate significant differences ($P < 0.05$).

tent in response to supplementation of media with any concentration of LA FFA (Fig. 2D).

*Cell viability.* No difference was seen in cell viability as measured by formazan production by cells grown in ALA or LA up to 50 µg/mL when compared with cells grown in control media (data not shown). There was, however, a significant decrease in cell viability at concentrations of both ALA and LA greater than or equal to 100 µg/mL in both cell lines. This was most likely due to a toxic effect of these high concentrations of FFA, as no toxicity to the maximal added ethanol 0.5% (vol/vol) was demonstrated.

## DISCUSSION

This is the first direct comparison of the incorporation of ALA and LA into human respiratory epithelium *in vitro*. The level of incorporation of LA attained in these experiments is comparable to that reported by Smith and colleagues (15) who showed an increase in LA to 28% of choline phosphoglyceride fatty acids by A549 cells after incubation with albumin-bound LA at a concentration of 85 µM (molar ratio albumin/fatty acid, 1:1). Similarly, ALA at 30 µg/mL has been shown to be readily incorporated into the phosphatidylethanolamine and phosphatidylcholine fractions of human Y79 retinoblastoma cells to a level of 20% with an increase in all other n-3 fatty acids except DHA (16). At lower concentrations of supplemental ALA (10 µg/mL), Y79 cells were able to metabolize and incorporate significant quantities of DHA. This elongation to the 22-carbon LCPUFA DHA or n-6 docosapentaenoic acid from the substrate ALA or LA was not observed in either the A549 or 16HBE respiratory cell lines.

Although ALA concentration did increase substantially in both respiratory cell lines, the incorporation into the A549 cells was higher than that into the 16HBE cells at the same supplemental ALA FFA concentration. This may be due to a decrease in ALA uptake in the presence of higher concentrations of FBS in the culture medium of the 16HBE cells (10%) compared with that of the A549 cells (5%) as observed previously by Tranchant and colleagues (17) in a Caco-2 human intestinal cell line. Alternatively, this may be a property of 16HBE cells.

Addition of ALA and LA to media resulted in small curvilinear effects on EPA and AA levels, respectively, in A549 cells (Figs. 2A,B). Y79 retinoblastoma cells in the presence of ALA showed a similar curvilinear effect of EPA and DHA production and incorporation with increasing ALA concentration (16). The explanation for this curvilinear response of 18-carbon precursors on 20-carbon metabolites is complex. This relationship may be a result of additional substrate for

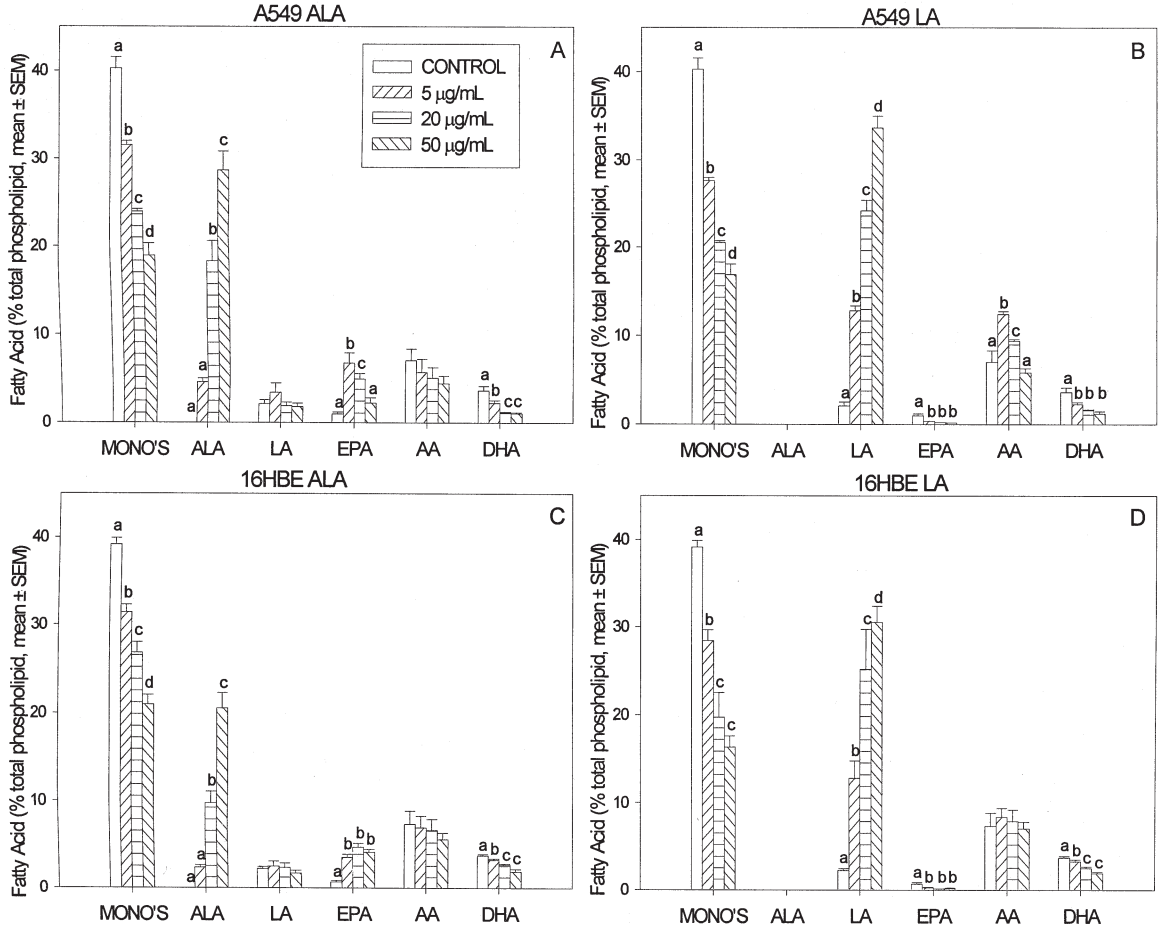


**FIG. 2.** Cell membrane phospholipid polyunsaturated fatty acids after incubation of respiratory cell lines A549 (A,B) and 16HBE (C,D) with differing concentrations of ALA and LA (C,D). Results from three or four experiments are expressed as a relative percentage of the total fatty acids (mean ± SEM). Points with different superscripts indicate significant differences ($P < 0.05$). MONO'S, monounsaturated fatty acids; EPA, eicosapentaenoic acid; AA, arachidonic acid; DHA, docosahexaenoic acid. For other abbreviations see Figure 1.

endogenous synthesis at lower ALA and LA levels offset by competition for incorporation between 18-carbon and 20-carbon fatty acids at the higher ALA and LA levels. Since neither metabolism nor incorporation was measured directly, these hypotheses remain untested. The 16HBE cells, while appearing to be unable to incorporate significant quantities of AA, maintain an increased level of EPA in their membrane even at increasing ALA levels. However, the effects on both 20-carbon LCPUFA are small relative to the changes in 18-carbon fatty acid incorporation achieved in both cell lines.

This study also demonstrates that for both alveolar and bronchial respiratory epithelial cells, incorporation of ALA and LA to levels of 20–33% of total phospholipid causes no measurable decrease in viable cell number and therefore in cell proliferation over the incubation period. There was, however, no stimulatory effect of LA on cell proliferation as shown by Schonberg and Krokan (18) in A549 cells and Usha and colleagues (19) with normal human respiratory epithelial cells incubated with concentrations of LA from 10–40 μM.

Our data support the concept that ALA is readily incorporated into membrane phospholipids, and the lack of incorporation in human and animal tissues *in vivo* may be due to the rapid metabolism or oxidation of this fatty acid to other n-3 PUFA in the liver. However, the relevance of these observations using transformed cell lines needs to be confirmed in normal epithelial cells.

This model provides the basis for examining the potential role for ALA and LA in the regulation of synthesis of mediators of immunity such as cytokines and eicosanoids in these respiratory epithelial cell lines.

## ACKNOWLEDGMENTS

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**TABLE 2**
**Changes in Cell Membrane Phospholipids with Maximal Incorporation of ALA and LA (50 µg/mL supplemental FFA) (% total fatty acids ± SEM, *n* = 3 or 4)**

| | A549[a] | | | 16HBE[a] | | |
|---|---|---|---|---|---|---|
| | Control | +ALA | +LA | Control | +ALA | +LA |
| Total SATS | 36.6 ± 0.5 | 34.7 ± 1.1 | 33.3 ± 1.1 | 39.4 ± 0.1 | 36.0 ± 1.1 | 34.5 ± 0.6[a] |
| Total MONOS | 40.3 ± 1.3 | 18.9 ± 1.4[b] | 16.9 ± 1.2[b] | 39.2 ± 0.7 | 21.0 ± 1.1[b] | 16.3 ± 1.3[b] |
| n-7 | 9.0 ± 0.6 | 5.1 ± 1.1[a] | 4.7 ± 1.0[a] | 11.3 ± 0.4 | 5.2 ± 0.3[b] | 4.7 ± 0.4[b] |
| n-9 | 32.2 ± 1.1 | 13.6 ± 0.9[b] | 14.1 ± 0.6[b] | 27.5 ± 0.3 | 14.8 ± 1.3[b] | 14.7 ± 1.3[b] |
| 18:2n-6 | 2.1 ± 0.4 | 1.8 ± 0.4 | 33.6 ± 1.3[b] | 2.2 ± 0.3 | 1.7 ± 0.3 | 30.6 ± 1.9[b] |
| 20:3n-6 | 1.0 ± 0.1 | 0.4 ± 0.1[a] | 1.2 ± 0.3 | 1.2 ± 0.1 | 0.7 ± 0.1[a] | 1.1 ± 0.1 |
| 20:4n-6 | 7.0 ± 1.3 | 4.4 ± 0.8 | 5.8 ± 0.5 | 7.4 ± 1.5 | 5.6 ± 0.7 | 7.1 ± 0.8 |
| 22:5n-6 | 0.4 ± 0.2 | 0.3 ± 0.1 | 0.2 ± 0.2 | 0.4 ± 0.2 | 0.4 ± 0.1 | 0.2 ± 0.1 |
| Total n-6 | 13.5 ± 0.2 | 7.8 ± 0.8[a] | 44.7 ± 1.1[b] | 12.4 ± 1.3 | 9.0 ± 0.8 | 42.0 ± 1.0[b] |
| 18:3n-3 | ND | 28.7 ± 2.1[b] | ND | ND | 20.6 ± 1.7[b] | ND |
| 20:5n-3 | 1.0 ± 0.2 | 2.2 ± 0.6 | 0.2 ± 0.0[b] | 0.7 ± 0.2 | 4.1 ± 0.3[b] | 0.2 ± 0.1[a] |
| 22:5n-3 | 0.8 ± 0.2 | 1.1 ± 0.2 | 0.6 ± 0.0 | 2.9 ± 0.6 | 1.9 ± 0.3 | 1.2 ± 0.3 |
| 22:6n-3 | 3.6 ± 0.5 | 1.0 ± 0.1[b] | 1.2 ± 0.2[b] | 3.7 ± 0.2 | 1.9 ± 0.3[b] | 1.9 ± 0.1[b] |
| Total n-3 | 5.4 ± 1.0 | 36.9 ± 2.7[b] | 2.0 ± 0.3[b] | 7.3 ± 1.0 | 33.1 ± 2.4[b] | 3.3 ± 0.4[a] |

[a]Superscript roman letters indicate significant differences from relevant controls. [a]$P < 0.01$. [b]$P < 0.001$. ND = not detectable. For other abbreviations see Table 1.


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# Modeling of α-Tocopherol Loss and Oxidation Products Formed During Thermoxidation in Triolein and Tripalmitin Mixtures

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**ABSTRACT:** The degradation of α-tocopherol and the formation of α-tocopherol and triacylglycerol oxidation products at high temperatures (150–250°C) over a heating period (0–4 h) for a model system ranging between triolein and tripalmitin were modeled by use of an experimental design. The oxidation products of α-tocopherol formed under these conditions were α-tocopherolquinone (1.4–7.7%) and epoxy-α-tocopherolquinones (4.3–34.8%). The results indicate a very high susceptibility of α-tocopherol to capture peroxyl radicals upon oxidation, leading to the formation of polar tocopherol oxidation products. Both α-tocopherolquinone and epoxy-α-tocopherolquinones were not stable upon prolonged heating and were further degraded to other unknown oxidation products. The kinetics of α-tocopherol oxidation were significantly influenced by the triolein/tripalmitin ratio. By increasing the level of triacylglycerol unsaturation the rate of α-tocopherol recovery after heating increased significantly from 2.2 to 44.2% whereas in the meantime triacylglycerol polymerization increased from 0 to 3.7%.

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Vegetable oils have different susceptibilities toward oxidative degradation due to differences in fatty acid unsaturation and varying contents of antioxidants. Triacylglycerols rich in polyunsaturated fatty acids are especially prone to oxidation. Tocopherols are the most important natural antioxidants present in vegetable oils (1). The antioxidant properties of tocopherols at low temperatures have been ascribed to the easy hydrogen transfer from their phenolic hydrogen to a peroxyl radical. Hydrogen transfer produces a tocopheroxyl radical, which might combine with another lipid peroxyl radical in a series of termination reactions yielding nonradical oxidation products (2–4).

$$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet} \quad [1]$$

$$LOO^{\bullet} + TO^{\bullet} \rightarrow TO\text{-}OOL \quad [2]$$

α-Tocopherol (**I**) (Scheme 1) is the main antioxidant for some major frying oils such as high-oleic sunflower, palm and olive oils. The number of studies dealing with the activity of **I** in the inhibition of oxidation reactions at low temperatures is extensive (e.g., 1,5,6), but studies at high temperatures are scarce (7–9). Rapid degradation of tocopherols under accelerated oxidation conditions (e.g., frying and microwave heating) has been reported (10,11).

Oxidation of **I** in model systems was shown to produce various oxidation products of which α-tocopherolquinone (**II**), 4a,5-epoxy-α-tocopherolquinone (**III**), 7,8-epoxy-α-tocopherolquinone (**IV**), α-tocopherol-α-tocopheroxyldimer (**V**), α-tocopherol-α-tocopherolquinonespirodimer (**VI**), α-tocopherolspirodimer (**VII**), and α-tocopherolspirotrimer (**VIII**) have been identified (1,12–17). However, little information is available on the identification and quantification of **I** degradation products formed during oxidation in a lipid matrix, and only **II** and epoxy-α-tocopherolquinone have been identified as the **I** oxidation products in heat-treated oils (18).

At the high temperatures of frying, it is of primary interest to know the evolution of **I** degradation in relation to the fatty acid oxidation process. Scattered research on the influence of triacylglycerol unsaturation on the **I** loss and triacylglycerol polymerization during frying is available. Tocopherols were reported to degrade faster in saturated than in unsaturated oils in simulated deep-frying experiments at 180°C but not at temperatures below 100°C (19–21). According to Reference 9, tocopherol losses were not different in fatty acid matrices of triolein (OOO) and trilinolein.

In this research, we studied the degradation of **I** at high temperatures in a model system of OOO and tripalmitin (PPP) using a statistical central composite design. Special emphasis was undertaken to study the oxidation products formed from both **I** and the triacylglycerols during the heating trials. The kinetics of **I** degradation and formation of identified oxidation products was studied as a function of the heating-time, heating-temperature, and composition of the triacylglycerol matrix.

## MATERIALS AND METHODS

*Materials and reagents.* Pure **I** and silica gel 60 for chromatography were purchased from Merck (Darmstadt, Germany), and technical-grade PPP and OOO were delivered by Sigma Chemical Company (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade solvents (petroleum ether, hexane, 1,4-dioxane, tetrahydrofuran, and methanol) were

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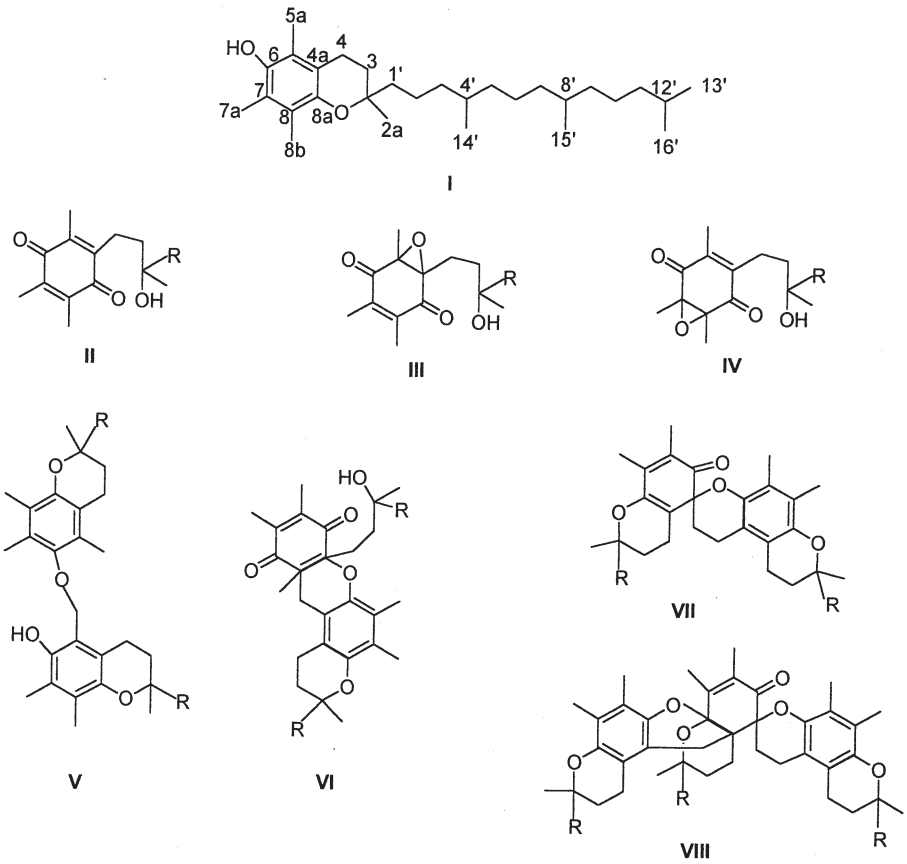
Abbreviations: FAME, fatty acid methyl ester; GC, gas chromatography; HPLC, high-performance liquid chromatography; HPSEC, high-performance size exclusion chromatography; IUPAC, International Union of Pure and Applied Chemistry; NMR, nuclear magnetic resonance; OOO, triolein; PPP, tripalmitin; UV, ultraviolet.




**SCHEME 1**

purchased from Merck. Water was distilled twice and additionally purified with activated char. All chemicals and reagents were of analytical grade and were used without further purification.

*Characterization of the technical OOO and PPP used in the study.* Technical-grade OOO and PPP were selected, owing to financial considerations, as the model triacylglycerols instead of highly purified standards. These materials were characterized by analysis of fatty acid composition, triacylglycerol distribution, and tocopherol content. Fatty acid composition was determined after derivatization to fatty acid methyl esters (FAME) with 2 N KOH in methanol according to the International Union of Pure and Applied Chemistry (IUPAC) Standard Method (22). Gas chromatography (GC) of FAME was performed on an HP 6890 chromatograph using HP Innowax capillary column (polyethylene glycol, 30 m × 0.25 mm internal diameter, film thickness 0.25 μm) (Hewlett-Packard, Avondale, PA) under the following temperature program: 180ºC (2 min), 4°C/min to 230°C. Samples were introduced to the column *via* a split injector (split ratio 1:40) at 250°C and the flow rate of hydrogen, used as carrier gas, was 1 mL/min. Temperature of both split injector and flame-ionization detector was 250ºC. The distribution of triacylglycerols was analyzed by GC on a fused-silica capillary column Restek RTX–65TG (35% dimethyl/65%diphenyl polysiloxane cross-bonded, 30 m length × 250 μm internal diameter, Hewlett-Packard) with programming from 300ºC (held for 1 min) to 360°C at 2ºC/min. The temperatures of the injector and detector were set at 370ºC. Samples were dissolved in hexane to a concentration of 1 mg/mL, and 1-μL portions were used for injection. The other fatty acids present in these samples (only identifiable components present in a concentration >1% are listed) are shown in Table 1. Technical-grade PPP (92.5% palmitate residues) contained small amounts of other saturated fatty acids, but the quality of technical OOO (73.1% oleate residues) was much inferior as it contained 5.8% linoleate residues, 8.5% saturated acyl residues, and 12.6% of a wide range of other acids present at <0.5% levels. We do not consider this quality problem as limiting to the conclusions of this study. No tocopherols could be detected in these materials.

*Synthesis of **I** oxidation products.* Several known oxidation products of **I** have been synthesized, purified, and authenticated as described below. Nuclear magnetic resonance (NMR) spectrometry was performed on a Jeol JNM-EX270 spectrophotometer (Tokyo, Japan). $^{1}$H NMR and $^{13}$C NMR spectra were recorded in deuterated solvents with tetramethylsilane as internal standard at 270 and 68 MHz, respectively.

α-Tocopherolquinone (**II**) was obtained by oxidation of **I** with ferric chloride (23,24), and **III** and **IV** were prepared according to a method modified from Csallany and Ha (25). Compounds **V** and **VI** were prepared by oxidation of **I** with *tert*-butylhydroperoxide (13,14), and compounds **VII** and **VIII** were prepared by oxidation of **I** with alkaline ferricyanide

**TABLE 1**
**Fatty Acid and Triacylglycerol Composition of Technical-Grade Triolein (OOO) and Tripalmitin (PPP) Used as Model Substrates**

| | OOO | PPP |
|---|---|---|
| Fatty acid composition (%) | | |
| Myristic (M, 14:0) | 2.4 | 1.3 |
| Palmitic (P, 16:0) | 4.6 | 92.5 |
| Stearic (S, 18:0) | 1.5 | 3.5 |
| Oleic (O, 18:1) | 73.1 | — |
| Linoleic (L, 18:2) | 5.8 | — |
| Others[a] | | |
| Triacylglycerol composition (%) | | |
| PPM | — | 3.1 |
| PPP | — | 80.4 |
| PPS | — | 10.1 |
| PSS | — | 1.9 |
| PPO | 6.3 | — |
| PPL | 3.3 | — |
| POO | 9.0 | — |
| POL | 10.6 | — |
| OOO | 48.7 | — |
| OOL | 11.6 | — |
| Others[b] | 10.5 | 4.5 |

[a]Several other fatty acids were present in minor amounts (<0.5%) except for arachidic acid, which was present in tripalmitin at a 1% level.
[b]Main minor compounds are diacylglycerols.

(12,15,26–28). All spectral data of the components synthesized matched the spectral data reported in the literature cited.

*Thermoxidation of model triacylglycerol mixtures*. A full-factorial Central Composite Design was selected to study the influence of the factors (temperature, heating time, and fatty acid composition expressed as % OOO) on the degradation of **I** and the formation of identified oxidation products from **I** and triacylglycerols. Five levels (−2, −1, 0, +1, +2) were used for each of the three factors: OOO (0, 25, 50, 75, 100%), temperature (150, 175, 200, 225, 250°C), and heating time (0, 1, 2, 3, 4 h). The initial **I** content in the different triacylglycerol mixtures was 1000 ppm. For the heat treatment, oil samples (5 g) were weighed in tubes (22 mm i.d.) and placed in an oven preheated at the desired temperature. No air bubbling was applied during the heating, and the tubes were left open. After heating, the samples were transferred in glass tubes, purged with nitrogen and sealed until the time for analysis.

*HPLC analysis of **I** and its oxidation products.* The contents of residual **I** in thermoxidized oils were analyzed directly by normal-phase HPLC, and the content of **I** and its oxidation products was analyzed, after extraction and concentration in methanol, by reversed-phase HPLC.

Normal-phase HPLC was conducted on equipment consisting of compact Bischoff model 2250 HPLC pump (Leonberg, Germany) connected to a Midas version 1.3 auto-injector (Spark Holland BV, AJ Emmen, The Netherlands) and a LaChrom L-7480 fluorescence detector (Merck-Hitachi, Tokyo, Japan). Data were collected and processed by the software of Chromeleon version 4.12 (Gynkotek GmbH, Germering, Germany). HPLC separations were performed on an Alltima 5 μm, 250 × 4.6 mm i.d. silica column (Alltech, Deerfield, IL). The mobile phase, hexane/1,4-dioxane (96:4, vol/vol), was pumped at a flow rate of 1.5 mL/min. Fluorimetric detection of the **I** peak was performed at an excitation wavelength of 294 nm and an emission wavelength of 326 nm, and quantification of the amount of **I** was made using external standard calibration.

Enrichment of the residual **I** and polar **I** oxidation products was performed by extraction of oil samples (2 g) with hot methanol (3 × 8 mL) followed by centrifugation (18). Upon cooling of the combined methanol extracts, some crystals of co-extracted PPP were observed. The extract was evaporated to dryness and redissolved in methanol (1 mL). The clear methanol fraction was removed and analyzed by reversed-phase HPLC, whereas the PPP crystals were dissolved in hexane (1 mL) and analyzed for residual **I** content by normal-phase HPLC using fluorescence detection. A direct analysis of residual **I** by normal-phase HPLC allowed us to calculate the recovery of the **I** extraction procedure. The recovery factor for **I** was 35 ± 4.6% ($n$ = 20), which is rather low but was very reproducible.

Reversed-phase HPLC of **I** and its oxidation products was performed on an HP series 1100 chromatograph equipped with a diode array detector (Hewlett-Packard). Separations were performed on an Econosil C18 5 μm, 250 × 4.6 mm internal diameter column (Alltech). The solvent mixture and applied solvent gradient were optimized to obtain a good separation, within 17 min, of **III**, **IV**, **II**, and residual **I** starting with methanol/water (80:20, vol/vol) and increasing to 100% methanol within 4 min. The **III**, **IV**, and **II** were quantified by their ultraviolet (UV) maximal adsorption at 275 nm, whereas **I** was analyzed at a wavelength of 292 nm. Quantification of **I** and the identified oxidation products was performed using standard **I** and the synthesized oxidation products for external calibration. The contents of **I** and its oxidation products were calculated using the recovery factor of **I** determined by normal-phase HPLC (*vide supra*). In the study, all concentrations are expressed as percentages, relative to the initial **I** concentration of 1000 ppm.

*Quantification of total polymers.* Polymeric triacylglycerols were quantified by high-performance size exclusion chromatography (HPSEC) following the IUPAC Standard Method (22). Conditions applied for HPSEC were as follows: A Rheodyne 7725i injector (Cotati, CA) with a 10-μL sample loop and a Waters 510 HPLC pump (Waters Associates, Milford, MA), two 100 and 500 Å Ultrastyragel columns (Waters Associates), 25 × 0.77 cm internal diameter, packed with a porous, highly cross-linked styrenedivinylbenzene copolymer (<10 mm), connected in series, and a refractive index detector (Hewlett-Packard) were used. Tetrahydrofuran was the mobile phase with a flow of 1 mL/min, and sample concentration was around 50 mg/mL in tetrahydrofuran.

*Quantification and distribution of polar compounds.* Nonpolar and polar fractions from 50 mg of sample were separated by solid-phase extraction using monostearin as internal standard in 1-g Sep-Pak cartridges (Waters). The nonpolar fraction containing unoxidized triacylglycerols was eluted with 15 mL of *n*-hexane/diethyl ether (90:10, vol/vol), and the second fraction, comprising total polar compounds, was eluted with 15 mL of diethylether. The efficiency of the separation by adsorption chromatography was checked by thin-layer chroma-

tography using *n*-hexane/diethylether/acetic acid (80:20:1, by vol) for development of plates and exposure to iodine vapor to reveal the spots. The polar fraction obtained was dissolved in tetrahydrofuran to a concentration of 15 mg/mL for analysis by HPSEC using the conditions described above for the direct analysis of polymers. Quantification of polar compounds was achieved through the internal standard method (29). The experiments having a triacylglycerol composition with PPP concentration higher than 50% could not be analyzed as these samples were not soluble in the solvent used for the elution of unoxidized triacylglycerols (*n*-hexane/diethylether, 90:10, vol/vol), thus decreasing the efficiency of separation.

*Selection of experimental model equation.* A full-factorial Central Composite Design was selected to study the influence of the different factors (time, temperature, and triacylglycerol composition expressed as % OOO) on the responses (residual **I**, formation of **I** oxidation products, and total triacylglycerol polymer content). Establishment of the response surface quadratic models was done with Design Expert® (Stat-Ease, Minneapolis, MN) by evaluation of the analysis of variance. The best model (linear or quadratic) was selected by interpretation of the Sequential Model Sum of Squares, which was highly significant ($P < 0.0001$) and showed no lack of fit ($P > 0.05$). The complete quadratic model contains 10 different terms (linear, quadratic, and interaction terms), which makes handling the model equations very complicated in practice. Therefore, a reduced quadratic model was derived by elimination of the nonsignificant individual factors ($P > 0.05$).

## RESULTS AND DISCUSSION

In accordance with expectations, heating of the triacylglycerol mixtures caused a marked reduction in residual **I** level and increased the formation of total polar materials, mainly polymers and oxidized monomers, from triacylglycerols (Table 2). These results indicate a very fast degradation of **I** upon heating, giving rise to a recovery between 1.5 and 44.2% of **I**. Analytical data concerning triacylglycerol polymer content indicate a low total polymerization level ranging between 0.1 and 3.7%, except for samples containing 50 and 75% OOO heated at 250 and 225°C, respectively, where higher polymerization levels (>10%) were found. The results from the sample that did not receive heat treatment clearly indicate that the total polar fraction is mainly composed of diacylglycerols and free fatty acids initially present in the technical-grade OOO and PPP (Table 1). Depending on the experimental conditions, polymers and oxidized triacylglycerol monomers are formed to different extents, ranging between 0.2 and 11.1% and 0.6 and 6.1%, respectively.

Upon oxidation, **I** is known to degrade to a wide range of oxidation products (Scheme 1), but some are not easy to analyze owing to the lack of strong fluorescence of the chro-

**TABLE 2**
**Distribution of α-Tocopherol, Epoxy-tocopherolquinones, α-Tocopherolquinone, and Polar Compounds in Heated Model Samples**

| | Experimental conditions | | | α-Tocopherol and α-tocopherol oxidation products[a] (% of initial α-tocopherol) | | | | | | Polymers[b] | Distribution of polar compounds[c] (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment number | Time (h) | Temp. (°C) | Triolein (%) | α-T NP | α-T RP | 4a,5-ETQ | 7,8-ETQ | α-TQ | Total | (% total) | Total polar material | Polymers | OTAGM | DAG + FFA |
| 1 | 2 | 200 | 50 | 5.3 | 6.6 | 14.9 | 5.3 | 3.2 | 30.1 | 0.2 | 10.8 | 0.4 | 1.2 | 9.2 |
| 2 | 2 | 250 | 50 | 2.1 | 2.7 | 1.2 | 3.1 | 2.2 | 9.2 | 10.9 | 28.0 | 11.1 | 7.0 | 9.9 |
| 3 | 4 | 200 | 50 | ND | 1.5 | 19.5 | 4.3 | 3.9 | 29.3 | 3.2 | 16.3 | 3.4 | 3.5 | 9.4 |
| 4 | 0 | 200 | 50 | 100.0 | 100.0 | ND | ND | ND | 100.0 | ND | 9.2 | 0.1 | 0.2 | 8.9 |
| 5 | 2 | 200 | 50 | 6.2 | 8.1 | 19.2 | 4.7 | 5.3 | 37.2 | 0.3 | 11.3 | 0.3 | 1.6 | 9.4 |
| 6 | 2 | 200 | 50 | 8.5 | 5.7 | 19.6 | 7.0 | 5.2 | 37.5 | 0.2 | 11.0 | 0.3 | 1.5 | 9.2 |
| 7 | 2 | 150 | 50 | 23.5 | 21.2 | 15.8 | 6.4 | 5.9 | 49.3 | ND | 10.1 | 0.2 | 0.6 | 9.3 |
| 8 | 1 | 175 | 75 | 48.2 | 43.6 | 13.8 | 5.4 | 5.2 | 68.1 | 1.4 | 11.6 | 1.6 | 1.1 | 8.9 |
| 9 | 3 | 225 | 75 | 1.4 | 1.8 | 6.2 | ND | 2.9 | 11.0 | 11.0 | 27.0 | 10.8 | 6.1 | 10.1 |
| 10 | 3 | 175 | 25 | 0.2 | 0.9 | 27.8 | 7.0 | 2.7 | 38.3 | 0.2 | MV | MV | MV | MV |
| 11 | 2 | 200 | 50 | 2.0 | 3.1 | 17.6 | 5.3 | 5.2 | 31.2 | 0.2 | 11.3 | 0.4 | 1.3 | 9.6 |
| 12 | 3 | 175 | 75 | 15.0 | 12.3 | 24.4 | 9.2 | 7.7 | 53.7 | 2.3 | 15.4 | 2.2 | 3.4 | 9.8 |
| 13 | 1 | 225 | 75 | 30.1 | 26.6 | 17.3 | 5.1 | 4.9 | 53.8 | 3.2 | 17.2 | 3.4 | 4.1 | 9.7 |
| 14 | 1 | 225 | 25 | 6.6 | 7.6 | 11.6 | 6.7 | 2.3 | 28.1 | 0.4 | MV | MV | MV | MV |
| 15 | 2 | 200 | 50 | 5.6 | 7.9 | 19.8 | 4.6 | 4.8 | 37.2 | 0.4 | 11.8 | 0.5 | 1.9 | 9.4 |
| 16 | 2 | 200 | 50 | 4.3 | 4.7 | 17.6 | 4.7 | 4.9 | 31.9 | 0.1 | 11.1 | 0.3 | 1.1 | 9.7 |
| 17 | 1 | 175 | 25 | 20.7 | 25.1 | 5.8 | 4.1 | 2.4 | 37.4 | ND | MV | MV | MV | MV |
| 18 | 3 | 225 | 25 | ND | 0.7 | 3.4 | 2.4 | 1.4 | 8.0 | 3.1 | MV | MV | MV | MV |
| 19 | 2 | 200 | 100 | 45.1 | 44.2 | 15.4 | ND | 6.4 | 66.0 | 3.7 | 15.6 | 3.4 | 2.7 | 9.5 |
| 20 | 2 | 200 | 0 | 1.8 | 4.8 | 6.6 | 3.5 | ND | 14.9 | ND | MV | MV | MV | MV |

[a]α-T NP, percent residual α-tocopherol by normal-phase high-performance liquid chromatography (HPLC); α-T RP, percent residual α-tocopherol by reversed-phase HPLC; 2,3-ETQ and 5,6-ETQ, percentages of 4a,5- and of 7,8-epoxy-tocopherolquinones by reversed-phase HPLC; α-TQ, percent of tocopherolquinone by reversed-phase HPLC; Total, sum of α-tocopherol, 2,3- and 5,6-ETQ, and α-TQ determined by reversed-phase HPLC; ND, not detected.
[b]Total polymers analyzed directly by high-performance size exclusion chromatography (HPSEC).
[c]Distribution of polar compounds (separated by solid-phase extraction) as analyzed by HPSEC (for details, see Materials and Methods section). MV, missing value (see Results and Discussion section); OTAGM, oxidized triacylglycerol monomers; DAG, diacylglycerols; FFA, free fatty acids.

manol structure. Direct analysis of these tocopherol oxidation products was therefore not possible, and an enrichment step was necessary. Enrichment was performed by extraction of residual **I** and its polar oxidation products with hot methanol (18). Several peaks were present in the reversed-phase HPLC chromatogram of the methanol extract (Fig. 1). The components eluting at 10.4, 10.7, 12.5, and 15.5 min were identified as **IV**, **III**, **II** and **I**, respectively, because their retention times matched those of **I** and chemically synthesized oxidation products. These results are in accordance with the report of Murkovic *et al.* (18), who found marked formation of epoxy-α-tocopherolquinones during thermoxidation. Identification of the other peaks was not possible, but these may include oxidation products from OOO.

As mentioned in the Materials and Methods section, a recovery factor of 35 ± 4.6% was determined for the methanol extractability of **I** using normal-phase HPLC. A good correlation ($R^2 = 0.9916$) was obtained between the residual **I** concentration analyzed by normal-phase and that analyzed by reversed-phase HPLC using the determined recovery factor. This indicates that the applied extraction procedure had a good reproducibility and was influenced neither by the con-

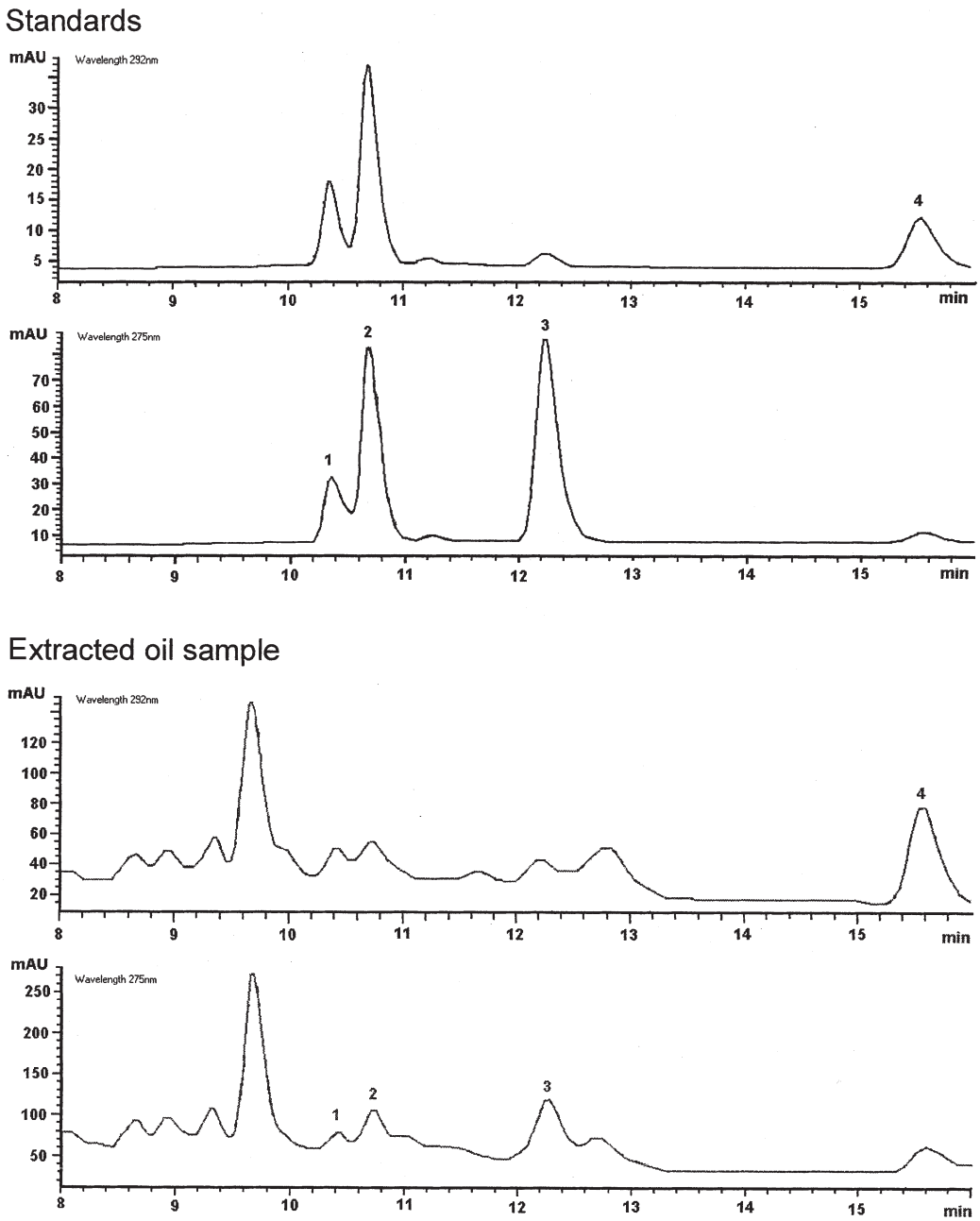



**FIG. 1.** Reversed-phase high-performance liquid chromatograms of standards and extracted oil samples with an ultraviolet detector at 275 nm for oxidation products and 292 nm for residual α-tocopherol. 7,8-Epoxy-α-tocopherolquinone (1), 4a,5-epoxy-α-tocopherolquinone (2), α-tocopherolquinone (3), and α-tocopherol (4).

centration of the oxidation products nor by the composition of the triacylglycerol matrix; this also allows further interpretation of the quantitative results. A similar recovery factor as determined for **I** was assumed for the **I** oxidation products. Although this assumption might not be perfectly right, only small variations in recovery factors were expected without major effects on the conclusions of this study. Epoxy-α-tocopherolquinones were the major identifiable **I** oxidation products present in the concentration range 4.3–34.6% of initial **I** concentration, whereas **II** was present in a much lower concentration range, 1.4–7.7%. Upon extraction of the oil with methanol, only the polar **I** oxidation products should be extracted leaving the nonpolar **I** oxidation products (**V**–**VII**) behind in the oil. Normal-phase HPLC analysis of the extracted oil residues with UV detection (250–292 nm) or fluorescence detection (excitation 294 nm, emission 326 nm) gave no positive detection of **V**, **VI**, **VII**, and **VIII**. Whether this result is due to the absence or instability of these products warrants further investigation.

The formation of **III**, **IV**, and **II** suggests these as degradation products of **I** peroxides formed at the 5, 7, and 8a positions, respectively. The origin of the peroxyl radicals that combine with α-tocopheroxyl to form the precursor adducts discussed above is not known since both peroxyl radicals and hydroperoxides are very unstable at these high temperatures. Moreover, the fast degradation of **I** in a reaction mixture containing only PPP (Experiment No. 20, Table 2) does not support participation of peroxyl radicals from fatty acid moieties. Degradation of **I** might have occurred by reaction of resonance forms of tocopheroxyl radicals with oxygen, although this reaction was found to have a very low rate at low temperatures (30). The predominance of **III** over **IV** is in accordance with the work of Nilsson *et al*. (31) showing increased reactivity at the 5- compared to the 7-position. Breakdown of these peroxides will generate alkoxyl radicals; in the case of 5- and 7-adducts, these radicals will cyclize to epoxy radical species with carbon-centered radicals at the 8a position, which capture oxygen and form 8a-hydroperoxyl derivatives. Further degradation of 8a-hydroperoxy-4a,5-epoxy-α-tocopherol, 8a-hydroperoxy-7,8-epoxy-α-tocopherol, and 8a-hydroperoxy-α-tocopherol will generate **III**, **IV**, and **II**, respectively.

The data in Table 2 were fitted into response surface quadratic models, and the "best" models (linear, quadratic) were selected by evaluating the Sequential Model Sum of Squares and choosing the significant model ($P < 0.0001$) having a good lack of fit test ($P > 0.05$). An overview of the lack of fit probabilities and the model correlation coefficients for the different analyses is listed in Table 3. As indicated by these results, within the range of the experimental study, the following models could be established:

$$\alpha\text{-tocopherol (\%)} = 187.88 - 43.57x - 1.22y + 0.25z + 3.87x^2 + 2.181 \times 10^{-3}y^2 + 7.200 \times 10^{-3}z^2 + 0.12xy - 0.13xz - 1.970 \times 10^{-3}yz \quad [3]$$

$$\text{total epoxy-}\alpha\text{-tocopherolquinones (\%) (4a,5- + 7,8-epoxy-}\alpha\text{-tocopherolquinone)} = -271.49 + 74.29x + 2.19y + 0.79z - 0.4x^2 - 4.196 \times 10^{-3}y^2 - 4.402 \times 10^{-3}z^2 - 0.34xy - 0.071xz - 7.549 \times 10^{-4}yz \quad [4]$$

$$\alpha\text{-tocopherolquinone (\%)} = -7.52 + 8.67x + 0.021y + 0.12z - 0.66x^2 - 5.368 \times 10^{-4}z^2 - 0.028xy \quad [5]$$

$$\text{triacylglycerol polymer (\%)} = 88.51 - 8.27x - 0.85y - 0.23z + 1.925 \times 10^{-3}x^2 + 0.047xy + 1.440 \times yz \quad [6]$$

where $x$ = thermoxidation time (h); $y$ = temperature (°C); $z$ = technical triolein (%).

By applying the above listed model equations the influence of the different factors on the kinetics of residual **I**, formation of **I** oxidation products, and triacylglycerol polymerization can be represented in perturbation graphs where one factor is varied within the established model borders while keeping the other two factors constant at their center point (Fig. 2). The time factor had a major influence on the degradation of **I** (Fig. 2A). After heating the triacylglycerol mixture containing 50% OOO for 1 h at 200°C, the **I** content decreased to 20%; after 3 h no residual **I** was present. Within the studied time interval, little influence of heating time on the concentrations of **III**, **IV**, and **II** was observed. This is indicative for an equilibrium between the formation of new epoxy-α-tocopherolquinone and their further degradation to unknown oxidation products. Murkovic *et al.* (18) reported a notable decrease in epoxy-α-tocopherolquinone concentration after 4 h heating at 220°C.

The influence of temperature on the degradation of **I** (Fig. 2B) can be evaluated by considering the decrease in residual **I** content, after heating the triacylglycerol mixture of 50% OOO for 2 h, at 175 and 225°C to 12.1 and 2.0%, respectively. By increasing the temperature above 175°C a gradual decrease in the epoxy-α-tocopherolquinone concentration is observed, whereas the level of **II** seems not to be affected by temperature. This indicates degradation of the epoxy-α-tocopherolquinone at high temperatures to unidentified oxidation products. At moderate

**TABLE 3**
**Overview of the Statistical Data of the Models Established for the Different Analyses**

| Analysis[a] | Model quadratic | Lack of fit | Correlation ($R^2$) |
|---|---|---|---|
| α-Tocopherol NP | ($P < 0.0001$) | 0.2365 | 0.9851 |
| α-Tocopherol RP | ($P < 0.0001$) | 0.0463 | 0.9706 |
| α-Tocopherol-quinone-epoxide | ($P < 0.0001$) | 0.2960 | 0.9814 |
| α-Tocopherolquinone | ($P < 0.0001$) | 0.3025 | 0.8658 |
| Total polymers | ($P < 0.0001$) | 0.0625 | 0.8881 |

[a]NP, normal phase HPLC; RP, reversed phase HPLC.

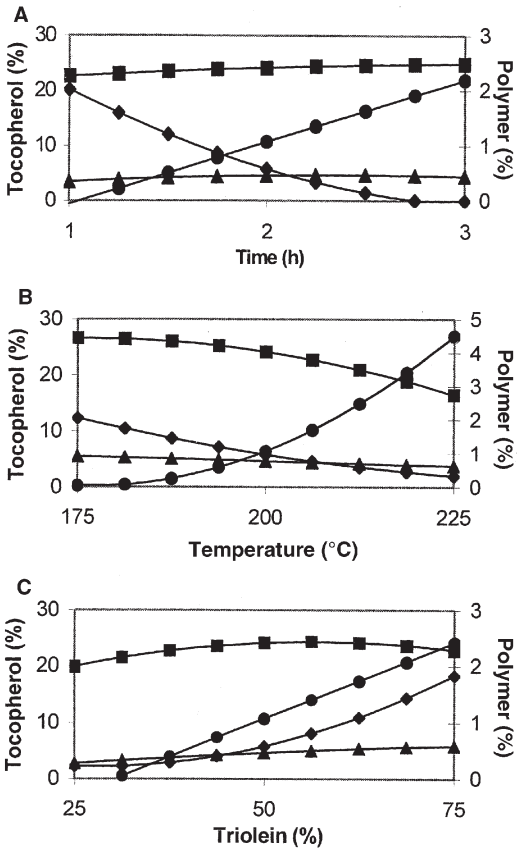



**FIG. 2.** Influence of time, temperature and triacylglycerol composition on evolution of α-tocopherol (◆), epoxy-α-tocopherolquinone (■), and α-tocopherolquinone (▲) concentrations relative to an initial α-tocopherol concentration of 1000 ppm; total triacylglycerol polymer content (●). (A) Temperature 200°C, triolein 50%; (B) time 2 h, triolein 50%; (C) time 2 h, temperature 200°C.

temperatures (175–200°C), low triacylglycerol polymer contents are formed (0–1%). However, small temperature increments above 200°C greatly accelerated the rate of polymerization, leading to a polymer content of 10.9% at 250°C.

The influence of the triacylglycerol composition on the degradation kinetics of **I** is very interesting (Fig. 2C). By increasing triacylglycerol unsaturation, the rate of **I** oxidation decreased although the triacylglycerol polymerization increased. When triacylglycerol mixtures containing 25:75, 50:50, 75:25, and 100:0 (w/w) of OOO/PPP were heated for 2 h at 200°C, the residual **I** concentrations were 2.2, 5.8, 18.3, and 44.2% and the total polymer percentages were 0, 0.3, 2.4, and 3.7%, respectively. These results are in agreement with Jorge *et al.* (19,20), who reported faster degradation of **I** in less unsaturated oils. These seemingly paradoxical results can be explained by considering involvement of peroxyl and alkoxyl radicals. As mentioned, the adduct products of α-tocopheroxyl and peroxyl radicals are unstable, and upon degradation they form the epoxy-tocopherones and tocopherolquinone after generation of alkoxyl radicals, which initiate new chain reactions. Alkoxyl radicals are not selective in their reactions, and unsaturated fatty acids will compete with **I** in these reactions. Saturated fatty acids, on the other hand, will stay inert, and only **I** will be oxidized by alkoxyl and peroxyl radicals. In line with this explanation is the finding of Barrera-Arellano (9) that **I** losses in OOO and trilinolein were independent of the fatty acid unsaturation, i.e., the difference in oxidizability between oleate and linoleate might not be sufficient to influence the rate of **I** oxidation significantly.

**TABLE 4**
**Model Calculated Values Indicating Interaction Between Variables (time, temperature, and OOO content)[a]**

| Time (h) | Temp. (°C) | Triolein (%) | Concentration (%) α-T | ETQ | TQ | TPol |
|---|---|---|---|---|---|---|
| Time and temperature (*x* and *y*) | | | | | | |
| 1 | 175 | 50 | 29.7 | 16.6 | 3.7 | 0.1 |
| 1 | 225 | 50 | 13.5 | 23.4 | 3.3 | 2.2 |
| 3 | 175 | 50 | 2.6 | 35.8 | 6.0 | 0 |
| 3 | 225 | 50 | 0 | 8.7 | 2.9 | 6.8 |
| Time and triolein % (*x* and *z*) | | | | | | |
| 1 | 200 | 25 | 13.5 | 16.6 | 1.6 | 0 |
| 1 | 200 | 75 | 35.9 | 23.1 | 4.7 | 1.3 |
| 3 | 200 | 25 | 0 | 22.4 | 2.6 | 0.8 |
| 3 | 200 | 75 | 8.51 | 21.8 | 5.7 | 3.5 |
| Temperature and triolein % (*y* and *z*) | | | | | | |
| 2 | 175 | 25 | 7.5 | 21.9 | 3.6 | 0 |
| 2 | 175 | 75 | 26.0 | 25.8 | 6.7 | 0.5 |
| 2 | 225 | 25 | 0 | 12.7 | 1.9 | 2.2 |
| 2 | 225 | 75 | 13.3 | 14.7 | 5.0 | 6.8 |

[a]OOO, triolein; α-T, α-tocopherol; ETQ, epoxy-α-tocopherolquinone; TQ, α-tocopherolquinone; TPol, total polymer content.

Interaction terms between time and temperature (*x* and *y*), time and triacylglycerol composition (*x* and *z*), and temperature and triacylglycerol composition (*y* and *z*) are present in the model equations for the levels of **I**, epoxy-α-tocopherolquinone, **II**, and triacylglycerol polymers. Owing to the presence of interaction terms, some of the previously observed effects will be highlighted at a position away from the center point. Some calculated values indicating these interactions are listed in Table 4. The slowest rate of tocopherol oxidation and lowest levels of oxidation products are found at low temperatures in an unsaturated triacylglycerol matrix. As indicated, temperature and triacylglycerol composition have a strong influence on the residual **I** content, whereas their influence on the level of epoxy-α-tocopherolquinone and **II** is much smaller. Extremely fast degradation of tocopherols is observed at high temperatures in a saturated triacylglycerol matrix. However, under these conditions the identified tocopherol oxidation products will quickly degrade to further unknown oxidation products. For unsaturated triacylglycerol levels, the polymerization is mainly determined by time and is strongly dependent on the applied temperature. At 200°C the linear increase in polymer content is 1.07%/h whereas at 225°C the linear increase in polymer content is 2.3%/h.

The results of this study suggest a very high susceptibility of **I** to oxidize during thermoxidation and to form **II**, **III,** and **IV** as oxidation products. The mechanism behind the conversion of **I** to oxygenated products is not fully understood and might in-

volve direct reaction with oxygen. Both **II** and **III**, **IV** were not stable, and upon prolonged heating at high temperatures a further degradation to other unknown oxidation products was observed. The rate of **I** oxidation decreased by competitive oxidation of unsaturated triacylglycerols and was significantly increased by elevated temperatures. At high temperatures, the protection provided to unsaturated fatty acids by **I** is reduced due to increased instability of this antioxidant. Further studies to confirm this hypothesis are under way in our laboratory.

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# The Occurrence of Geometric Polyprenol Isomers in the Rubber-Producing Plant, *Eucommia ulmoides* Oliver

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**ABSTRACT:** The chain length and geometric isomerism of polyprenols from *Eucommia ulmoides* Oliver were analyzed using supercritical fluid chromatography. After intensive effort to establish separation conditions for geometric isomers, a phenyl-bonded silica gel-packed column was found that cleanly separated poly-*trans* and -*cis* prenols. The presence of long-chain poly-*trans* prenols (>9 mers) was confirmed for the first time in plants. *Trans* isomers were found in the leaf, seed coat, and root, but not in the bark and seed. Poly-*trans* prenols in this plant may act as intermediates for *trans*-polyisoprene biosynthesis.

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More than 2000 plant species are known to produce rubber (1). The latex of the Para rubber tree, *Hevea brasiliensis*, contains large amounts of polyisoprenes that can be processed to give elastic natural rubber. A woody plant, *Palaquium gutta*, produces hard rubber, or gutta-percha. It is assumed that polyisoprene is formed by a polymerization chain reaction between an allyl substrate, e.g., geranylgeranyl pyrophosphate, and a large number of isopentenyl pyrophosphate (IPP) molecules (2). However, chain-length regulation mechanisms and the geometry of the natural polyisoprene chains have not been fully elucidated.

Besides the rubber fractions, polyprenols with relatively small molecular weights, such as dolichols, are found in almost all organisms; the latter play an important role in glycoprotein biosynthesis (3). Interestingly, dolichols are found in a variety of organisms. We have been investigating polyisoprenoid biosynthesis. As the first step in studying rubber biosynthesis, it is necessary to characterize the polyprenol fraction, which contains low molecular weight polyisoprenoids. A detailed analysis of polyprenols provides many important clues to their biosynthesis, leading to understanding of rubber biosynthesis.

Polyprenols are linear polymers of $C_5$ isoprene units with a primary alcohol group at the terminal end. In 1956, solanesol was first isolated from tobacco as a novel unsaturated alcohol, and the geometry of all of the double bonds was elucidated to be *trans* (4). Many studies have been accumulated on the structure and the chain length of polyprenols (5–7). However, all of the structures of polyprenols so far reported are tri- or di-*trans* poly-*cis* except for the case of solanesol. Profiles of polyprenol contents in plants have been also analyzed from the chemotaxonomic point of view. The results indicate that individual plant species are characterized by the presence of a variety of polyprenols with different polymerization degrees and by the separation patterns.

In the course of elucidating polymerization mechanisms of polyisoprenoids in plants, we have established a new method for analyzing polyprenol fractions. To develop high-resolution analysis of polyprenols, a variety of chromatographic conditions were tested. Previously, Swiezewska *et al.* (8) reported a conventional polyprenol separation method using reversed-phase high-performance liquid chromatography (HPLC). Their findings showed that *Prunus kurilensis* has a broad distribution pattern of polyprenols with two distribution peaks centering at 19 and 34 mers. In comparison, *Cornus mas* has a unimodal and comparatively narrow distribution.

By using similar separation conditions with a $C_{18}$ reversed-phase column, polyprenols from rubber-producing plants were analyzed. Among the plants examined, the *n*-hexane-soluble fraction of the woody plant, *Eucommia ulmoides* Oliver (Hardy rubber tree), showed an unusual separation profile, suggesting the presence of polyprenol geometric isomers.

In this paper, we report the successful separation of polyprenol isomers by supercritical fluid chromatography (SFC), together with the characterization of the polyprenol components by means of spectral analyses.

## MATERIALS AND METHODS

*Materials.* Prenol C80-110 was purchased from Sigma Chemical Company and carbon dioxide (99.9%) from Daiwa Youzai (Osaka, Japan). Ethanol, for use as a modifier for SFC, was obtained from Wako Chemicals (Osaka, Japan) and was of >99.5% purity. For HPLC-analyses, HPLC grade methanol, 2-propanol, and *n*-hexane were used (Wako Chemicals). Water was purified with a Millipore Milli-Q system (Bedford, MA).

Plant samples of *E. ulmoides* were collected in July 2000 at the Hitachi Zosen Corporation experimental station (Habu 2264-1 Innoshima, Hiroshima, Japan).

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Abbreviations: HPLC, high-performance liquid chromatography; IPP, isopentenyl pyrophosphate; MS, mass spectrometry; NMR, nuclear magnetic resonance; SFC, supercritical fluid chromatography; UV, ultraviolet.

*Analysis by HPLC.* HPLC of polyprenols was performed on a reversed-phase column, Inertsil ODS-3 (250 × 4.6 mm i.d.; particle size, 5 μm; pore size, 100 Å; surface area, 450 $m^2$/g; GL Sciences, Tokyo, Japan), using a dual pump apparatus (Hitachi L7100; Hitachi, Tokyo, Japan), and an ultraviolet (UV) detector (set at 210 nm, L7420; Hitachi). A column oven (set at 40°C; L7300, Hitachi), a degasser (L7610, Hitachi), and a data station (SIC-480II data station; System Instruments, Tokyo, Japan) were used. For elution, a gradient was applied from an initial methanol 2-propanol/water mix (60:40:5, by vol) in pump A to 100% *n*-hexane/2-propanol (70:30, vol/vol) in pump B. The solvent flow rate was 1 mL/min and the end of the gradient was reached after 40 min.

*Analysis by SFC.* SFC analyses were performed on Inertsil ODS-3 (250 × 4.6 mm i.d.; particle size, 5 μm; pore size, 100 Å; surface area, 450 $m^2$/g; GL Sciences), Inertsil Ph-3 (250 × 4.6 mm i.d.; particle size, 5 μm; pore size, 100 Å; surface area, 450 $m^2$/g; GL Sciences) and Develosil C30-UG (250 × 4.6 mm i.d.; particle size, 5 μm; pore size, 140 Å; surface area, 300 $m^2$/g; Nomura Chemical, Aichi, Japan) columns using a Super-201 Chromatograph (JASCO, Tokyo, Japan). The system consisted of two pumps, one for delivery of liquid $CO_2$ as a mobile phase (flow rate = 3.0 mL/mim) and the other for delivering ethanol as a modifier. The ethanol flow rate (0.8 mL/min) was increased to 2.0 mL/min within the experimental time of 30 min. The fluid pressure was controlled at 19.6 MPa by back-pressure regulator. The column temperature was set to 130°C (ODS-3 column) and 100°C (Ph-3 column). Chromatograms were recorded using a UV detector (950-UV, JASCO) operating at a wavelength of 210 nm. For preparative SFC, Inertsil Ph-3 (250 × 10 mm i.d.; particle size, 5 μm; pore size, 100 Å; surface area, 450 $m^2$/g; GL Sciences) was used with the following flow rates for $CO_2$: 8.0 mL/min; ethanol: 2.3 to 5.5 mL/min within 30 min, and 5.5 mL/min thereafter.

*Evaluation of analysis.* The resolution ($R_s$) between octadecaprenol (prenol 18) and nonadecaprenol (prenol 19) was calculated using SIC-480II.

*Extraction and isolation of polyprenols from* E. ulmoides. *Eucommia ulmoides* leaf, bark, seed, seed coat, and root were heated in an oven at 95°C for 3 h and then ground for 1 min using a blender. The ground parts (2 g) were then treated with alkali (40 mL of 11.2% ethanolic potassium hydroxide containing 2% pyrogallol) for 2.5 h at 100°C. The saponifiable lipid was extracted with *n*-hexane, and then the resulting concentrate was weighed. The saponifiable lipid portion (5 mg) was loaded onto a Silica gel Sep-Pak column (Waters, Milford, MA). The column was washed thoroughly with 30 mL of *n*-hexane/ ethyl ether (98:2, vol/vol) and nonpolar lipids containing free polyprenols were eluted with 50 mL of *n*-hexane/ethyl ether (85:15, vol/vol). The samples for reversed-phase HPLC were pretreated with $C_{18}$ Sep-Pak (Waters). Polyprenols were eluted with *n*-hexane/2-propanol (7:3, vol/vol).

A large-scale preparation for spectral analysis was made by preparative SFC using samples 10–20 times larger than those at analytical scale.

*Nuclear magnetic resonance (NMR) and mass spectrometry (MS) measurements.* Mass spectra were determined in the field-desorption mode with a JMS-DX303 spectrometer (JEOL, Tokyo, Japan). $^1$H NMR spectra were obtained with a Varian Unity Inova 750 MHz NMR spectrometer (Varian, Oxford, United Kingdom) at 50°C in deuterated benzene, with trimethylsilane as an internal standard.

## RESULTS AND DISCUSSION

*SFC analysis of polyprenols from* E. ulmoides. *Eucommia ulmoides* Oliver (Hardy rubber tree) is an elm-like deciduous tree native to central and southern China that can grow to 18 m tall. This plant produces fibrous rubber, called *EU*-Rubber, which accumulates in all parts but the seed (Fig. 1). This unique rubber is thermoplastic and is similar to gutta-percha in term of chemical and physical properties (9). This information prompted us to examine the key intermediate compounds that play important roles in *EU*-Rubber biosynthesis.

We focused on the *n*-hexane-soluble components of the dried samples. Under alkaline hydrolysis condition, the polyprenol content was markedly increased, suggesting that the terminal alcohol was esterified. The crude sample obtained by silica gel chromatography was analyzed using a $C_{18}$ reversed-phase column. A unimodal separation profile was seen in the samples of bark and seed. Polyprenols with polymerization degrees of 15–21 were found in the bark and 15–23 mers in the seed (Fig. 2). However, in the leaf, seed coat, and root, overlapping peaks presumably corresponding to 15–20 mers, appeared as congested peaks around $R_t$ 20–25 min, which made it difficult to separate and identify components with closely similar structures (Fig. 3A).

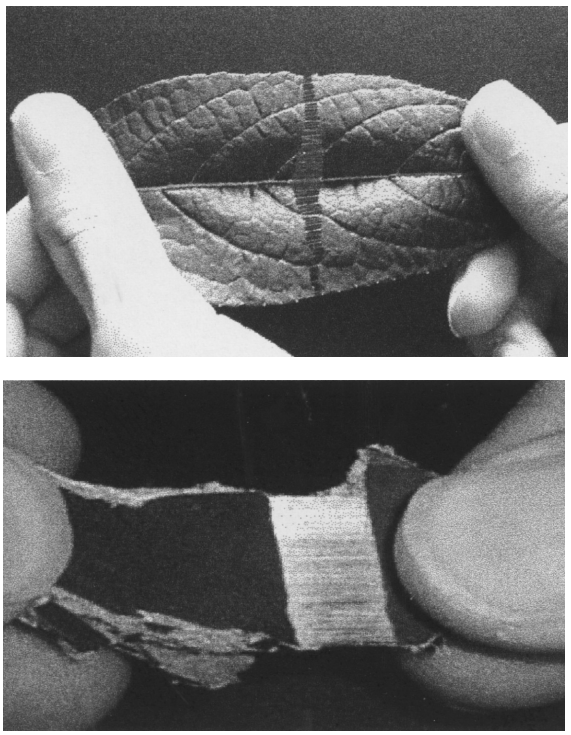


**FIG. 1.** Fibrous rubber of *Eucommia ulmoides.* (A) Leaf, (B) Bark.

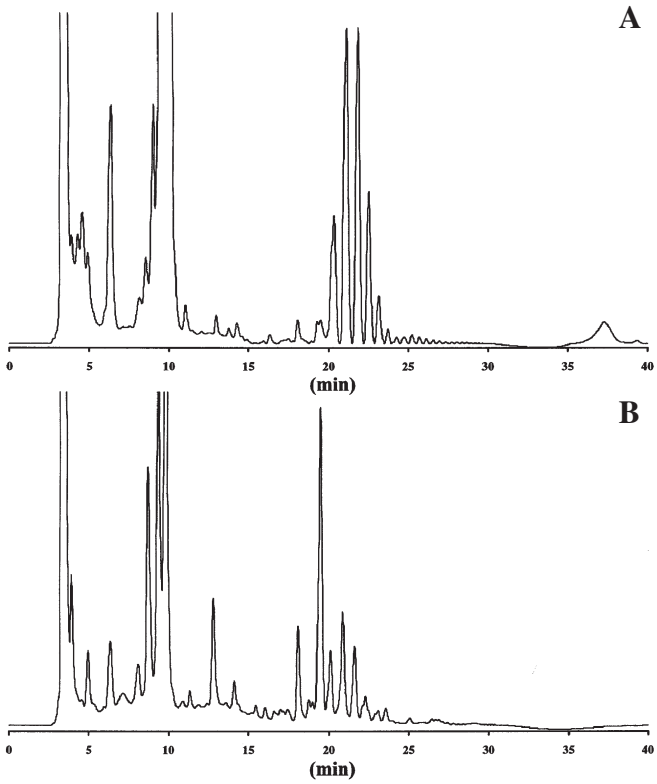


**FIG. 2.** Reversed-phase high-performance liquid chromatogram of polyprenol mixtures. (A) Bark sample, (B) seed sample. Conditions: programmed elution of methanol/propanol/water (60:40:5) to *n*-hexane/isopropanol (70:30) within 40 min, 1.0 mL/min, 40°C, ultraviolet detection at 210 nm. The determination of polymerization degree was made by cochromatography with authentic polyprenols.

We previously investigated the separation potential of SFC for waxy compounds and established a reliable separation condition (10). The resolution ($R_s$) of separation between the two peaks for prenol-18 and -19 was *ca*. two times larger than that using conventional reversed-phase HPLC.

SFC coupled with a $C_{18}$ reversed-phase column gave excellent baseline separation and enabled us to separate as many as 30 polyprenol components in the leaf sample (Fig. 3B). However, several polyprenols that eluted between $R_t$ 15 and 25 min were partly overlapped and were not satisfactorily separated. To improve separation, we investigated several reversed-phase columns and solvent systems. A reversed-phase column, Inertsil Ph-3, combined with supercritical carbon dioxide containing ethanol as a mobile phase, showed excellent separation (Fig. 4 B) and enabled us to conduct preparative SFC and obtain each component as a single compound. An improvement of the separation profiles by use of phenyl-type columns enabled us to confirm the presence of minor geometric isomers appearing as prominent peaks, each of which was eluted at equal time intervals in the leaf, root, and seed coat.

The Para rubber tree is known to produce *cis*-polyisoprene with a molecular weight of hundreds of thousands and to contain poly-*cis* prenols with polymerization degrees of more than 50 (11). These facts confirm that polyprenols play important roles in the *cis*-polyisoprene biosynthesis. It is also suggested that *E. ulmoides* should contain long-chain poly-*trans* prenols.

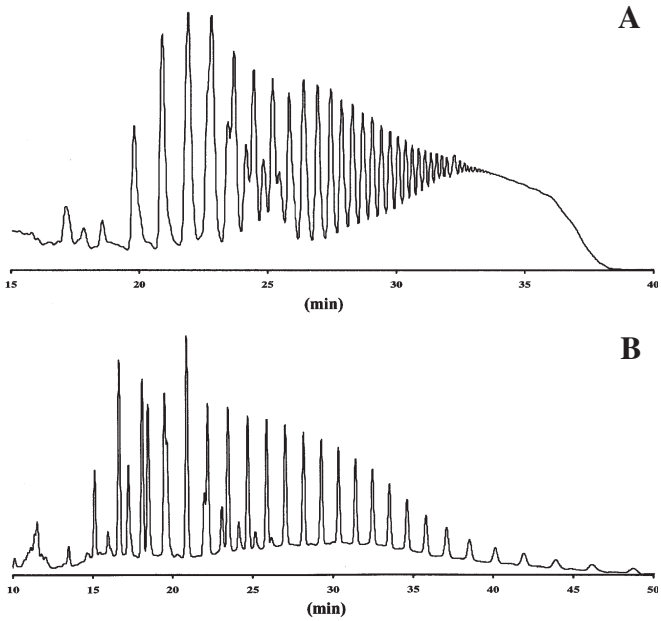


**FIG. 3.** Analysis of polyprenol mixtures from *Eucommia ulmoides* leaves by (A) reversed-phase high-performance liquid chromatography (HPLC) and (B) supercritical fluid chromatography (SFC). Conditions are described in the Materials and Methods section.

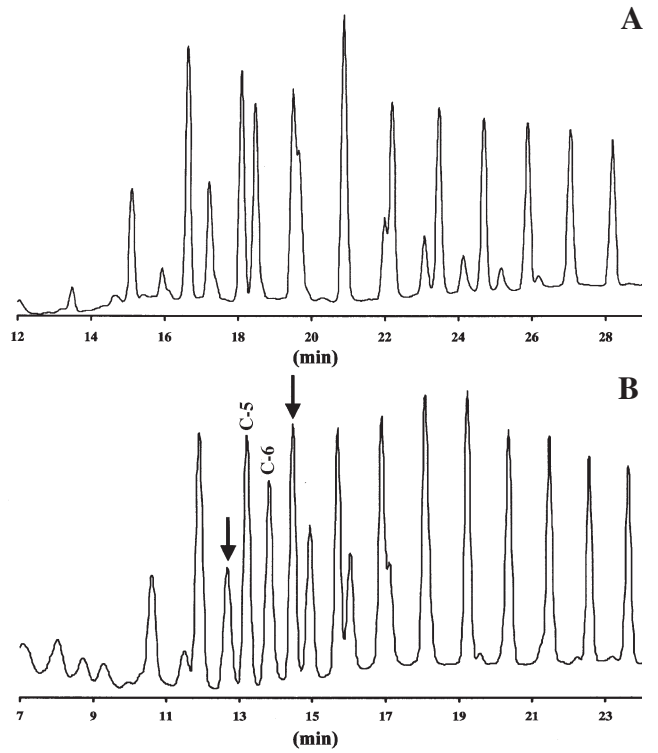


**FIG. 4.** Separation of polyprenols from *Eucommia ulmoides* leaves by SFC with (A) ODS-3 column, (B) Ph-3 column. Conditions: mobile phase ($CO_2$) flow, 3.0 mL/min; modifier (ethanol) flow, 0.8–2.0 mL/min within 30 min; top presssure, 19.6 MPa; detection, ultraviolet, 210 nm. The pair of peaks labeled with arrows were the same molecular weight. C-5: Compound 5, C-6: Compound 6. For abbreviation see Figure 3.

*Structural elucidation of polyprenols.* A detailed comparison of separation profiles allowed us to tentatively assign every other peak appearing at certain intervals to *trans* isomers. Only when the baseline peak separation of each component was achieved could we determine the number of isoprene units together with the content of the individual component in the leaf polyprenol fraction by spectroscopic analysis. Identification of the components of *E. ulmoides* supported the presence of unknown geometric isomers with all-*trans* double bonds.

Molecular weight analysis of the baseline-separated peaks was carried out by means of field desorption MS, and the same molecular weights were seen for the pair of peaks shown as arrows in Figure 4B, which indicated the presence of geometrical isomers.

In order to identify the geometry of the double bonds as well as the chain length of each component, every possible peak corresponding to *ca.* 10–30 mers of polyprenols was isolated using SFC. Two compounds (C-5 and C-6) from the leaf sample were separated by preparative SFC (Fig. 4B), and the collected samples were subjected to NMR analysis.

Geometric isomers can be identified by the assignment of methyl proton signals in $^1$H NMR. Methyl groups on *cis*- and *trans*-polyisoprene generally appear at different chemical shifts; *cis*: 1.76 ppm and *trans*: 1.64 ppm in benzene-$d_6$ (12). The α and ω end terminals can be assigned by high-resolution NMR.

In the $^1$H NMR spectrum of the collected sample (C-5), the signal at 1.635 ppm was assigned to the methyl groups in the central isoprene units of the *trans*-*trans*-*trans* sequence (Fig. 5; underlines indicate the observation site, i.e., the middle). The signals at 1.577 and 1.684 ppm were assigned to the methyls of the terminal dimethylallyl. The signals at 1.503 and 1.600 ppm correspond to the methyls of the *trans* (α)-terminal units, *trans*-α(*t*)-OH and *trans*-*trans*-α(*t*), respectively. These indicated that C-5 was an all-*trans* polyprenol, and this polyprenol had 15 isoprene units, as calculated from the integral value. This is the first report of the occurrence of a long- chain all-*trans* polyprenol, though solanesol (9 mers) from *Nicotiana*

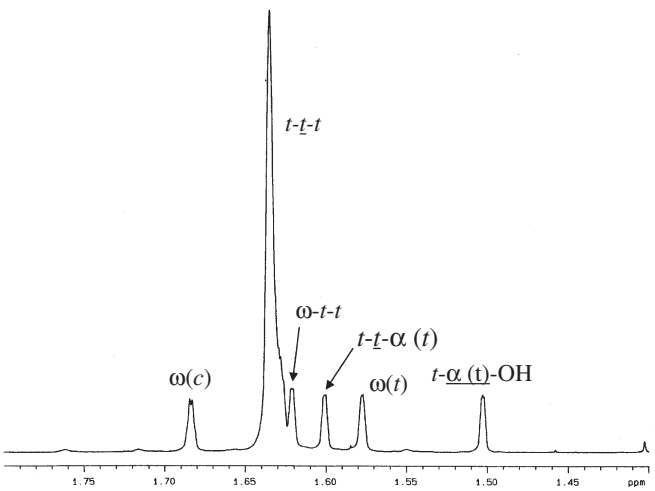


**FIG. 5.** $^1$H Nuclear magnetic resonance spectrum of C-5 in $C_6D_6$.

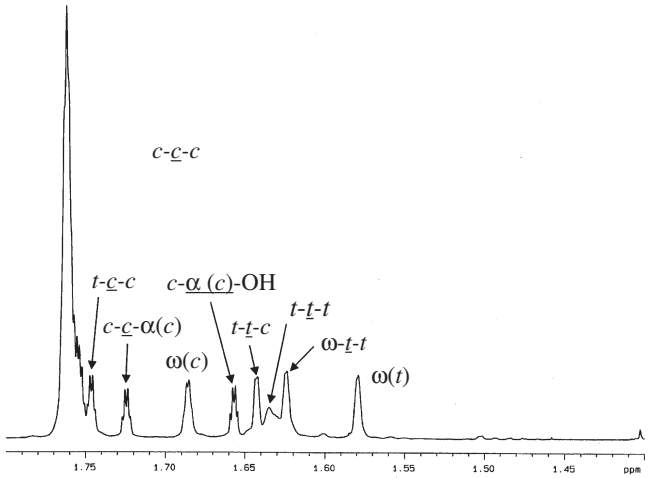


**FIG. 6.** $^1$H Nuclear magnetic resonance spectrum of C-6 in $C_6D_6$.

had previously been the only known all-*trans* polyprenol with polymerization degrees of more than five isoprene units.

In the $^1$H NMR spectrum of C-6, the methyls of the dimethylallyl terminal resonated at 1.579 and 1.685 ppm (Fig. 6). The signal at 1.762 ppm was assignable to the methyls in the central isoprene unit of the *cis*-*cis*-*cis* sequence. The signals at 1.746 and 1.724 ppm were from *cis* units. The signal at higher magnetic field (1.724 ppm) was due to the sequence *cis*-*cis*-*cis*(α), and the signal at lower magnetic field (1.776 ppm) was ascribable to the sequence *trans*-*cis*-*cis*, based on accumulated data (12). The signal at 1.657 ppm was assigned to *cis*-*cis*(α)-OH. The two signals at 1.624 and 1.642 ppm were from *trans* units, i.e., those in ω-*trans*-*trans* and *trans*-*trans*-*cis* sequences. By comparison with previously published data (12), the signal at 1.642 ppm was ascribable to a *trans*-*trans*-*cis* sequence, and the signal at 1.624 ppm to an ω-*trans*-*trans* sequence. Consequently, C-6 was identified as a tri-*trans* poly-*cis* prenol with 17 isoprene units.

Precise chain lengths were determined by field desorption-MS analysis, and the molecular weight of each peak was elucidated as shown in Table 1. The results suggested that *cis* and *trans* geometric isomers eluted at equal intervals. Based on NMR and MS analyses, the distribution of the chain length of the polyprenols was determined, as shown in Figure 7.

In *E. ulmoides*, polymerization degrees of poly-*cis* prenols are thus between 15 and 20 mers, and this is similar to those in other organisms (5,13). In contrast, *trans* forms showed a broad distribution range of 13 to 37 mers, or more.

Analyses of polyprenols that originated from the bark, seed, seed coat, and root were performed in a similar manner to the leaf sample analysis. Each sample had a characteristic distribution of polyprenols (Fig. 8). Both poly-*trans* and poly-*cis* prenols existed in the seed coat and root samples. In contrast, there was no poly-all-*trans* prenol in the seed and bark sample but rather tri-*trans* poly-*cis* prenols .

The poly-*trans* prenols had a broad distribution. However, the poly-*cis* prenols having peak maxima of 16 and 17 mers had a comparatively narrow distribution. In our previous study of *EU-*

**TABLE 1**
**Field Desorption-Mass Spectrometric Analysis of Polyprenols from *Eucommia ulmoides* Leaves[a]**

| Fraction no. | MW | DP | Configuration |
|---|---|---|---|
| 1 | 902 | 13 | Poly-*trans* |
| 2 | 1038 | 15 | Poly-*cis* |
| 3 | 970 | 14 | Poly-*trans* |
| 4 | 1106 | 16 | Poly-*cis* |
| 5 | 1038 | 15 | Poly-*trans* |
| 6 | 1174 | 17 | Poly-*cis* |
| 7 | 1106 | 16 | Poly-*trans* |
| 8 | 1242 | 18 | Poly-*cis* |
| 9 | 1174 | 17 | Poly-*trans* |
| 10 | 1310 | 19 | Poly-*cis* |
| 11 | 1242 | 18 | Poly-*trans* |
| 12 | 1378 | 20 | Poly-*cis* |
| 13 | 1310 | 19 | Poly-*trans* |
| 14 | 1378 | 20 | Poly-*trans* |

[a]MW, moecular weight; DP, degree of polymerization.

Rubber, *trans*-polyisoprene was found in all parts except the seed (14). Our SFC analyses suggested that poly-*trans* prenols, presumed to be biosynthetic intermediates of *trans*-polyisoprene, occurred in all of the *EU*-Rubber-producing tissues except the bark, where only *EU*-Rubber with high molecular weights was found. In preliminary *in vitro* polymerization experiments using $^{14}$C-labeled IPP, radioactivities were found not only in the rubber fraction but also in the polyprenol fraction (low molecular weight polyisoprene fraction). This result strongly supported the idea that polyprenols are biosynthetic intermediates in polyisoprene biosynthesis. The lack of poly-*trans* prenols in the bark samples supports the presence of an effective polymerization process of the intermediate polyprenols, which quickly react with IPP to form fibrous structures. The chain elongation mechanism leading to higher molecular weight products might take place effectively in the bark, and thus, low molecular weight polyprenols do not remain in the bark. Our ongoing isotopic experiments should provide conclusive results for this point.

We have shown that the content and molecular weight distribution of *EU*-Rubber differ in the various parts of *E. ulmoides* and are site-specific (13). This study revealed that *trans* geometric intermediates were detected in all the tissues but the bark, and that the molecular weight distribution of polyprenols is tissue-specific, suggesting that the regulation mechanisms of polyisoprene elongation differ in these tissues. In order to elucidate the regulation mechanisms for the chain elongation and geometrical isomerization of polyisoprenes, an effective and precise analysis of the polyprenol and rubber fractions is essential.

Our SFC analytical method for polyprenol derivatives should be helpful in promoting biosynthetic work on rubber, since this technique is applicable to the separation of close structural relatives of rubber components other than polyprenols and lipophilic hydrocarbon analogs.

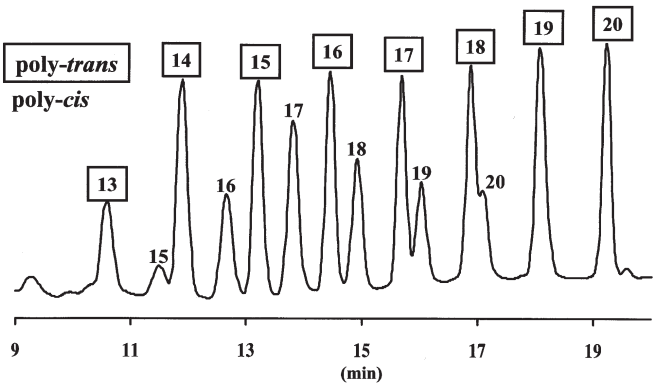


**FIG. 7.** Identification of polyprenols from *Eucommia ulmoides* leaves. Poly-*trans* prenol: box; poly-*cis*-prenol: unboxed. The numbers represent polymerization degrees in the chromatographic trace of SFC. For abbreviation see Figure 3.

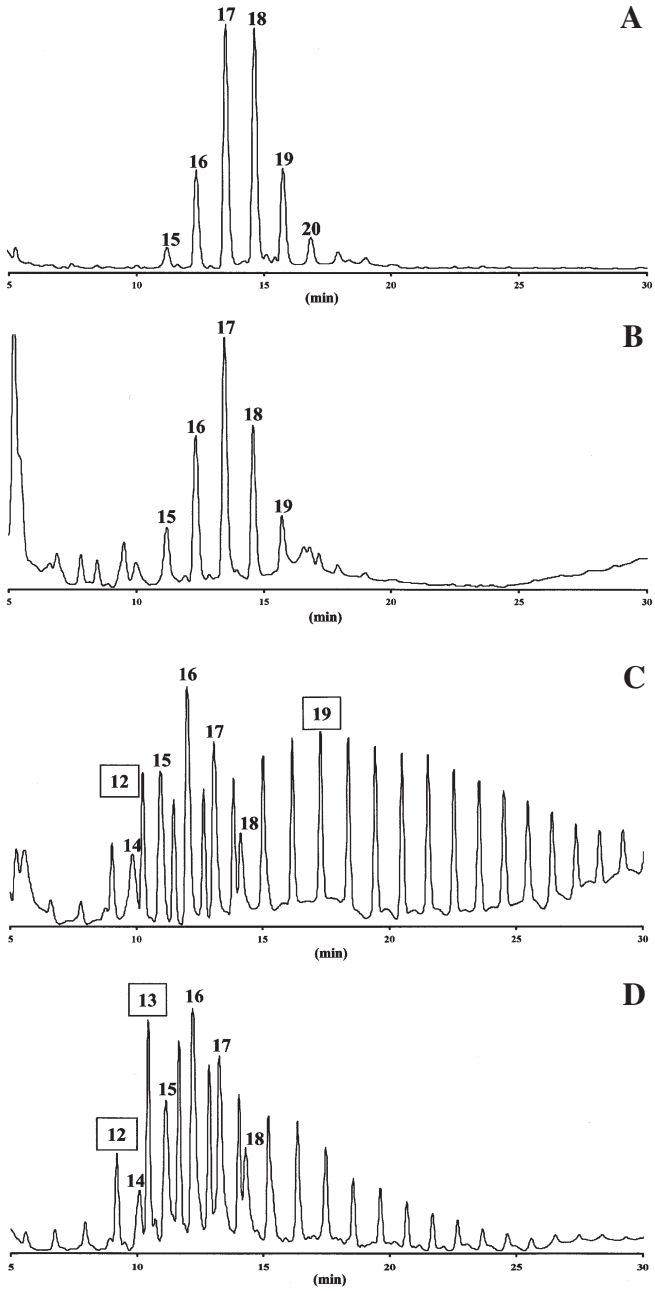


**FIG. 8.** Distribution of polyprenols in *Eucommia ulmoides*. (A) Bark, (B) seed, (C) root, and (D) seed coat. Poly-*trans* prenol: box; poly-*cis* prenol: unboxed. SFC analytical conditions were the same as in the case of leaf samples. For abbreviation see Figure 3.

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# Positional Distribution of Highly Unsaturated Fatty Acids in Triacyl-*sn*-glycerols of *Artemia* Nauplii Enriched with Docosahexaenoic Acid Ethyl Ester

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**ABSTRACT:** This paper presents the positional distribution of fatty acids in triacyl-*sn*-glycerols (TAG) of *Artemia* nauplii used in aquaculture as a live food for marine fish larvae. The nauplii were enriched with docosahexaenoic acid (DHA) ethyl ester (EE) in the form of gelatin-acacia microcapsules for 4, 18, and 24 h. TAG of the initial, enriched, and unenriched *Artemia* nauplii were subjected to stereospecific analysis. A remarkable increase of DHA content in the enriched *Artemia* TAG confirmed the view that DHA-EE is effectively assimilated and incorporated into the TAG fraction of *Artemia* nauplii. TAG of the nauplii enriched with 25 mg/L of DHA-EE contained DHA at concentrations of 5.9–6.8, 4.3–6.0, and 14.3–22.3 mol% in the *sn*-1, *sn*-2, and *sn*-3 positions, respectively. When the nauplii were enriched with 100 mg/L of DHA-EE, proportions of DHA in the *sn*-1, *sn*-2, and *sn*-3 positions were 5.2–8.6, 3.9–6.0, and 12.2–25.4 mol%, respectively. In all of the enriched *Artemia*, DHA was preferentially located in the *sn*-3 position followed in sequence by the *sn*-1 and *sn*-2 positions. The lower content of DHA in the *sn*-1 and *sn*-2 positions was consistent with low content of this acid in 1,2-diacyl-*sn*-glycerophospholipids. When fish larvae are reared on *Artemia* nauplii enriched with EE-type DHA oil, the larvae feed on DHA esterified in TAG with a positional distribution pattern similar to that of marine mammals (*sn*-3 >> *sn*-1 > *sn*-2) rather than that of fish or marine invertebrates (*sn*-2 >> *sn*-3 > *sn*-1).

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Longer-chain n-3 highly unsaturated fatty acids (HUFA) such as docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are essential dietary components for marine fish larvae (1–3). Various marine fish larvae exhibit high mortalities and abnormalities, such as underdeveloped swim bladders, scoliosis and depigmentation, when reared on diets either devoid or containing very low levels of n-3 HUFA (2,4). These essential fatty acids must be supplied in the diet to ensure good growth and survival of cultured marine fish larvae.

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Abbreviations: DHA, docosahexaenoic acid; EE, ethyl ester; EPA, eicosapentaenoic acid; HPLC, high-performance liquid chromatography; HUFA, highly unsaturated fatty acids; MAG, monoacyl-*sn*-glycerols; PC, phosphatidylcholines; PE, phosphatidylethanolamines; TAG, triacyl-*sn*-glycerols; TL, total lipids.

*Artemia* nauplii are used in aquaculture as an important live food for fish larvae. However, the nauplii are an incomplete food source for many marine fish larvae because of their low level of essential n-3 HUFA and of DHA in particular (1,5–11). The nauplii are usually enriched with diets rich in n-3 HUFA, such as commercially available oil-based emulsions (e.g., Ref. 10), selected microalgae (11,12), algal-derived products (13), HUFA-modified yeast (9,11), or microparticulate diets (14,15), prior to being fed to fish larvae. Sargent *et al*. (1) reviewed recent procedures for optimizing the presentation of dietary HUFA to marine fish larvae in relation to the advantages and disadvantages of using single-cell eukaryotic organisms or purified fish oils as enrichment diet of live foods including *Artemia* nauplii.

Enrichment diets are evaluated mostly based on their capability to increase the content of n-3 HUFA in *Artemia* nauplii (e.g., 8–11,16–19). Enrichment of *Artemia* is often considered as a single filling of the gut with the enrichment diet. However, the increase in fatty acid content of *Artemia* nauplii after enrichment is due not only to a retention of the enrichment diet in the gut but also to an assimilation of fatty acids into lipid reserves as the result of ingestion, digestion, and metabolic conversions. Coutteau and Mourente (20) assessed lipid class compositions and their content of DHA and EPA in *Artemia* nauplii enriched with a lipid emulsion in which fatty acid ethyl ester (EE) provided a level of 30% n-3 HUFA. Enrichment of *Artemia* with the lipid emulsions resulted in an increase of triacyl-*sn*-glycerol (TAG) content. The low levels of EE recovered from *Artemia* after 24 h of enrichment with the emulsion containing EE demonstrated an efficient metabolic conversion into TAG. The TAG fraction concentrated >91% of the DHA and >64% of the total EPA present. These results showed that the n-3 HUFA and particularly DHA are effectively assimilated and incorporated into the TAG fraction during n-3 HUFA enrichment of *Artemia*. Takeuchi *et al*. (21) also concluded that fatty acid ethyl or methyl esters containing 43% of n-3 HUFA were very quickly converted to TAG and incorporated into the TAG fraction in *Artemia* nauplii. Recently, Navarro *et al.* (22) presented incontestable evidence of incorporation by using radiolabeled fatty acid EE for enrichment of *Artemia*.

The present study reveals positional distributions of n-3 HUFA in TAG of DHA-enriched *Artemia* nauplii, i.e., con-

tents of DHA esterified in the *sn*-1, *sn*-2, and *sn*-3 positions of TAG. The aim of this work was to characterize the mechanisms whereby ingested DHA is incorporated into TAG of *Artemia* nauplii and to determine the TAG form in which the essential DHA is available for fish larvae. For this purpose, EE of DHA (DHA-EE) with a high purity was selected as the simplest DHA-containing oil, and directly given to *Artemia* nauplii in the form of gelatin-acacia microcapsules.

## EXPERIMENTAL PROCEDURES

*Preparation of enrichment diet.* Gelatin-acacia microcapsules were used as DHA enrichment diet for *Artemia* nauplii, because fatty acid-related emulsifiers, such as Tween 80 (23,24) and natural phospholipids (17,18), are not necessary for their preparation.

The microcapsules were prepared by a method based on those described by Green and Schleicher (25) and Southgate and Lou (16) after some modifications as follows. A 10% (w/w) solution of gelatin (type A, from porcine skin, approx. 175 bloom; Sigma Chemical Co., St. Louis, MO) and a 10% (w/w) solution of acacia (gum arabic from acacia tree; Sigma Chemical Co.) were made up in distilled water at 40°C. Ten milliliters of these solutions were mixed and maintained at 40°C. DHA-EE (1 g; purity, >99%; Shiseido Co., Tokyo, Japan) was added to the gelatin-acacia solution, and the mixture was homogenized using a two-blade blender for 60 s. Distilled water (60 mL) was then added slowly to the homogenate drop by drop with constant stirring at 40°C. The mixture was poured into 500 mL of distilled water at 0°C, and the resulting microcapsule suspension was placed in a refrigerator for 2 h. The microcapsules had a mean diameter of 0.88 ± 0.47 μm ($n$ = 100; based on particle number) and were used in suspension without further treatment.

Artemia *enrichments. Artemia* cysts (Great Salt Lake, UT; Senju Seiyaku Co., Itami, Japan) were incubated in 2% (w/w) salinity artificial seawater at 25°C under continuous illumination and aeration for 24 h. Nauplii were separated from empty cysts, washed in a fresh portion of 2% salinity artificial seawater, and placed in clean, aerated 2% salinity artificial seawater. Enrichment was carried out with 24-h-old *Artemia* nauplii at a density of 100,000 nauplii/L in 10 L tanks containing well-aerated 2% (w/w) salinity artificial seawater at 20°C. Microcapsules containing DHA-EE were added to the tanks at 25, 50, and 100 mg/L on weight bases of DHA-EE. After the microcapsules were added to the culture tanks, enriched *Artemia* nauplii samples (2 L of the artificial seawater) were taken on a nylon mesh at 4, 18, and 24 h, washed in distilled water, weighed, and stored at –35°C for lipid analysis. Under the same conditions, unenriched *Artemia* samples were obtained after 4, 18, and 24 h starvation without adding the microcapsules. *Artemia* nauplii were also taken just before the start of enrichment as an initial sample.

*Isolation of TAG.* Total lipids (TL) were extracted from *Artemia* nauplii by the method of Bligh and Dyer (26). TAG were isolated from other lipids by preparative thin-layer chromatography on Silicagel 60G plates (0.5 mm thickness; Merck, Darmstadt, Germany) with hexane/diethyl ether (80:20, vol/vol) for development.

*Fatty acid analysis.* Fatty acid methyl esters were prepared by reacting 0.5 mg of TAG in a mixture of 1,2-dichloromethane (0.6 mL), methyl acetate (25 μL), and 1 M sodium methoxide/methanol solution (25 μL) at room temperature overnight. After adding acetic acid (9 μL) and removing the solvents, the products were taken up in hexane. Fatty acid methyl esters were analyzed by gas–liquid chromatography on a Shimadzu GC-14A gas chromatograph (Shimadzu Co., Kyoto, Japan) equipped with a capillary column Omegawax 320 (30 m × 0.32 mm i.d., 0.25 μm film thickness; Supelco Inc., Bellefonte, PA) and a flame-ionization detector. Column temperature was programmed from 180 to 240°C (1°C/min), and injector and detector temperatures were 250 and 260°C, respectively. Helium was the carrier gas. Peak area percentages were measured with a Shimadzu C-R6A integrator.

*Stereospecific analysis.* Positional distribution of n-3 HUFA in the TAG of *Artemia* nauplii was determined by stereospecific analysis of TAG. The method for stereospecific analysis of natural oil TAG (27–30) was used after modifications as follows. TAG (5 mg), mixed with trinonadecanoylglycerol (0.5 mg), were dissolved in 0.23 mL of dry diethyl ether, and ethyl magnesium bromide in dry diethyl ether (0.1 mL of 1 M solution) was added. The mixture was shaken for 25 s, and then 2 mL of acetic acid/diethyl ether (1:200, vol/vol) followed by water (1 mL) was added to stop the reaction. The ether layer was washed once with 2% aqueous sodium bicarbonate, then washed with water, and dried over anhydrous sodium sulfate. After removal of the solvent at ambient temperature, all of the products were dissolved in dry toluene (0.5 mL) and reacted with 3,5-dinitrophenylisocyanate (40 mg) in the presence of dry pyridine (5 μL) for 1 h at ambient temperature. Dry 1-propanol (20 μL) was added to stop the reaction, the bulk of the solvent was then removed in a stream of nitrogen, and the residual toluene solution filtered through a small cotton-wool plug. The resulting bis-3,5-dinitrophenylurethane derivatives of isomeric or enantiomeric 1-, 2-, and 3-monoacyl-*sn*-glycerols (MAG) were isolated from other products by high-performance liquid chromatography (HPLC) with a Shimadzu LC-6A pump, a Jasco 875-UV ultraviolet spectrophotometric detector (Japan Spectroscopic Co., Tokyo, Japan), and a Shimadzu C-R6A integrator. A column of Sumichiral OA-4100 (25 cm × 4.6 mm i.d., 5 μm particles; Sumitomo Chemical Co., Osaka, Japan) was used for isolation of *sn*-1- plus *sn*-2-MAG and *sn*-3-MAG fractions, and then one of Sumichiral OA-4000 (25 cm × 4.6 mm i.d., 5 μm particles) for separation of *sn*-1-MAG and *sn*-2-MAG fractions. Both HPLC separations were done with hexane/1,2-dichloromethane/ethanol (40:12:3, by vol) as mobile phase at a flow rate of 1.5 mL/min at –10°C. Detection was at 254 nm. Constituent fatty acids of the *sn*-1-, *sn*-2-, and *sn*-3-MAG derivative fractions were analyzed by reversed-phase HPLC without further derivatization. HPLC was done with a Hitachi L-6200 pump (Hitachi Co., Tokyo,

Japan), a Hitachi L-4200 ultraviolet spectrophotometric detector, and a Shimadzu C-R6A integrator. A column of Capcellpak C18 UG120 S-3 (15 cm × 4.6 mm i.d., 3 μm particles; Shiseido Co.) was used with acetonitrile and water as mobile phase at a flow rate of 1.0 mL/min. A linear gradient of 70% acetonitrile/30% water to 100% acetonitrile was generated over 90 min. Column temperature was held at 10°C for 35 min and then immediately changed to 30°C. Assignments of each fatty acid to the *sn*-1-, *sn*-2-, and *sn*-3-positions of TAG were obtained from the peak area ratio of each MAG molecular species relative to monononadecanoylglycerol, formed from the trinonadecanoylglycerol internal standard, and the fatty acid composition of each position was calculated on the basis of the assignments.

## RESULTS

*Contents of TL and TAG in* Artemia *nauplii.* Contents of TL and TAG in *Artemia* nauplii (dry-weight base) enriched with DHA-EE are shown in Table 1 together with those of initial and unenriched *Artemia*. Enrichment of *Artemia* with DHA-EE resulted in an increase of TL content from 20.2% (0 h) to 25.0–26.3% (4 or 18 h) and subsequent decrease to 20.3–22.6% (24 h). TAG content also increased from 7.7% (0 h) to 8.5–9.5% (4 h) and then decreased to 6.6–7.7% (24 h). Changes in TL and TAG contents were similar among the three enrichments with 25, 50, and 100 mg/L of DHA-EE with exception of a faster decrease of TL content observed for enrichment with 25 mg/L of DHA-EE. Unenriched *Artemia* showed a consistent decrease of TL content from 20.2 to 12.4%. This was due to a decrease of TAG content from 7.7 to 1.3%. Although the TAG content of enriched *Artemia* was not high compared with that of the initial sample, the enriched nauplii contained markedly higher amounts of TAG than the unenriched nauplii. Differences in TAG contents between the enriched and unenriched *Artemia* nauplii were 3.1–4.1% at 4 h, 5.6–6.4% at 18 h, and 5.3–6.4% at 24 h.

*Fatty acid compositions of* Artemia *nauplii TAG.* Table 2 shows the fatty acid compositions of TAG in *Artemia* nauplii. The principal fatty acids at more than 3 mol% of the total fatty acids were 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-7, 18:2n-6, and 18:3n-3 in the initial and unenriched *Artemia* nauplii. Proportions of EPA (20:5n-3) and DHA (22:6n-3) were 1.9–2.4 and ≤0.2 mol%, respectively. Changes in the proportions of major fatty acids were similar among the *Artemia* enriched with DHA-EE at the three concentrations. In all of the enrichments, concentrations of DHA increased with enrichment period (especially 0–4 h), reached maxima after 18 h, and dropped slightly during 18–24 h. The maximal concentrations of DHA were 11.4, 12.2, and 13.2 mol% in the enrichment with 25, 50, and 100 mg/L of DHA-EE, respectively. The levels of EPA consistently increased from 2.4 to 4.1–4.2 mol% during the enrichment period. The levels of other major fatty acids such as 16:0, 16:1n-7, 18:1n-9, 18:1n-7, 18:2n-6, and 18:3n-3 decreased during the enrichment period (especially 0–18 h). In the unenriched *Artemia* TAG, fatty acid composition was almost steady, although slight decreases of 18:3n-3 and 18:2n-6 levels were observed during 0–24 h. Therefore, enriched *Artemia* showed significantly higher proportions of DHA and EPA than unenriched *Artemia*, and lower proportions of all other fatty acids.

*Positional distributions of fatty acids in TAG of* Artemia *nauplii.* Table 3 shows the positional distributions of principal fatty acids in TAG in *Artemia* nauplii enriched with DHA-EE at 25 and 100 mg/L together with those of initial and unenriched *Artemia*. TAG of *Artemia* enriched with 50 mg/L of DHA-EE were not subjected to stereospecific analysis, because of the similarity in positional distributions between the *Artemia* enriched with 25 and 100 mg/L of DHA-EE as described below.

In the initial *Artemia*, the predominant saturated and monounsaturated fatty acids (16:0, 18:0, and 18:1) were preferentially esterified in the *sn*-1 position followed in sequence by the *sn*-3 and *sn*-2 positions (i.e., *sn*-1 > *sn*-3 > *sn*-2 positions). The most prominent polyunsaturated fatty acid (18:3n-3) was concentrated in the *sn*-2 position followed in sequence by the *sn*-3 and *sn*-1 positions (i.e., *sn*-2 >> *sn*-3 > *sn*-1 positions), whereas 18:2n-6 was primarily located in the *sn*-2 and *sn*-3 positions (i.e., *sn*-2 ≈ *sn*-3 > *sn*-1 positions). EPA was esterified in the *sn*-2 position followed by the *sn*-3 position (i.e., *sn*-2 > *sn*-3 > *sn*-1 positions). The *sn*-1 position was high in 18:1 and 16:0 followed by 18:0 and 18:3n-3; the *sn*-2 position in 18:3n-3 and 18:1 followed by 18:2n-6; and the *sn*-3 position in 18:1, 18:3n-3, and 16:0 followed by 16:1n-7 and 18:2n-6.

In the unenriched *Artemia* TAG, the *sn*-1 position showed fatty acid compositions consistently similar to that in the initial *Artemia*. In the *sn*-2 position, 18:3n-3 decreased from 62.5 mol% to 59.1 mol% (24 h), and in contrast 18:1 increased from 10.2 mol% to 13.2 mole % (24 h). Decreases of 18:2n-6 and 18:3n-3 were also observed in the *sn*-3 position.

Enriched *Artemia* showed increases of DHA in all three positions of TAG. The nauplii enriched with 25 mg/L of DHA-EE contained DHA at concentrations of 5.9–6.8, 4.3–6.0, and 14.3–22.3 mol% in the *sn*-1, *sn*-2, and *sn*-3 posi-

**TABLE 1**
**Contents of Total Lipids (TL) and Triacyl-*sn*-glycerols (TAG) in *Artemia* Nauplii[a] Enriched and Unenriched with Docosahexaenoic Acid Ethyl Ester (DHA-EE) (%, dry-weight basis)**

| | Initial | Unenriched | | | 25 mg/L[b] | | | 50 mg/L[c] | | | 100 mg/L[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | 4 h | 18 h | 24 h | 4 h | 18 h | 24 h | 4 h | 18 h | 24 h | 4 h | 18 h | 24 h |
| Total lipids | 20.2 | 16.2 | 14.7 | 12.4 | 25.0 | 22.0 | 20.3 | 26.0 | 26.3 | 21.6 | 25.5 | 26.2 | 22.6 |
| Triacyl-*sn*-glycerols | 7.7 | 5.4 | 2.0 | 1.3 | 8.5 | 7.6 | 7.0 | 9.5 | 8.1 | 6.6 | 8.5 | 8.4 | 7.7 |

[a]*Artemia* nauplii enriched with [b]25, [c]50, and [d]100 mg of DHA-EE per liter of rearing artificial seawater.

**TABLE 2**
**Fatty Acid Composition of TAG in *Artemia* Nauplii[a] Enriched and Unenriched with DHA-EE (mol%)**

| | Initial | Unenriched | | | 25 mg/L[b] | | | 50 mg/L[c] | | | 100 mg/L[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 h | 4 h | 18 h | 24 h | 4 h | 18 h | 24 h | 4 h | 18 h | 24 h | 4 h | 18 h | 24 h |
| 14:0 | 1.2 | 1.3 | 1.2 | 1.3 | 0.9 | 0.8 | 0.8 | 1.0 | 0.9 | 0.8 | 1.0 | 0.7 | 0.8 |
| Iso-15:0 | 1.3 | 1.5 | 1.6 | 1.7 | 1.1 | 1.0 | 1.0 | 1.2 | 1.1 | 1.0 | 1.2 | 1.0 | 1.0 |
| Anteiso-15:0 | 0.6 | 0.1 | 0.7 | 0.9 | 0.5 | 0.4 | 0.5 | 0.5 | 0.4 | 0.5 | 0.5 | 0.4 | 0.4 |
| 15:0 | 0.5 | 0.6 | 0.6 | 0.6 | 0.4 | 0.4 | 0.4 | 0.5 | 0.4 | 0.4 | 0.5 | 0.4 | 0.4 |
| 15:1 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 |
| Iso-16:0 | 0.8 | 0.9 | 1.0 | 1.1 | 0.7 | 0.7 | 0.7 | 0.8 | 0.7 | 0.7 | 0.8 | 0.7 | 0.7 |
| 16:0 | 13.0 | 13.5 | 12.9 | 13.3 | 11.5 | 10.6 | 10.7 | 11.6 | 10.6 | 10.6 | 11.7 | 10.2 | 10.3 |
| 16:1n-9 | 0.8 | 1.2 | 1.2 | 1.2 | 1.2 | 1.1 | 1.1 | 1.1 | 1.1 | 1.0 | 1.1 | 1.1 | 1.0 |
| 16:1n-7 | 4.2 | 4.4 | 4.2 | 4.4 | 3.8 | 3.5 | 3.5 | 3.9 | 3.4 | 3.4 | 3.9 | 3.3 | 3.3 |
| 16:1n-5 | 0.2 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.2 | 0.2 | 0.2 |
| Iso-17:0 | 0.9 | 1.0 | 1.1 | 1.2 | 0.8 | 0.8 | 0.9 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| 16:2n-6 | 1.6 | 1.7 | 1.9 | 2.1 | 1.5 | 1.4 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.4 | 1.4 |
| 17:0 | 1.0 | 1.1 | 1.1 | 1.1 | 1.0 | 0.9 | 0.9 | 1.0 | 1.0 | 1.0 | 1.0 | 0.9 | 0.9 |
| 17:1n-8 | 1.5 | 1.5 | 1.5 | 1.5 | 1.3 | 1.2 | 1.3 | 1.4 | 1.3 | 1.2 | 1.4 | 1.2 | 1.2 |
| 16:3n-3 | 0.8 | 0.7 | 0.6 | 0.6 | 0.6 | 0.6 | 0.5 | 0.6 | 0.5 | 0.6 | 0.7 | 0.6 | 0.5 |
| 17:2 | 0.3 | 0.3 | 0.2 | 0.2 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 18:0 | 3.3 | 3.4 | 3.7 | 3.8 | 3.1 | 3.1 | 3.2 | 3.2 | 3.1 | 3.2 | 3.1 | 3.1 | 3.2 |
| 18:1n-9 | 18.8 | 19.5 | 19.6 | 19.7 | 17.2 | 16.4 | 16.7 | 17.3 | 16.2 | 16.5 | 17.4 | 16.3 | 16.3 |
| 18:1n-7 | 6.0 | 5.9 | 6.0 | 6.1 | 5.7 | 5.4 | 5.5 | 5.4 | 5.1 | 5.2 | 5.6 | 5.3 | 5.2 |
| 18:2n-6 | 6.1 | 5.8 | 5.8 | 5.4 | 5.6 | 5.2 | 5.2 | 5.6 | 5.2 | 5.2 | 5.7 | 5.1 | 5.1 |
| 18:3n-6 | 0.9 | 0.7 | 0.7 | 0.6 | 0.7 | 0.7 | 0.6 | 0.7 | 0.6 | 0.6 | 0.8 | 0.6 | 0.6 |
| 18:3n-3 | 27.0 | 26.1 | 25.8 | 24.8 | 24.9 | 24.1 | 24.4 | 24.9 | 23.7 | 24.0 | 25.0 | 23.5 | 23.7 |
| 18:4n-3 | 2.6 | 2.2 | 1.9 | 1.7 | 2.4 | 2.1 | 2.0 | 2.4 | 2.1 | 2.0 | 2.4 | 2.1 | 2.0 |
| 20:0 | 0.1 | 0.2 | 0.2 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| 20:1n-9 | 0.4 | 0.5 | 0.5 | 0.6 | 0.3 | 0.4 | 0.4 | 0.3 | 0.3 | 0.4 | 0.3 | 0.4 | 0.4 |
| 20:4n-6 | 0.9 | 0.8 | 0.9 | 0.8 | 0.8 | 0.9 | 1.0 | 0.9 | 0.9 | 0.9 | 0.9 | 0.9 | 1.0 |
| 20:3n-3 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| 20:4n-3 | 0.5 | 0.5 | 0.4 | 0.4 | 0.5 | 0.5 | 0.4 | 0.5 | 0.4 | 0.4 | 0.5 | 0.5 | 0.4 |
| 20:5n-3 | 2.4 | 2.0 | 2.1 | 1.9 | 2.9 | 3.7 | 4.1 | 2.9 | 3.8 | 4.1 | 2.8 | 3.9 | 4.2 |
| 22:0 | 0.1 | 0.1 | 0.2 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| 22:5n-3 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 | 0.3 | 0.4 | 0.1 | 0.3 | 0.4 | 0.1 | 0.3 | 0.4 |
| 22:6n-3 | 0.1 | 0.1 | 0.0 | 0.2 | 8.1 | 11.4 | 10.2 | 7.5 | 12.2 | 11.4 | 7.0 | 13.2 | 12.5 |
| Others[e] | 1.5 | 1.5 | 1.7 | 1.5 | 0.9 | 0.9 | 0.9 | 0.9 | 1.0 | 1.0 | 1.1 | 0.9 | 1.0 |

[a]*Artemia* nauplii enriched with [b]25, [c]50, and [d]100 mg of DHA-EE per liter of rearing artificial seawater.
[e]Less than 0.1 mol%. For abbreviations see Table 1.

tions, respectively. When *Artemia* nauplii were enriched with 100 mg/L of DHA-EE, the proportions of this fatty acid in the *sn*-1, *sn*-2, and *sn*-3 positions were 5.2–8.6, 3.9–6.0, and 12.2–25.4 mol%, respectively. The increase of DHA in the *sn*-3 position was larger than that in the *sn*-1 and *sn*-2 positions. In all of the enriched *Artemia*, DHA was preferentially esterified in the *sn*-3 position. Smaller amounts of DHA were found in the *sn*-1 position followed by the *sn*-2 position. This acid was associated with the *sn*-3 >> *sn*-1 > *sn*-2 positions. During the enrichment with DHA-EE, the levels of EPA also increased in the three positions. This fatty acid increased from 0.7, 3.7, and 2.8 mol% (0 h) to 1.5–2.2, 3.9–5.0, and 3.2–5.1 mol% in the *sn*-1, *sn*-2, and *sn*-3 positions in *Artemia* enriched with 25 mg/L of DHA-EE, respectively. The nauplii enriched with 100 mg/L of DHA-EE had 1.6–2.3, 3.7–5.4, and 3.0–5.2 mol% of EPA in the *sn*-1, *sn*-2, and *sn*-3 positions, respectively. At the end of enrichment period (24 h), EPA was preferentially located in both the *sn*-2 and *sn*-3 positions followed by the *sn*-1 position. Distribution pattern of EPA after the 24-h enrichment (i.e., *sn*-2 ≈ *sn*-3 > *sn*-1 positions) was different from that of initial and unenriched *Artemia* (i.e., *sn*-2 > *sn*-3 > *sn*-1 positions).

Due to the considerable increase of DHA and EPA levels, some specific fatty acids changed in percentage in each position. In the *sn*-1 position, 16:0 decreased from 24.2 to 19.7 and 18.3 mol% during 18 h of enrichment with 25 and 100 mg/L of DHA-EE, respectively. The proportion of 18:1, the most prominent fatty acid in this position, was consistent. In the *sn*-2 position, 18:3n-3 showed a decrease from 62.5 to 55.5 and 53.8 mol% (18 h), whereas a slight increase was observed for 16:0. In the *sn*-3 position, all fatty acids other than DHA and EPA showed decreases during the 18-h enrichment.

## DISCUSSION

*Incorporation of DHA to* Artemia *nauplii TAG.* An increase of TL and TAG contents has been observed during enrichment of *Artemia* with DHA-containing EE (e.g., 18,20,21,31). In the study of Coutteau and Mourente (20), enrichment of *Artemia* with EE-type oil resulted in an increase of TL from 20.0 to 28.7% of dry matter due to the accumulation of neutral lipids, primarily TAG (from 8.2% in freshly hatched *Artemia* to 15.8% in 24-h enriched *Artemia*). McEvoy *et al.* (18) obtained, after 18-h enrichment with Super Selco (a com-

**TABLE 3**
**Positional Distribution of Fatty Acids in TAG of *Artemia* Nauplii Enriched and Unenriched with DHA-EE (mol%)**

| | | | 14:0 | 16:0 | 16:1n-7[a] | 18:0[a] | 18:1n-9[a] | 18:2n-6 | 18:3n-3 | 20:5n-3 | 22:6n-3 | Others |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial | 0 h | *sn*-1 | 0.9 | 24.2 | 3.8 | 7.0 | 35.8 | 3.5 | 6.3 | 0.7 | 0.1 | 17.9 |
| | | *sn*-2 | 0.1 | 2.5 | 4.3 | 0.3 | 10.2 | 7.3 | 62.5 | 3.7 | 0.0 | 9.1 |
| | | *sn*-3 | 2.4 | 12.1 | 7.2 | 3.7 | 27.7 | 7.6 | 14.0 | 2.8 | 0.2 | 22.5 |
| | | Total | 1.2 | 13.0 | 5.1 | 3.7 | 24.8 | 6.1 | 27.0 | 2.4 | 0.1 | 16.7 |
| Unenriched | 4 h | *sn*-1 | 1.0 | 24.5 | 4.0 | 7.3 | 35.8 | 3.4 | 5.8 | 0.7 | 0.1 | 17.5 |
| | | *sn*-2 | 0.2 | 3.4 | 4.9 | 0.5 | 12.1 | 7.3 | 62.4 | 3.4 | 0.0 | 5.8 |
| | | *sn*-3 | 2.7 | 11.9 | 6.8 | 4.1 | 27.6 | 6.8 | 12.2 | 2.1 | 0.3 | 25.5 |
| | | Total | 1.3 | 13.5 | 5.3 | 4.0 | 25.4 | 5.8 | 26.1 | 2.0 | 0.1 | 16.5 |
| | 18 h | *sn*-1 | 1.0 | 23.4 | 3.6 | 7.6 | 35.7 | 3.4 | 6.1 | 0.6 | 0.0 | 18.6 |
| | | *sn*-2 | 0.3 | 2.6 | 4.6 | 0.4 | 12.3 | 7.2 | 61.3 | 3.5 | 0.0 | 7.9 |
| | | *sn*-3 | 2.5 | 12.0 | 7.1 | 4.7 | 27.8 | 6.8 | 12.1 | 2.1 | 0.0 | 24.9 |
| | | Total | 1.2 | 12.9 | 5.1 | 4.3 | 25.6 | 5.8 | 25.8 | 2.1 | 0.0 | 17.3 |
| | 24 h | *sn*-1 | 1.0 | 24.3 | 4.1 | 7.7 | 35.6 | 3.8 | 5.1 | 0.6 | 0.1 | 17.8 |
| | | *sn*-2 | 0.3 | 3.5 | 5.3 | 0.5 | 13.2 | 7.4 | 59.1 | 3.3 | 0.1 | 7.3 |
| | | *sn*-3 | 2.5 | 11.8 | 6.4 | 5.3 | 28.0 | 4.9 | 11.1 | 1.9 | 0.5 | 27.7 |
| | | Total | 1.3 | 13.3 | 5.2 | 4.5 | 25.8 | 5.4 | 24.8 | 1.9 | 0.2 | 17.7 |
| Enriched (25 mg/L) | 4 h | *sn*-1 | 0.7 | 21.2 | 3.7 | 6.5 | 35.3 | 3.4 | 5.6 | 1.5 | 6.0 | 16.0 |
| | | *sn*-2 | 0.1 | 3.1 | 4.2 | 0.7 | 10.1 | 7.4 | 59.0 | 3.9 | 4.3 | 7.4 |
| | | *sn*-3 | 1.9 | 10.0 | 6.2 | 3.0 | 23.0 | 6.2 | 10.5 | 3.2 | 14.3 | 21.7 |
| | | Total | 0.9 | 11.5 | 4.7 | 3.4 | 22.9 | 5.6 | 24.9 | 2.9 | 8.1 | 15.1 |
| | 18 h | *sn*-1 | 0.6 | 19.7 | 3.5 | 7.0 | 36.0 | 3.3 | 5.8 | 1.9 | 6.8 | 15.3 |
| | | *sn*-2 | 0.2 | 3.9 | 4.1 | 0.6 | 9.5 | 6.7 | 55.5 | 4.9 | 6.0 | 8.6 |
| | | *sn*-3 | 1.7 | 8.2 | 5.5 | 2.9 | 20.1 | 5.5 | 9.5 | 4.4 | 22.3 | 19.9 |
| | | Total | 0.8 | 10.6 | 4.4 | 3.5 | 21.8 | 5.2 | 24.1 | 3.7 | 11.4 | 14.5 |
| | 24 h | *sn*-1 | 0.7 | 19.6 | 3.7 | 7.2 | 35.5 | 3.4 | 6.0 | 2.2 | 5.9 | 15.8 |
| | | *sn*-2 | 0.1 | 4.1 | 4.2 | 0.5 | 10.0 | 6.8 | 57.2 | 5.0 | 4.6 | 7.5 |
| | | *sn*-3 | 1.5 | 8.1 | 5.6 | 3.1 | 20.5 | 5.5 | 9.5 | 5.1 | 20.9 | 20.2 |
| | | Total | 0.8 | 10.7 | 4.5 | 3.6 | 22.2 | 5.2 | 24.4 | 4.1 | 10.2 | 14.4 |
| Enriched (100 mg/L) | 4 h | *sn*-1 | 1.0 | 21.3 | 3.9 | 6.6 | 35.1 | 3.6 | 7.3 | 1.6 | 5.2 | 14.5 |
| | | *sn*-2 | 0.2 | 3.7 | 4.4 | 0.7 | 10.2 | 7.2 | 56.7 | 3.7 | 3.9 | 9.2 |
| | | *sn*-3 | 1.9 | 10.1 | 6.1 | 3.0 | 23.6 | 6.5 | 10.7 | 3.0 | 12.2 | 23.1 |
| | | Total | 1.0 | 11.7 | 4.8 | 3.5 | 23.0 | 5.7 | 25.0 | 2.8 | 7.0 | 15.5 |
| | 18 h | *sn*-1 | 0.7 | 18.3 | 3.6 | 6.7 | 35.3 | 3.5 | 7.8 | 2.3 | 8.6 | 13.4 |
| | | *sn*-2 | 0.1 | 4.4 | 4.1 | 0.8 | 10.0 | 6.7 | 53.8 | 5.1 | 6.0 | 9.0 |
| | | *sn*-3 | 1.4 | 7.7 | 5.1 | 2.9 | 19.1 | 5.1 | 8.7 | 4.4 | 25.3 | 20.3 |
| | | Total | 0.7 | 10.2 | 4.2 | 3.5 | 21.5 | 5.1 | 23.5 | 3.9 | 13.2 | 14.2 |
| | 24 h | *sn*-1 | 0.7 | 18.5 | 3.6 | 7.1 | 35.2 | 3.5 | 7.2 | 2.3 | 7.1 | 14.8 |
| | | *sn*-2 | 0.1 | 4.5 | 4.1 | 0.5 | 9.6 | 6.6 | 54.9 | 5.4 | 5.8 | 8.5 |
| | | *sn*-3 | 1.5 | 7.4 | 5.2 | 3.0 | 19.2 | 5.1 | 9.1 | 5.2 | 25.4 | 19.0 |
| | | Total | 0.8 | 10.3 | 4.3 | 3.6 | 21.5 | 5.1 | 23.7 | 4.2 | 12.5 | 14.0 |

[a]Values for 16:1n-7, 18:0, and 18:1n-9 contain minor amounts of 20:4n-6, 20:1, and 18:1n-7, respectively. For abbreviations see Table 1.

mercially available enrichment diet consisting mainly of EE), a TL content in *Artemia* of 16.8% of which 11.4% (on a dry-weight basis) was TAG. These authors started their enrichment with 6-h-old *Artemia* that contained 12.3% of TL and 7.2% of TAG. Takeuchi *et al*. (21) reported two sets of data showing an increase of TL in *Artemia* from 33.0 and 24.4% to 45.2 and 31.9% and of TAG from 12.0 and 3.8% to 22.9 and 10.9% after 24 h of enrichment with EE-type oil, respectively.

The present study also showed increases of TL and TAG contents during the enrichment with DHA-EE in a manner similar to those reported (Table 1). The contents and increments of TL and TAG differed somewhat from those described above. Such variability can be explained by differences in populations and harvests of *Artemia*, starvation period before enrichment, and other enrichment protocols, such as duration, dosage, concentration and type for the enrichment diet, and physical and chemical conditions.

Coutteau and Mourente (20) also determined the distribution of DHA and EPA over the major neutral and polar lipid classes of n-3 HUFA-enriched *Artemia*. Over an 18-h enrichment period, the levels of DHA and EPA increased from 0.2 and 2.7% to 12.5 and 6.7% of total fatty acids in TAG, respectively. The TAG fraction contained 91 and 64% of the total amount of DHA (22.3 mg/g dry weight) and EPA (17.8 mg/g dry weight) present in the enriched *Artemia*, respectively. Navarro *et al.* (22) enriched *Artemia* nauplii for 24 h with radiolabeled fatty acid EE and analyzed the distribution of radioactivity in lipid classes of the enriched *Artemia*. At the end of the enrichment period, about 80% of radiolabeled DHA and EPA were recovered in the TAG fraction.

In the present study, the levels of DHA in *Artemia* TAG

**TABLE 4**
**Proportional Distribution of Docosahexaenoic Acid (DHA) Among the *sn*-1, *sn*-2, and *sn*-3 Positions of TAG in *Artemia* Nauplii Enriched with DHA-EE (% of DHA present)**

| | 25 mg/L[a] | | | 100 mg/L[b] | | |
|---|---|---|---|---|---|---|
| | 4 h | 18 h | 24 h | 4 h | 18 h | 24 h |
| *sn*-1 | 24.4 | 19.3 | 18.7 | 24.6 | 21.5 | 18.5 |
| *sn*-2 | 17.5 | 17.1 | 14.6 | 18.3 | 15.0 | 15.2 |
| *sn*-3 | 58.1 | 63.6 | 66.7 | 57.1 | 63.4 | 66.2 |

[a,b]*Artemia* nauplii enriched with [a]25 and [b]100 mg of DHA-EE per liter of rearing artificial seawater. For abbreviations see Table 1.

drastically increased from 0.1 mol% in the initial to 11.4–13.2 mol% in 18-h enriched *Artemia* (Table 2). Distribution of DHA between TAG and other lipid classes was not known, because lipid class compositions and their content of DHA were not determined in the present study. In spite of the lack of this information, the drastic increase of DHA in TAG confirms the view that EE-type DHA is effectively assimilated and incorporated into the TAG fraction of *Artemia* nauplii (20–22).

In the enriched *Artemia* TAG, the levels of EPA also increased from 2.4 to 4.1–4.2 mol% (Table 2). *Artemia* nauplii have the ability to retroconvert DHA to EPA (22). The consistent increase of EPA demonstrates that the retroconversion took place during the enrichment with DHA-EE in the present study.

*Positional distribution of DHA characteristic of* Artemia *nauplii.* Brockerhoff *et al*. (32) studied the positional distribution of fatty acids in TAG of many aquatic animals and described the general tendencies observed for their distribution. In invertebrates, squid (liver), periwinkle (whole body), lobster (hepatopancreas), and scallop (hepatopancreas), polyunsaturated fatty acids were preferentially located in the *sn*-2 position, saturated acids, 16:0 and 18:0, in the *sn*-1 position, and longer-chain acids in the *sn*-3 position. In fish, the *sn*-1 position attracted saturated and monounsaturated acids; the *sn*-2 position polyunsaturated and shorter-chain acids; and the *sn*-3 position longer-chain acids. The *sn*-2 position was also rich in 16:0. In marine mammals, shorter-chain acids accumulated in the *sn*-2 position but the polyunsaturated fatty acids occupied the *sn*-3 position, and then the *sn*-1 position.

The distribution pattern of fatty acids in TAG of initial and unenriched *Artemia* nauplii (Table 3) was not very different from the general tendency for invertebrates. The total unsaturation of the *sn*-2 position was very high. The contents of n-3 HUFA were low in both the initial and unenriched *Artemia* TAG. Instead of EPA and DHA, a common polyunsaturated fatty acid, 18:3n-3, was prominent in the *sn*-2 position. Saturated fatty acids, 16:0 and 18:0, also showed a distribution pattern similar to the general tendency for invertebrates.

Brockerhoff *et al*. (32) pointed out the general tendency of DHA, 22:5, and EPA to be preferentially esterified in the *sn*-2 position in fish and invertebrate TAG, and in the *sn*-3 position in marine mammal TAG. Litchfield (33,34) discussed the relationships between the percentage of DHA esterified at each position and the total percentage of DHA in fish, invertebrates, and marine mammals and showed that the positional distribution of DHA can be predicted by the following proportionality equations: for fish and invertebrate TAG,

$$y_1 = 0.28x;\ y_2 = 2.06\ x;\ \text{and}\ y_3 = 0.66\ x \qquad [1]$$

and for marine mammal TAG,

$$y_1 = 0.94x;\ y_2 = 0.22x;\ \text{and}\ y_3 = 1.84x \qquad [2]$$

where $x$ shows the mole percentage of DHA in the total TAG and $0 < x < 30$; and $y_1$, $y_2$, and $y_3$ show the mole percentages of DHA in the *sn*-1, *sn*-2, and *sn*-3 positions, respectively. These relationships essentially indicate that about 10, 70, and 20% of DHA were esterified in the *sn*-1, *sn*-2, and *sn*-3 positions of fish and invertebrate TAG, and about 30, 10, and 60% of it were in the *sn*-1, *sn*-2, and *sn*-3 positions of marine mammal TAG.

Proportional distributions of DHA among the *sn*-1, *sn*-2, and *sn*-3 positions of the enriched *Artemia* nauplii TAG are shown in Table 4. The general tendency for fish and invertebrate TAG did not hold for *Artemia* nauplii. In the enriched *Artemia*, a preference of DHA for the *sn*-2 position was not observed. Less than 18% of DHA was esterified in the *sn*-2 position of the enriched *Artemia*, whereas 19–25 and 57–67% of this acid were located in the *sn*-1 and *sn*-3 positions, respectively. Such a distribution pattern resembles that reported for marine mammal TAG rather than invertebrates and fish TAG. The positional distribution of DHA in the DHA-enriched *Artemia* nauplii is found to be characterized by considerably higher assignment of DHA to the *sn*-3 position than to the *sn*-2 position, and therefore cannot be predicted by Litchfield's prediction rule (Eqs. 1,2; Refs. 33,34) for fish and aquatic invertebrates.

Owing to the enrichment of DHA and simultaneous retroconversion, the distribution pattern of EPA changed in the enriched *Artemia* nauplii (Table 3). Decreases in preference of this acid in the *sn*-2 position also support the view that the general tendency for fish and invertebrates does not hold for the DHA-enriched *Artemia*.

*Biosynthesis of DHA-containing TAG in* Artemia *nauplii*. The present study revealed the positional distribution of n-3 HUFA and other major fatty acids in TAG of *Artemia* nauplii. As far as the authors know, there has been no prior report on the positional distribution. In all of the *Artemia* nauplii en-

riched with DHA-EE, DHA was preferentially located in the *sn*-3 >> *sn*-1 > *sn*-2 positions of TAG. This distribution pattern was obvious even in the early period (4 h) of the enrichment. The increase of the DHA content in the enriched *Artemia* is attributable primarily to the increase of this acid in the *sn*-3 position of TAG.

Lesser amounts of DHA in polar lipids of enriched *Artemia* were also reported by Coutteau and Mourente (20). When the proportional distribution of DHA among major lipid classes was calculated from their data, phosphatidylcholines (PC) and phosphatidylethanolamines (PE) contained only 2.3 and 1.4% of this acid present in enriched *Artemia*, respectively. Czesny *et al*. (35) presented fatty acid profiles of neutral and polar lipid fractions of *Artemia* nauplii enriched with EE-type n-3 HUFA concentrate. The concentration of DHA in the polar lipid fraction (0.3 wt% of total fatty acids) was much lower than that in the neutral lipid fraction (5.0 wt% of total fatty acids). Preliminary experiments for the present study also showed low contents of DHA in polar lipids as follows: 1.0% of total fatty acids in PC fraction (28 wt% of TL) and 3.2% in PE (11%) vs. 8.9% in TAG (38%). Such low contents of DHA in 1,2-diacyl-*sn*-glycerophospholipids are consistent with the low content of this acid in the *sn*-1 and *sn*-2 positions of *Artemia* TAG. This consistency indicates that synthesis of DHA-containing TAG goes through the *sn*-3 glycerophosphate pathway in *Artemia* nauplii. Preference of exogenous DHA for the *sn*-3 position of TAG indicates that most of the DHA is esterified to 1,2-diacyl-*sn*-glycerols formed from 1,2-diacyl-*sn*-glycerophosphate at the final stage of the pathway.

This study revealed the incorporation of DHA in the *sn*-3 position of *Artemia* nauplii TAG in preference to the *sn*-1 and *sn*-2 positions. A lesser preference for the *sn*-1 and *sn*-2 positions confirms the fact that the preponderance of DHA is not incorporated into glycerophospholipids but into TAG. Glycerophospholipids have been found to be superior to TAG for fish larvae as a source of essential fatty acids and energy due to their better digestibility (36). However, without any modifications to present technology, it seems essentially difficult to boost DHA content in the phospholipids by enrichment with DHA-EE. When marine fish larvae are reared on *Artemia* nauplii enriched with EE-type of DHA oil, the larvae feed on DHA esterified in TAG with a positional distribution pattern similar to that of marine mammals rather than fish or invertebrates.

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# Determination of Stereochemical Configuration of the Glycerol Moieties in Glycoglycerolipids by Chiral Phase High-Performance Liquid Chromatography

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**ABSTRACT:** This study reports a simple and sensitive method for determining the absolute configuration of the glycerol moieties in glycoglycerolipids. The method is based on chiral phase high-performance liquid chromatography (HPLC) separations of enantiomeric di- and monoacylglycerols released from glycosyldi- and monoacylglycerols, respectively, by periodate oxidation followed by hydrazinolysis. The released di- and monoacylglycerols were chromatographed as their 3,5-dinitrophenylurethane (3,5-DNPU) and bis(3,5-DNPU) derivatives, respectively. The derivatives were separated on two chiral phases of opposite configuration, (*R*)- and (*S*)-1-(1-naphthyl)ethylamine polymers for diacylglycerols and *N*-(*R*)-1-(1-naphthyl)ethylaminocarbonyl-(*S*)-valine and *N*-(*S*)-1-(1-naphthyl)ethylamino-carbonyl-(*R*)-valine for monoacylglycerols. Clear enantiomer separations, which permit the assignment of the glycerol configuration, were achieved for *sn*-1,2(2,3)-diacyl- and *sn*-1(3)-monoacylglycerols generated from linseed oil triacylglycerols by partial Grignard degradation on all the chiral stationary phases employed. Using the method, we have determined the glycerol configuration in the glycosyldiacylglycerols (monogalactosyl-, digalactosyl-, and sulfquinovosyldiacylglycerols) and glycosylmonoacylglycerols (monogalactosyl-, digalactosyl-, and sulfoquinovosylmonoacylglycerols) isolated from spinach leaves and the coralline red alga *Corallina pilulifera*. The results clearly showed that the glycerol moieties in all the glycoglycerolipids examined have *S*-configuration (*sn*-1,2-diacyl- and *sn*-1-monoacylglycerols). The new method demonstrates that chiral phase HPLC provides unambiguous information on the configuration of the glycerol backbone in natural glycosyldi- and monoacylglycerols, and that the two-step liberation of the free acylglycerols does not compromise glycerol chirality.

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It is well known that monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), and sulfoquinovosyldiacylglycerols (SQDG) are major membrane constituents in plant chloroplasts and that they have also been found in terrestrial and marine organisms (1–3). In addition to various functions as membrane components, glycosyldi- and monoacylglycerols have recently been shown to have some pharmaceutical and biological activities (4–12).

The stereochemical configuration of the glycerol moieties in MGDG, DGDG, and SQDG can be assigned by physical methods, such as X-ray crystallography, $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy, optical rotatory dispersion, and circular dichroism (3). These techniques are powerful tools for determining the glycerol configuration, but they are not simple or practical to use. Recently, an enzymatic method, which uses diacylglycerol kinase from *Escherichia coli* and ATP, was reported for assigning the C2 configuration of the diacylglycerols released from $^{14}C$-labeled glycolipids (13). The method is sensitive and gives clear results as the enzyme has a high enantioselectivity for phosphorylating *sn*-1,2-diacylglycerols, but the radio-labeled compounds are somewhat troublesome to prepare and use. Thus, there is considerable interest in chromatographic methods.

Kim *et al*. (14) reported a highly sensitive method for determination of the absolute configuration of the glycerol moiety in glycosyldiacylglycerols by normal-phase high-performance liquid chromatography (HPLC) with fluorescent detection. The original glycosyldiacylglycerols were deacylated, methylated, and hydrolyzed to yield *sn*-1,2(2,3)-di-*O*-methylglycerols. The enantiomeric di-*O*-methylglycerols were reacted with (*S*)-(+)-2-*tert*-butyl-2-methyl-1,3-benzodioxol-4-carboxylic acid chloride, and the resulting diastereomeric derivatives were resolved on a conventional silica column. The method gives almost complete resolution of the diastereomeric derivatives at the level of a few picomoles, but the derivatization procedures including the synthesis of the chiral derivatization reagent seem to be complex and time-consuming. In addition, the method gives no information on the molecular species of glycosyldiacylglycerols.

We report here a simpler and more practical HPLC method for assigning the glycerol configuration in glycosyldi- and monoacylglycerols. The method is based on chiral-phase HPLC separations of enantiomeric di- and monoacylglycerols released from glycosyldi- and monoacylglycerols, respectively, by the frequently used periodate oxidation followed by hy-

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Abbreviations: DGDG, digalactosyldiacylglycerol; DGMG, digalactosylmonoacylglycerol; DNPU, dinitrophenylurethane; ECN, equivalent carbon number; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; MGDG, monogalactosyldiacylglycerol; MGMG, monogalactosylmonoacylglycerol; MS, mass spectrometry; NMR, nuclear magnetic resonance; SQDG, sulfoquinovosyldiacylglycerol; SQMG, sulfoquinovosylmonoacylglycerol; TLC, thin-layer chromatography.

drazinolysis. Although the enantiomer separations of di- and monoacylglycerols have already been reported by chiral and normal-phase HPLC (15–17), these methods have not been applied to the di- and monoacylglycerol moieties in glycosyldi- and monoacylglycerols.

## MATERIALS AND METHODS

*Chemicals.* Standard samples of MGDG, DGDG, and SQDG from plant leaves were obtained from Lipid Products (Redhill, United Kingdom). Silica gel 60 (70–230 mesh) for column chromatography, Silica gel 60 $F_{254}$ aluminum sheets for analytical thin-layer chromatography (TLC), and Silica gel $60F_{254S}$ plates for preparative TLC (20 × 20 cm, 0.25-mm thick) were obtained from Merck (Darmstadt, Germany). 3,5-Dinitrophenyl isocyanate was a product of Sumika Analysis Service (Osaka, Japan). Linseed oil was a commercial product from Nacalai Tesque (Kyoto, Japan). HPLC-grade solvents, *n*-hexane, dichloromethane and ethanol, were obtained from Kanto Chemicals (Tokyo, Japan). All other chemicals and solvents were of reagent grade or better quality and were also obtained from Kanto Chemicals.

*Isolation of glycosyldi- and monoacylglycerols from spinach leaves and* Corallina pilulifera. Spinach leaves were purchased from a local supermarket in Hakodate, Japan. The red alga *C. pilulifera* was collected in the intertidal zone of Oshoro Bay, Otaru, Hokkaido, Japan, during May to October 1997 and 1998. The leaf and algal lipids were extracted by the method of Bligh and Dyer (18) and with methanol (12), respectively. The leaf lipids (250 mg) were subjected to silica gel column chromatography, which facilitated further purification by TLC. The lipid classes were separated by sequential elution with chloroform, methanol, and water into four fractions: 1000 mL of chloroform (I), 300 mL of chloroform/methanol (9:1, vol/vol) (II), 300 mL of chloroform/methanol/water (65:25:4, by vol) (III), and 300 mL of chloroform/methanol/water (65:35:8, by vol) (IV). Fraction II contained MGDG, which were further purified by TLC with a solvent system of *n*-hexane/ethyl acetate/methanol/water (20:25:5:1, by vol). Fraction III contained both DGDG and SQDG, as did Fraction IV. Therefore, DGDG and SQDG were isolated from the combined Fractions III and IV by TLC with a solvent system made up of chloroform/methanol/water/ethyl acetate/2-propanol (5:2:1:5:5, by vol). The $R_f$ values of the MGDG, DGDG, and SQDG on TLC using these solvent systems were 0.35, 0.27, and 0.16, respectively, which were in good agreement with those of authentic standards. After spraying with 2′,7′-dichlorofluorescein reagent, bands were visualized under ultraviolet (UV) irradiation. The MGDG was recovered from the adsorbent with chloroform/methanol (2:1, vol/vol); DGDG and SQDG were recovered with chloroform/methanol/water (6:4:1, by vol). On analytical TLC, purified glycoglycerolipids showed characteristic dark red-colored spots when sprayed with an orcinol-sulfonic acid reagent and subjected to heating (19).

The methanol extracts (46 g) of *C. pilulifera* were partitioned between water and 1-butanol, and the organic phase was evaporated to dryness. The 1-butanol portion (6.1 g) was further partitioned between methanol and *n*-hexane. The methanol portion (42 mg) was separated into five fractions (V–IX) by preparative TLC with a solvent system of chloroform/methanol/water/ethyl acetate/2-propanol (5:2:1:5:5, by vol) to give MGDG ($R_f$ 0.61) (V), monogalactosylmonoacylglycerol MGMG ($R_f$ 0.39) (VI), DGDG ($R_f$ 0.27) (VII), SQDG ($R_f$ 0.16) (VIII), and a mixture of digalactosylmonoacylglycerol (DGMG) and sulfoquinovosylmonoacylglycerol SQMG ($R_f$ 0.10) (IX). Fraction IX was further separated into two portions by preparative TLC with a solvent system of 2-propanol/water/25% ammonia solution (72:11:17, by vol) to give SQMG ($R_f$ 0.39) and DGMG ($R_f$ 0.23). Structures of these glycosylmonoacylglycerols were confirmed by $^1H$ and $^{13}C$ NMR spectrometry, as described elsewhere (12).

*Release of di- and monoacylglycerols from glycoglycerolipids*. Glycosyldi- (MGDG, DGDG, and SQDG) and monoacylglycerols (MGMG, DGMG, and SQMG) were subjected to two consecutive reactions, periodate oxidation and fission of glycosidic linkage with 1,1-dimethylhydrazine, as described by Heinze *et al*. (20) for glycosyldiacylglycerols. The method releases free di- and monoacylglycerols from glycosyl diacylglycerols and monoacylglycerols, respectively. Briefly, glycoglycerolipid (3 mg) and $HIO_4{\cdot}4H_2O$ (46 mg) were dissolved in methanol (1 mL) and kept at room temperature for 90 min in the dark. Then chloroform (4 mL) and 0.45% NaCl solution (1.5 mL) were added and shaken vigorously. After brief centrifugation, the lower phase was evaporated under nitrogen gas. The residue was dissolved with 1,1-dimethylhydrazine (6.5 μL) in chloroform/water/2-propanol/acetic acid (6:7:2:3, by vol) and kept in the dark at 25°C for 4 h for DGDG and DGMG, and 20 h for MGDG, MGMG, SQDG, and SQMG. After adding *n*-hexane (3 mL), the mixture was washed twice with 50 mM $KH_2PO_4$ solution (2 mL) and dried over anhydrous $Na_2SO_4$. The residue obtained after removal of the solvent contained di- and monoacylglycerols released from glycosyldi- and monoacyglycerols, respectively.

*Preparation of derivatives.* The di- and monoacylglycerols released were immediately converted into their 3,5-dinitrophenylurethane (3,5-DNPU) derivatives without prior isolation from the reaction products to minimize acyl migration. The reaction products and 3,5-dinitrophenyl isocyanate (5 mg) were dissolved into dry toluene (0.5 mL) in the presence of dry pyridine (30 μL), and the solution was stirred at 30°C for 3 h (21,22). The reaction was quenched with methanol (1 mL). After removal of the solvent, the crude urethane derivatives were purified by TLC on silica gel GF (Merck), using *n*-hexane/1,2-dichloroethane/ethanol (40:10:3, by vol) for 3,5-DNPU derivatives of diacylglycerols (22) and *n*-hexane/1,2-dichloro-ethane/ethanol (40:15:5, by vol) for bis(3,5-DNPU) derivatives of monoacylglycerols as the developing solvents (23). Bands were visualized under UV irradiation, and the adsorbent containing the derivatives was scraped off and extracted with diethyl ether.

*Preparation of standard di- and monoacylglycerols from linseed oil triacylglycerols. sn*-1,2(2,3)-Diacylglycerols and *sn*-

1(3)-monoacylglycerols were generated by partial Grignard degradation from linseed oil triacylglycerols and were purified by boric acid TLC as described previously (24). The 3,5-DNPU and bis(3,5-DNPU) derivatives were prepared as described above.

*Mass spectrometry (MS).* Flow injection electrospray ionization (ESI)-MS was carried out in the negative ion mode with a LCQ ion-trap mass spectrometer (Thermo Separation Products, San Jose, CA). The 3,5-DNPU and bis(3,5-DNPU) derivatives of di- and monoacylglycerols dissolved in chloroform/methanol (2:1, vol/vol, *ca.* 0.1 mg/mL) were introduced directly into the ESI probe by flow injection (10 μL/min). The heated capillary temperature was 200°C and the spray voltage was 4.2 kV. The nitrogen sheath gas was set at 30 arb (arbitrary units) by the software. The mass spectrum was taken in the mass range 150–2000 *m/z*. The negative ESI-MS spectra of the two derivatives gave a prominent pseudomolecular ion $[M - H]^-$.

*Chiral-phase HPLC.* Chiral-phase HPLC was performed on a Shimadzu LC-6A instrument (Shimadzu, Kyoto, Japan) equipped with columns containing (*R*)- and (*S*)-1-(1-naphthyl)ethylamine (25 cm × 4.6 mm i.d.) packed with 5-μm particles (YMC-Pack A-K03 and A-L03, respectively; YMC, Kyoto, Japan) for 3,5-DNPU derivatives of diacylglycerols derived from MGDG, DGDG, and SQDG, and columns containing *N*-(*R*)-1-(1-naphthyl)ethylaminocarbonyl-(*S*)-valine (25 cm × 4 mm i.d., Sumichiral OA-4100; Sumika Chemical Analysis Service, Osaka, Japan) and *N*-(*S*)-1-(1-naphthyl)ethylaminocarbonyl-(*R*)-valine (25 cm × 4.6 mm i.d., Sumichiral OA-4100R) packed with 5-μm particles for bis(3,5-DNPU) derivatives of monoacylglycerols derived from MGMG, DGMG, and SQMG. The analyses were done isocratically using a mixture of *n*-hexane/dichloromethane/ethanol (40:10:1, by vol) for the diacylglycerol derivatives (22) and *n*-hexane/dichloromethane/ethanol (40:12:3, by vol) for the monoacylglycerol derivatives (21) as the mobile phases at a constant flow rate of 1 mL/min. The column temperature was kept at 10°C. Peaks were monitored with a Shimadzu SPD-6A variable wavelength detector set at 254 nm, and chromatograms were recorded on a Shimadzu Chromatopack C-R3A.

*Lipase hydrolysis and fatty acid analysis.* The positional distribution of fatty acids at the *sn*-1 and *sn*-2 positions in glycosyldiacylglycerol molecules was determined by specific hydrolysis with *Rhizopus delemar* lipase (Seikagaku Kogyo, Tokyo, Japan), as described by Fisher *et al*. (25). The fatty acids released from the *sn*-1 position and lyso-compounds were converted into methyl esters by heating at 90°C for 2 h in a 5% HCl/methanol solution. Fatty acid composition was determined by open-tubular gas chromatography on a Rascot Silar 5CP column (50 m × 0.25 mm i.d.; Nihon Chromato Works, Tokyo, Japan). The column temperature was 180°C isothermally. The injector and flame-ionization detector temperatures were set at 230°C. Hydrogen was used as the carrier gas at a constant flow of 1 mL/min. The split ratio was 1:50. Peaks were identified by comparison with known fatty acids from marine organisms (26).

## RESULTS AND DISCUSSION

The determination of absolute configuration of the glycerol moieties in glycoglycerolipids in their intact form has not been achieved by any HPLC modes including chiral-phase HPLC. The enantiomer separations of both *sn*-1,2(2,3)-diacylglycerols and *sn*-1(3)-monoacylglycerols, however, have already been established by chiral- and normal-phase HPLC (15–17). In this study therefore we applied the chiral-phase HPLC techniques to assign the glycerol configuration in the di- and monoacylglycerols released from glycosyldi- and monoacylglycerols. To obtain unambiguous conclusions, we employed two chiral stationary phases having opposite configurations in this study, which caused a reversal in the order of elution of enantiomeric di- and monoacylglycerols.

*Glycosyldiacylglycerols.* Figure 1 shows the chiral-phase HPLC profiles of the 3,5-DNPU derivatives of the diacylglycerols released from MGDG, DGDG, and SQDG of spinach leaves by periodate oxidation followed by hydrazinolysis, and of standard *sn*-1,2(2,3)-diacylglycerols generated from linseed oil triacylglycerols by partial Grignard degradation, on a column containing (*R*)-1-(1-naphthyl)ethylamine (A-K03). At 10°C, the linseed oil diacylglycerols were clearly resolved into two groups, representing the *sn*-1,2- and *sn*-2,3-enantiomers (Fig. 1D). Previous work established a faster elution of *sn*-1,2-enantiomers compared to *sn*-2,3-enantiomers on A-K03 (22). Each enantiomer group was split into six peaks, which were eluted in order of decreasing equivalent carbon numbers (ECN) of the molecules (22). When analysis was done at 28°C, some peak overlapping was observed between *sn*-1,2-enantiomers of low ECN values and *sn*-2,3-enantiomers of high ECN values, such as *sn*-1,2-dilinolenoylglycerol (18:3-18:3, ECN = 24) and *sn*-2,3-palmitoyloleoylglycerol (16:0-18:1, ECN = 32) (22). The separation factors of the *sn*-1,2- and *sn*-2,3-enantiomers at 10 and 28°C were 1.55 and 1.44, respectively. Apparently a lower column temperature employed in this study improved the enantiomer resolution. The effect of column temperature on the enantiomer resolution of diacylglycerols by chiral phase HPLC has been discussed elsewhere (27). The diacylglycerols released from the MGDG, DGDG, and SQDG of spinach leaves eluted within 20 min after injection with partial resolution of molecular species, all of which were *sn*-1,2-enantiomers (Figs. 1A–C). Any *sn*-2,3-enantiomers, if present, would appear after 20 min. Individual peaks were tentatively identified on the basis of the ECN concept, fatty acid compositions at the *sn*-1 and *sn*-2 positions in MGDG, DGDG, and SQDG molecules (Table 1), and a comparison of the molecular species separation obtained by reversed-phase HPLC for the intact MGDG and DGDG from spinach leaves (28). The major molecular species of the MGDG, DGDG, and SQDG were 18:3-18:3 (ECN = 24)/18:3-16:3 (ECN = 22), 18:3-18:3 (ECN = 24), and 16:0-18:3 (ECN = 28), respectively. Although no reverse isomer resolution was obtained for 18:3-16:3 and 16:0-18:3 under the HPLC conditions (29), it has been established that the 16:3 acid is mostly located at the *sn*-2 position and the 18:3 acid is

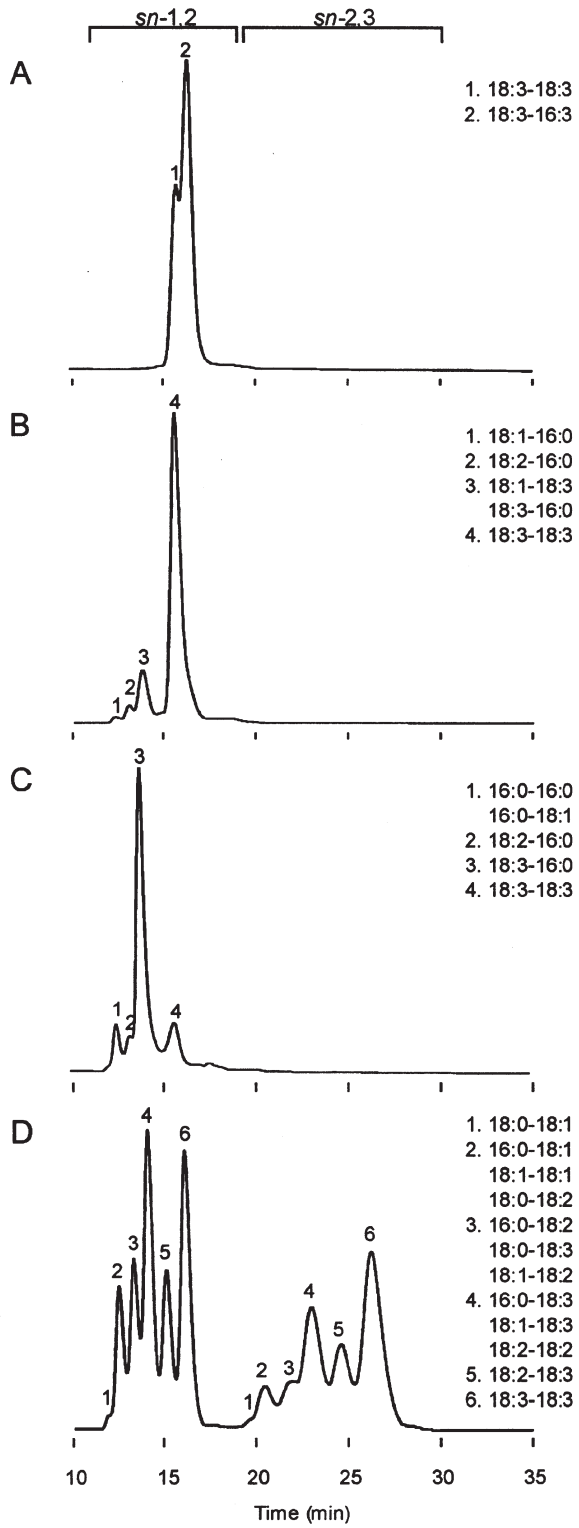


**FIG. 1.** Chiral-phase high-performance liquid chromatographic (HPLC) profiles of the 3,5-dinitrophenylurethane (DNPU) derivatives of diacylglycerols released from monogalactosyldiacylglycerol (MGDG) (A), digalactosyldiacylglycerol (DGDG) (B), and sulfoquinovosyldiacylglycerol (SQDG) (C) of spinach leaves, and of standard *sn*-1,2(2,3) diacylglycerols derived from linseed oil triacylglycerols by partial Grignard degradation (D) on a column containing (*R*)-1-(1-naphthyl)ethylamine polymer (A-K03; YMC, Kyoto, Japan).

**TABLE 1**
**Fatty Acid Composition of Glycoglycerolipids[a] Isolated from Spinach Leaf (mol%)**

| | MGDG | | DGDG | | SQDG | |
|---|---|---|---|---|---|---|
| Fatty acid | *sn*-1 | *sn*-2 | *sn*-1 | *sn*-2 | *sn*-1 | *sn*-2 |
| 14:0 | 2.3 | 1.5 | 1.6 | 2.2 | 6.1 | 6.0 |
| 15:0 | 1.0 | 0.5 | 0.6 | 0.6 | 2.2 | 2.4 |
| 16:0 | 6.2 | 6.4 | 7.9 | 12.1 | 37.8 | 60.0 |
| 16:1n-9 | 1.4 | 0.5 | 1.8 | 1.4 | 6.9 | 7.0 |
| 16:1n-7 | 0.4 | 0.3 | — | — | 0.7 | — |
| 16:3n-3 | 4.7 | 47.6 | 1.5 | 4.6 | 1.0 | — |
| 18:0 | 1.5 | 2.9 | 2.1 | 1.6 | 8.4 | 7.1 |
| 18:1n-9 | 1.6 | 2.5 | 2.1 | 2.1 | 2.1 | 1.8 |
| 18:1n-7 | 0.8 | 0.4 | 4.0 | — | 1.3 | — |
| 18:2n-6 | 0.9 | 1.7 | 2.3 | 2.2 | 3.4 | 0.8 |
| 18:3n-6 | 0.2 | — | 0.3 | 0.8 | 0.7 | 0.7 |
| 18:3n-3 | 79.0 | 35.7 | 75.8 | 72.4 | 29.4 | 14.2 |

[a]MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

located at both *sn*-1 and *sn*-2 positions in the galactolipid molecules (2,3). Figure 2 shows the chiral phase HPLC profiles of the 3,5-DNPU derivatives of the diacylglycerol moieties in the spinach glycosyldiacylglycerols and of the linseed oil diacylglycerols on a column containing (*S*)-1-(1-naphthyl)ethylamine (A-L03), which has a configuration opposite to that of A-K03. Like the A-K03 column, the A-L03 column also gave complete resolution between the *sn*-1,2- and *sn*-2,3-enantiomers of the linseed oil diacylglycerols at 10°C, but with a reversal in the elution order (Fig. 2D). The enantiomeric diacylglycerols released from the MGDG, DGDG, and SQDG of spinach leaves also appeared in the *sn*-1,2-enantiomer region of the A-L03 column, but in a reversed order of elution (Figs. 2A–C). These results confirm that the glycerol moieties in the glycosyldiacylglycerols obtained from spinach leaves have *S* configuration (*sn*-1,2-diacylglycerols), as established previously (1). Furthermore, the results demonstrate that the periodate/methylhydrazine degradation does not compromise the chirality of the released acylglycerols.

Figure 3 shows the chiral-phase HPLC profiles of the 3,5-DNPU derivatives of diacylglycerols released from MGDG, DGDG, and SQDG of the red alga *C. pilulifera*, and of standard *sn*-1,2(2,3)-diacylglycerols generated from linseed oil triacylglycerols by partial Grignard degradation, on the A-K03 column. Like the spinach leaf diacylglycerols, the *C. pilulifera* diacylglycerols from the glycosyldiacylglycerols were eluted within 20 min after injection, and their elution orders were reversed from those seen on A-L03 (chromatograms not shown). These results clearly show that all the diacylglycerols released from the MGDG, DGDG, and SQDG of *C. pilulifera* consist only of *sn*-1,2-enantiomers (*S* configuration), which had not been established previously. On the basis of the ECN concept and the fatty acid composition of the *sn*-1 and *sn*-2 positions in the MGDG, DGDG, and SQDG molecules (Table 2), the partially resolved peaks were tentatively identified as follows: 16:0-18:1 (ECN = 32), 16:0-18:2 (ECN = 30), 20:5-16:0 (ECN = 26), 20:5-20:4 (ECN = 22), and 20:5-20:5 (ECN = 20) for MGDG (Fig. 3A); 16:0-18:1 (ECN = 32), 16:0-18:2 plus 16:1-

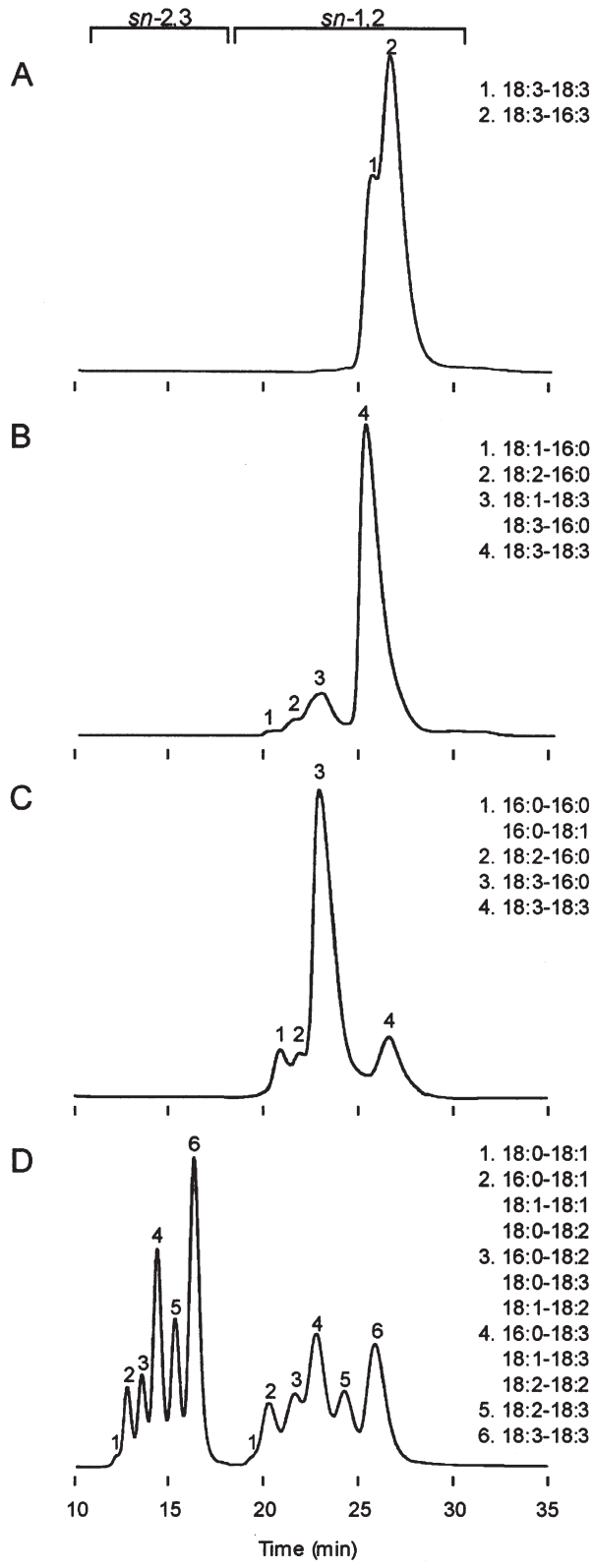


**FIG. 2.** Chiral-phase HPLC profiles of the 3,5-DNPU derivatives of diacylglycerols released from MGDG (A), DGDG (B), and SQDG (C) of spinach leaves, and of standard *sn*-1,2(2,3) diacylglycerols derived from linseed oil triacylglycerols by partial Grignard degradation (D) on a column containing (*S*)-1-(1-naphthyl)ethylamine polymer (A-L03; YMC). For abbreviations and manufacturer see Figure 1.

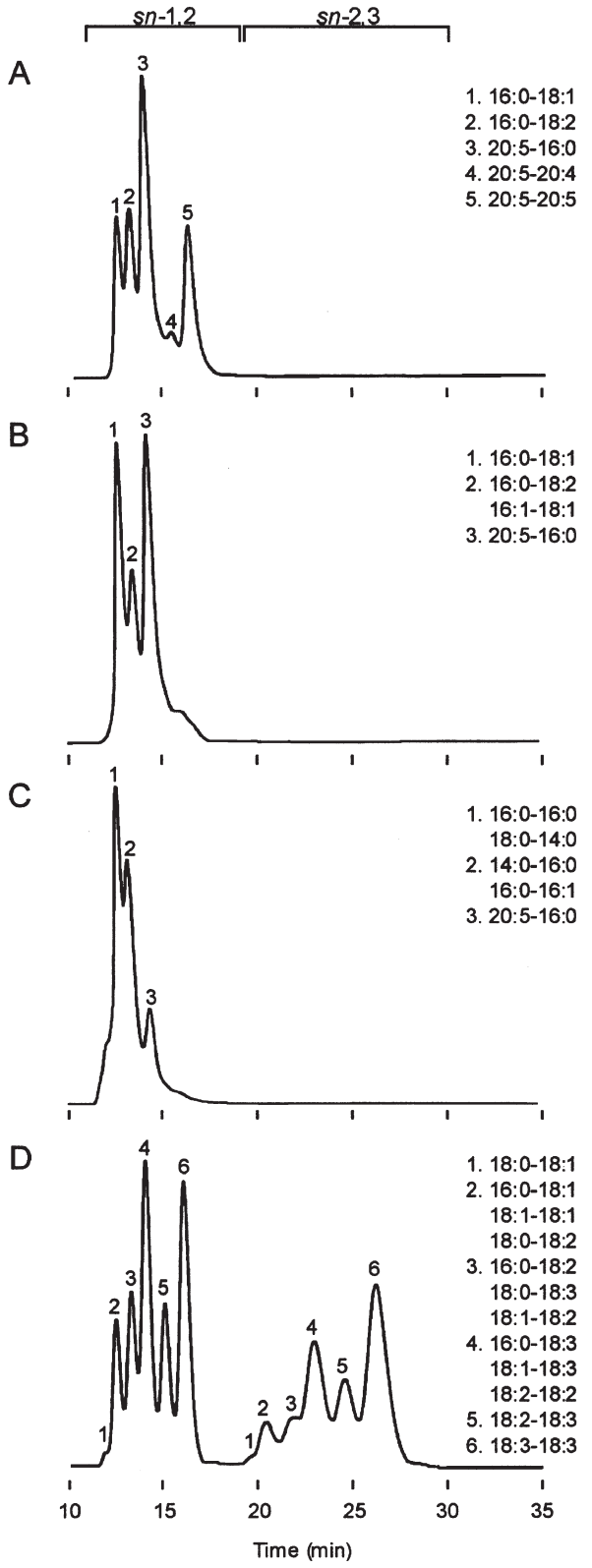


**FIG. 3.** Chiral-phase HPLC profiles of the 3,5-DNPU derivatives of diacylglycerols released from MGDG (A), DGDG (B), and SQDG (C) of the red alga *C. pilulifera*, and of standard *sn*-1,2(2,3) diacylglycerols derived from linseed oil triacylglycerols by partial Grignard degradation (D) on a column containing (*R*)-1-(1-naphthyl)ethylamine polymer (A-K03). For abbreviations and manufacturer see Figure 1.

18:1 (ECN = 30), and 20:5-16:0 (ECN = 26) for DGDG (Fig. 3B); and 16:0-16:0 plus 18:0-14:0 (ECN = 32), 14:0-16:0 plus 16:0-16:1 (ECN-30), and 20:5-16:0 (ECN = 26) for SQDG (Fig. 3C). The negative ESI-MS spectra of the 3,5-DNPU derivatives from MGDG, DGDG, and SQDG, which gave a prominent pseudomolecular ion $[M - H]^-$, also supported the occurrence of these molecular species. The molecular species of the glycosyldiacylglycerols in *C. pilulifera* are similar to those in the marine red alga *Porphyra yezoensis*, although the most predominant species of SQDG in the latter alga is 20:5-16:0 (30). The A-L03 column gave effective resolution of the reverse isomers of *sn*-1,2-diacylglycerols having very different pairs of acyl groups (29), but the occurrence of *sn*-1,2-palmitoyleicosapentaenoylglycerol (16:0-20:5) in the *C. pilulifera* glycosyldiacylglycerols could not be confirmed from the present chromatograms because of extensive overlapping among molecular species.

*Glycosylmonoacylglycerols.* The occurrence of the glycosylmonoacylglycerols (MGMG, DGMG, and SQMG) in the red alga *C. pilulifera* suggests hydrolysis of the glycosyldiacylglycerols by acyl hydrolases in the tissue. Notable amounts of free fatty acids, in which eicosapentaenoic acid was a major constituent, were also found in the algal lipids (31). The acyl hydrolase from the green alga *Dunaliella salina* chloroplasts preferentially attacks the *sn*-1 position of MGDG (32). The acyl hydrolase from potato tubers also attacks the *sn*-1 position in preference to the *sn*-2 position of MGDG (33). The enzymes from runner-bean leaves hydrolyze the acyl groups at both positions of MGDG and DGDG to form the corresponding galactosylglycerols and free fatty acids (34). The monoacylglycerols released from the MGMG, DGMG, and SQMG of *C. pilulifera* mainly consisted of *sn*-1-isomers (*sn*-1-monoacylglycerols, each 95%), which suggest that the enzymes preferentially hydrolyze the acyl group at the *sn*-2 position. In this study, however, we isolated the glycosylmonoacylglycerols from the alga using column chromatography and TLC on silicic acid, which could have isomerized some of the acyl groups at the *sn*-2 position to the *sn*-1 position (3). Therefore, the *sn*-1-isomers found in this study may contain appreciable amounts of the isomerized *sn*-2-isomers. The occurrence of both isomers in the red alga *C. pilulifera* suggests that acyl hydrolases in this tissue attack the *sn*-1 and *sn*-2 positions directly as already observed in higher plants and the green alga (32–34). In order to obtain clear HPLC chromatograms, small amounts of the *sn*-2-isomers (each 5%) were removed from the monoacylglycerols released from the MGMG, DGMG, and SQMG by silicic acid TLC after derivatization, and the remaining *sn*-1-isomers were used for determining the glycerol configuration. If the *sn*-2-isomers are not removed by TLC, they will interfere with the *sn*-1-isomers on OA-4100 and the *sn*-3-isomers on OA-4100R.

Figure 4 shows the chiral-phase HPLC profiles of the bis(3,5-DNPU) derivatives of the monoacylglycerols released from the MGMG, DGMG, and SQMG of the red alga *C. pilulifera* by periodate oxidation followed by hydrazinolysis, and of standard *sn*-1(3)-monoacylglycerols generated from linseed oil triacylglycerols by partial Grignard degradation, on a

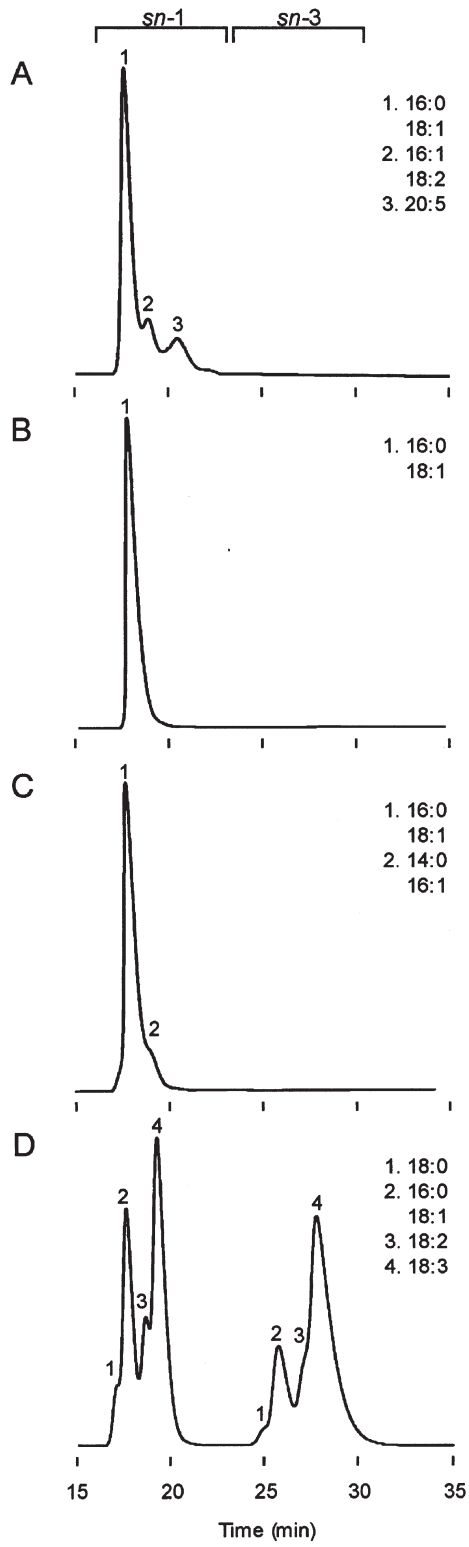


**FIG. 4.** Chiral-phase HPLC profiles of the bis(3,5-DNPU) derivatives of monoacylglycerols released from MGMG (A), DGMG (B), and SQMG (C) of the red alga *C. pilulifera*, and of standard *sn*-1(3)-monoacylglycerols derived from linseed oil triacylglycerols by partial Grignard degradation (D) on a column containing *N*-(*R*)-1-(1-naphthyl)ethylaminocarbonyl-(*S*)-valine (OA-4100; Sumika Chemical Analysis Service, Osaka, Japan). For abbreviations see Figure 1.

**TABLE 2**
**Fatty Acid Composition of Glycoglycerolipids[a] Isolated from the Red Alga *Corallina pilulifera* (mol%)**

| | MGDG | | DGDG | | SQDG | | MGMG | DGMG | SQMG |
|---|---|---|---|---|---|---|---|---|---|
| Fatty acid | *sn*-1 | *sn*-2 | *sn*-1 | *sn*-2 | *sn*-1 | *sn*-2 | *sn*-1 | *sn*-1 | *sn*-1 |
| 12:0 | 0.7 | 0.2 | 1.4 | — | — | — | — | — | — |
| 14:0 | 1.4 | 0.7 | 7.9 | 3.1 | 22.2 | 1.9 | 1.0 | 1.1 | 9.6 |
| 15:0 | 0.5 | 0.3 | 2.5 | 1.3 | 2.2 | 0.7 | 0.4 | 0.4 | 0.8 |
| 16:0 | 16.1 | 35.6 | 57.0 | 70.0 | 52.0 | 92.5 | 61.1 | 91.2 | 72.5 |
| 16:1n-9 | 0.5 | 0.6 | 5.0 | 3.3 | 3.7 | 1.0 | 1.0 | 0.3 | 0.4 |
| 16:1n-7 | 1.5 | 1.1 | 2.7 | 1.9 | 3.4 | 0.5 | 2.2 | 0.7 | 2.8 |
| 16:1n-5 | 0.2 | 0.3 | 1.4 | — | — | 0.2 | — | — | 0.3 |
| 16:2n-6 | 0.9 | 0.5 | — | — | 0.2 | — | — | — | — |
| 17:0 | 0.1 | 0.2 | 1.3 | 0.4 | 0.8 | 0.3 | 0.2 | 0.2 | 0.2 |
| 16:3n-3 | 0.2 | 0.3 | — | — | — | 0.1 | 0.2 | — | — |
| 16:4n-3 | — | — | — | — | — | — | 0.4 | — | — |
| 18:0 | 1.1 | 0.8 | 4.9 | 1.0 | 3.1 | 0.6 | 0.9 | 0.7 | 1.1 |
| 18:1n-9 | 1.7 | 3.2 | 5.1 | 11.6 | 2.3 | 0.7 | 12.7 | 4.1 | 1.4 |
| 18:1n-7 | 0.3 | 0.3 | 0.8 | 0.7 | 1.5 | 0.4 | 1.0 | 0.3 | 1.8 |
| 18:2n-6 | 1.6 | 2.3 | 2.0 | 4.2 | 1.1 | 0.4 | 4.8 | 1.0 | 0.5 |
| 18:3n-6 | 0.2 | 0.2 | — | 0.9 | 0.3 | 0.1 | 0.5 | — | 0.8 |
| 18:3n-3 | 1.7 | 0.4 | — | — | — | 0.1 | 0.5 | — | — |
| 18:4n-3 | 2.0 | 1.4 | — | — | — | — | 0.6 | — | — |
| 20:1n-9 | — | — | — | — | 1.2 | — | — | — | 2.0 |
| 20:2n-6 | — | — | — | — | 0.4 | — | 0.6 | — | 0.4 |
| 20:4n-6 | 4.2 | 5.4 | 1.1 | 1.6 | 1.5 | 0.5 | 2.8 | — | 0.9 |
| 20:5n-3 | 64.9 | 46.0 | 6.9 | — | 3.1 | — | 9.1 | — | 2.1 |
| 22:1n-9 | 0.2 | 0.2 | — | — | 1.0 | — | — | — | 2.4 |

[a]MGMG, monogalactosylmonoacylglycerol; DGMG, digalactosylmonoacylglycerol; SQMG, sulfoquinovosylmonoacylglycerol; for other abbreviations see Table 1.

column containing *N*-(*R*)-1-(1-naphthyl)ethylaminocarbonyl-(*S*)-valine (OA-4100). The linseed oil monoacylglycerols were clearly resolved into two groups, representing the *sn*-1- and *sn*-3-enantiomers (Fig. 4D). Previous study established faster elution of the *sn*-1-enantiomers when compared to the *sn*-3-enantiomers (23). Like the 3,5-DNPU of diacylglycerols, the bis(3,5-DNPU) derivatives of linseed oil monoacylglycerols were also eluted from the column according to their ECN values. Thus, the *sn*-1-enantiomers were partially resolved into four peaks, which were tentatively identified as follows: peak 1 (ECN = 18), monostearoylglycerol (18:0); peak 2 (ECN = 16), monopalmitoyl- (16:0) plus monooleoylglycerols (18:1); peak 3 (ECN = 14), monolinoleoylglycerol (18:2), and peak 4 (ECN = 12), monolinolenoylglycerol (18:3). All the monoacylglycerols released from the MGMD (Fig. 4A), DGMG (Fig. 4B), and SQMG (Fig. 4C) of *C. pilulifera* appeared in the *sn*-1-enantiomer region with partial resolution (MGMG and SQMG) or complete overlap (DGMG) of molecular species, which were attributed to 16:0 plus 18:1, 16:1 plus 18:2 and 20:5 (MGMG), 16:0 plus 18:1 (DGMG), and 16:0 plus 18:1 and 14:0 plus 16:1 (SQMG). Fatty acid compositions and negative ESI-MS spectra of each glycosylmonoacylglycerol also supported the peak identification (Table 2). No peaks were seen for the *sn*-3-enantiomers in the glycosylmonoacylglycerols (Figs. 4A–C). The bis(3,5-DNPU) derivatives of monoacylglycerols released from the *C. pilulifera* glycosylmonoacylglycerols and of the linseed oil monoacylglycerols were also resolved into two clearly distinguishable enantiomer groups but with a reversal in the elution order on a column containing *N*-(*S*)-1-(1-naphthyl)ethylaminocarbonyl-(*R*)-valine (OA-4100R), which has a configuration opposite that of OA-4100 (chromatograms not shown). Therefore, the results obtained with the two columns having opposite configurations clearly show that the glycerol moieties of all MGMG, DGMG, and SQMG of *C. pilulifera* have the *S*-configuration (*sn*-1-enantiomers), which had not been previously established.

In conclusion, this study reports the first successful application of chiral-phase HPLC to establishing glycerol chirality in complex natural glycosylacylglycerols. The method also provides information on the acyl group association in the glycosyldiacylglycerols. Further resolution of molecular species could be achieved by polar capillary gas–liquid chromatography or reversed-phase HPLC of the enantiomers isolated by chiral-phase HPLC, as well as by on-line mass spectrometry (17).

## ACKNOWLEDGMENTS

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# Quantification of Key Odorants Formed by Autoxidation of Arachidonic Acid Using Isotope Dilution Assay

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**ABSTRACT:** Six odor-active compounds generated by autoxidation of arachidonic acid (AA) were quantified by isotope dilution assay (IDA), i.e., hexanal (**1**), 1-octen-3-one (**2**), (*E,Z*)-2,4-decadienal (**3**), (*E,E*)-2,4-decadienal (**4**), *trans*-4,5-epoxy-(*E*)-2-decenal (**5**), and (*E,Z,Z*)-2,4,7-tridecatrienal (**6**). Compound **1** was the most abundant odorant with about 700 mg/100 g autoxidized AA, which corresponds to 2.2 mol% yield. Based on the odor activity values (ratio of concentration to odor threshold), odorants **3** (fatty) and **5** (metallic) showed the highest sensory contribution followed by **1** (green), **2** (mushroom-like), **6** (egg white-like), and **4** (fatty). For the first time, reliable quantitative results are reported for odorants **1–6** in autoxidized AA, in particular odorant **6**, which is a characteristic compound found in autoxidized AA. Synthesis of deuterated **6**, required for IDA, is described in detail. The formation of odorants **1–6** by autoxidation of AA is discussed with respect to the quantitative data.

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Arachidonic acid (AA, 20:4) is found in membrane phospholipids of all mammalian tissues and plays a role in the regulation of functional properties like fluidity, permeability, and activity of membrane-bound enzymes. Although it is not an essential fatty acid, AA has recently been shown to correlate with both intrauterine growth of preterm infants (1,2) and growth in the first year of life (3). There is an increasing interest in the production of highly purified oils rich in AA for infant formulas (4).

As a polyunsaturated fatty acid, AA is very susceptible to oxidation with atmospheric oxygen and thus brings about losses in nutritional quality. Oxidative degradation also gives rise to flavor deterioration, i.e., formation of stale and rancid off-flavors. Off-flavor caused by lipid oxidation has been the subject of numerous studies, mainly on unsaturated lipids containing oleic, linoleic, and linolenic acids (5,6). However, little work has been published on volatile compounds formed by autoxidation of AA or methyl arachidonate. Depending on the starting material, reaction conditions, and analytical techniques used, various volatile compounds were identified, such as hexanal (7–10), 2-heptenal (7–9), 2,4-decadienal (8,10), 2,4,7-tridecatrienal (8), 1-octen-3-ol (9), 1-octen-3-one (9), pentane (10), and methyl 5-oxopentanoate (10).

The volatile composition of autoxidized AA has recently been characterized with focus on odor-active constituents (11,12). Hexanal (**1**), 1-octen-3-one (**2**), (*E,Z*)-2,4-decadienal (**3**), (*E,E*)-2,4-decadienal (**4**), *trans*-4,5-epoxy-(*E*)-2-decenal (**5**), and (*E,Z,Z*)-2,4,7-tridecatrienal (**6**) revealed high sensory relevance among the 19 odorants detected by gas chromatography–olfactometry (GC–O). The aroma properties of the most potent odorants were in good agreement with the overall aroma of autoxidized AA, described as green, metallic, egg white-like, fatty, and fishy.

It is well known that results obtained by GC–O only roughly estimate the sensory relevance of odorants, mainly because of discrimination during isolation of volatiles. The isotope dilution assay (IDA) has been shown to be a sensitive, accurate, and reliable quantification technique in flavor research (13–15). This method involves spiking food materials with known amounts of a labeled substance prior to sample preparation and analysis by gas chromatography–mass spectrometry (GC–MS). In this way, losses can be accounted for because whatever changes occur in the natural substance also occur in the labeled version. IDA has also been applied to lipid degradation products and off-flavor studies (14,16,17).

In this paper we report quantitative data of key odorants generated by autoxidation of AA, using the IDA technique for five aldehydes and one ketone. Synthesis of fourfold deuterated **6** is also described.

## EXPERIMENTAL PROCEDURES

*Materials.* The following chemicals were obtained commercially: AA (99%), ethylmagnesium bromide, palladium on $CaCO_3$ (Lindlar's catalyst) (Aldrich, Buchs, Switzerland); deuterium gas (Carbagas, Lausanne, Switzerland); neutral aluminum oxide ($Al_2O_3$), hexanal, cuprous chloride (CuCl), manganese (IV) oxide ($MnO_2$), phosphorus tribromide ($PBr_3$), pyridine ($H_2O$ <0.005%), tetrahydrofuran ($H_2O$ <0.005%) (Fluka, Buchs, Switzerland); 2,4-decadienal (*E,E*: 95%; *E,Z*: 5%; Fontarom, Cergy Pontoise, France); deuterated chloroform ($C^2HCl_3$, 99.8%, Dr. Glaser AG, Basel, Switzerland); diethyl ether ($Et_2O$), hexane, pentane, silica gel

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Abbreviations: AA, arachidonic acid; CC, column chromatography; CI, chemical ionization; EI, electron ionization; GC, gas chromatography; GC–O, gas chromatography–olfactometry; HPET, hydroperoxyeicosatrienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPOD, hydroperoxyoctadecadienoic acid; IDA, isotope dilution assay; IS, internal standard; MS, mass spectrometry; NMR, nuclear magnetic resonance; OAV, odor activity value; PCI, positive chemical ionization; RI, retention index; SIM, selected ion monitoring.

60, sodium carbonate ($Na_2CO_3$), sodium hydrogen carbonate ($NaHCO_3$), anhydrous sodium sulfate ($Na_2SO_4$), sulfuric acid ($H_2SO_4$) (Merck, Darmstadt, Germany); 2-octyn-1-ol, (*E*)-2-penten-4-yn-1-ol (Lancaster, Morecambe, England); and 1-octen-3-one (Oxford, Brackley, United Kingdom).

Silica gel 60 and neutral aluminum oxide were treated as reported by Ullrich and Grosch (18). *trans*-4,5-Epoxy-(*E*)-2-decenal (**5**) and (*E*,*Z*,*Z*)-2,4,7-tridecatrienal (**6**) were synthesized as described earlier (12,19). The labeled internal standards [5,6-$^2H_2$]-hexanal (***d*-1**), [1-$^2H_{1;2}$,2-$^2H_{1;1}$]-1-octen-3-one (***d*-2**), [3,4-$^2H_2$]-(*E*,*E*)-2,4-decadienal (***d*-4**), and [4,5-$^2H_2$]-*trans*-4,5-epoxy-(*E*)-2-decenal (***d*-5**) were synthesized as previously reported (19–21).

*Autoxidation.* AA (500 mg) was dissolved in freshly distilled $Et_2O$ (50 mL) and placed into a 250-mL flask. The solvent was removed with a stream of nitrogen to obtain a thin layer of the lipid material. The flask was filled with oxygen and sealed. After storing the sample in the dark for 48 h at room temperature, the flask was flushed with nitrogen (1 min). The peroxide value was measured using the Fe test (22), indicating the presence of about 60 mol% of total peroxides.

*Clean-up.* Nonvolatile components were removed by column chromatography (CC) for quantitative analysis. $Et_2O$ (2 mL) was added to the reaction mixture after autoxidation. The solution was spiked with labeled internal standards dissolved in pentane. The homogenized mixture was applied onto a glass column (20 × 1 cm) packed with $Al_2O_3$ in pentane/$Et_2O$ (2:1, vol/vol). The column was maintained at 10°C by a cooling jacket. Compounds without a carboxylic group were eluted with 150 mL pentane/$Et_2O$ (2:1, vol/vol). The effluent was collected and concentrated to 2 mL using a Vigreux column (50 × 1 cm).

In order to further purify the samples for quantification, the concentrated effluent obtained from CC with $Al_2O_3$ was applied to a glass column (20 × 1 cm, with cooling jacket) packed with silica gel 60 in pentane. Elution was performed stepwise with 50 mL pentane/$Et_2O$ (98:2, vol/vol) and 150 mL pentane/$Et_2O$ (85:15, vol/vol). Only the second fraction was collected and concentrated to 0.5 mL for GC–MS analysis.

*Capillary GC.* This was performed with a Carlo Erba Mega 2 (Fisons Instruments, *via* Brechbühler, Schlieren, Switzerland) equipped with a cold on-column injector and a flame-ionization detector held at 230°C. Fused-silica capillary columns of low (DB-5), medium (DB-1701), and high (DB-Wax, DB-FFAP) polarity were used (J&W Scientific MSP Friedli, Koeniz, Switzerland), both 30 m × 0.32 mm with a film thickness of 0.25 µm. Helium (80 kPa) was used as carrier gas. The temperature program was 35°C (2 min), 40°C/min to 50°C (1 min), 6°C/min to 180°C, 10°C/min to 240°C (10 min). Linear retention indices (RI) were calculated according to van den Dool and Kratz (23).

*GC–MS.* Qualitative analysis was performed on a MAT 8430 mass spectrometer ( Finnigan, Bremen, Germany). Electron ionization (EI) mass spectra were generated at 70 eV. Chemical ionization (MS–CI) was performed at 150 eV with ammonia as the reagent gas. Further details of the GC–MS system and chromatographic conditions were described elsewhere (20). Relative abundances of the ions are given in percentages.

Quantitative analysis was performed on a Finnigan SSQ 7000 mass spectrometer coupled with an HP-5890 gas chromatograph. CI was carried out at 200 eV with isobutane as reagent gas. Samples were introduced *via* splitless injection at 250°C on a DB-1701 capillary column (30 m × 0.32 mm, film thickness 0.25 µm; J&W Scientific). Helium (90 kPa) was used as carrier gas. Temperature program: 20°C (1 min), 70°C/min to 60°C, 6°C/min to 180°C, 10°C/min to 240°C (10 min). Each sample (2 µL) was injected at least twice. Quantitative measurements were carried out in full scan or, if necessary, in the selected ion monitoring (SIM) mode measuring characteristic ions listed in Table 1.

*Determination of isotopic purity.* This was calculated from GC–MS data as recently described for deuterated aldehydes (20). Clusters of ions representing the species from $[M + 3]^+$ to $[M - 2]^+$ of both the deuterated standard and nonlabeled reference compound were measured in the positive chemical ionization (PCI) mode on the SSQ 7000 using SIM and isobutane as reagent gas (24,25). The nondeuterated substance was analyzed for isotope correction of the labeled compound. The deuterium distribution was calculated according to Rohwedder (25).

**TABLE 1**
**Parameters Used in the Quantification of Six Lipid-Derived Odorants by Isotope Dilution Assay**

| Analyte | Internal standard (IS) | Selected ions (*m/z*)[a] | | Linearity[b] ($r^2$) | Linear range[b] (ratio analyte/IS) |
|---|---|---|---|---|---|
| | | Analyte | IS | | |
| **1** | [5,6-$^2H_2$]-Hexanal (***d*-1**) | 101 | 103 | 0.999 | 0.05–19.0 |
| **2** | [1-$^2H_{1;2}$,2-$^2H_{1;1}$]-1-Octen-3-one (***d*-2**) | 127 | 129/130[c] | 0.999 | 0.05–9.0 |
| **3** | [3,4-$^2H_2$]-(*E*,*E*)-2,4-Decadienal (***d*-4**) | 153 | 155 | 0.999 | 0.05–9.0 |
| **4** | [3,4-$^2H_2$]-(*E*,*E*)-2,4-Decadienal (***d*-4**) | 153 | 155 | 0.999 | 0.05–9.0 |
| **5** | [4,5-$^2H_2$]-*trans*-4,5-Epoxy-(*E*)-2-decenal (***d*-5**) | 153 | 155 | 0.999 | 0.05–9.0 |
| **6** | [4,5,7,8-$^2H_4$]-(*E*,*Z*,*Z*)-2,4,7-Tridecatrienal (***d*-6**) | 193 | 197 | 0.999 | 0.05–9.0 |

[a]Chemical ionization was applied using isobutane as reagent gas. The ion pairs measured generally were the species $[M + H]^+$, except for **5** and ***d*-5** where $[M + H - O]^+$ was monitored.
[b]Linearity and linear range were obtained from the calibration graphs using selected ions (see the Experimental Procedures section).
[c]***d*-2** was composed of two major isotopomers (21). The sum of both ions (*m/z* 129 and 130) was used for establishing the calibration graph.

*IDA.* Defined amounts of labeled internal standard (IS) in solution were added to autoxidized AA before isolation of volatiles by CC. Calibration curves were obtained using mixtures of defined amounts of analyte and labeled IS (14). As recently described for **5** and ***d*-5** (19), nine mixtures **1**/***d*-1** were used, i.e., from 0.5 + 9.5, 1 + 9, 2 + 8, 3 + 7, and 5 + 5 to 7 + 3, 8 + 2, 9 + 1, and 9.5 + 0.5. Calibration curves were established for the odorants **1**, **2**, **4**, **5,** and **6**, while (*E*,*Z*)-2,4-decadienal (**3**) was quantified using that of **4**. The parameters used in the IDA of the five odorants are summarized in Table 1. Samples for establishing the calibration curves and for quantification were injected twice.

*Nuclear magnetic resonance (NMR) spectroscopy.* The sample for NMR spectroscopy was prepared in a WILMAD 528-PP 5 mm Pyrex NMR tube (Textronica AG, Oberreit, Switzerland), using as solvent $C^2HCl_3$ (0.7 mL) from a sealed vial. The NMR spectra were acquired on a Bruker AM-360 spectrometer, equipped with a quadrinuclear 5-mm probe head, at 360.13 MHz for $^1H$ and at 90.56 MHz for $^{13}C$ under standard conditions (20). All shifts are cited in ppm from the internal trimethylsilane standard. Since nondeuterated (*E*,*Z*,*Z*)-2,4,7-tridecatrienal had been previously elucidated in detail (12), one-dimensional spectra ($^1H$ NMR, $^{13}C$ NMR, proton decoupled and nondecoupled, distortionless enhancement by polarization transfer 135) were sufficient to characterize its deuterated analog.

*Synthesis of [4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-tridecatrienal (***d-6***).* The synthesis procedure used for (*E*,*Z*,*Z*)-2,4,7-tridecatrienal (12) was adapted to obtain ***d*-6** by partial deuteration of intermediate **10** as shown in Scheme 1. Experimental details for the first two steps leading to **10** are described elsewhere (12).

*[4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-Tridecatrien-1-ol (***d-11***).* This was prepared by partial deuteration of **10** in $CH_3O^2H$ at room temperature under normal pressure with Lindlar's catalyst following the conditions recently described (12). Monitoring of the reaction by GC–MS indicated ***d*-11** as the major compound with some under- and over-deuterated by-products. The product mixture was used in the next step without purification. MS(EI) *m/z* (% relative abundance): 82 (100), 83 (66), 81 (60), 97 (58), 96 (46), 167 (45), 70 (42), 69 (41), 95 (38), 84 (37), 57 (36), 85 (35), 79 (35), 56 (35), 71 (34), 80 (33), 55 (27), 198 (25, $M^+$), 110 (21), 109 (21), 108 (19), 122 (17), 123 (15), 138 (8), 137 (8), 155 (5). MS(CI), ammonia *m/z* (% relative abundance): 181 (100, $[M + H - H_2O]^+$), 198 (20, $M^+$ or $[M + NH_4 - H_2O]^+$), 216 (10, $[M + NH_4]^+$).

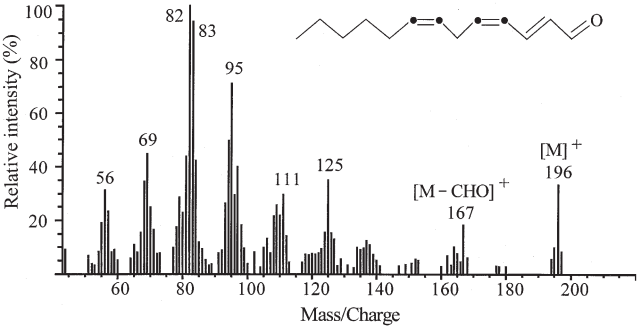



**FIG. 1.** Mass spectrum (electron ionization) of [4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-tridecatrienal (***d*-6**).

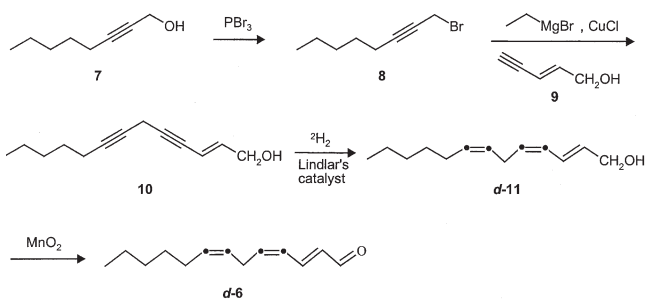
*[4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-Tridecatrienal (***d-6***).* The product mixture (1.0 g) containing ***d*-11** obtained in the previous step was oxidized with $MnO_2$ (10.0 g) in hexane. The oxidation, monitored by GC, was complete after 30 min. The target compound ***d*-6** was obtained with at least 80% (GC) purity by CC. GC: RI (DB-5) = 1581, RI (OV-1701) = 1731, RI (FFAP) = 2118, RI (DB-Wax) = 2105. MS(EI) of ***d*-6** is shown in Figure 1. MS(CI), ammonia *m/z* (% relative abundance): 197 (100, $[M + H]^+$), 214 (80, $[M + NH_4]^+$). The $^1H$ and $^{13}C$ NMR spectral data of ***d*-6** are given in Table 2.

## RESULTS AND DISCUSSION

*Synthesis of [4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-Tridecatrienal (***d-6***).* The IS ***d*-6** was synthesized in four steps (Scheme 1) in analogy to the nondeuterated compound **6** (12). Bromination of 2-octyn-1-ol (**7**) followed by coupling of the resulting 1-bromo-2-octyne (**8**) with the Grignard derivative of (*E*)-2-penten-4-yn-1-ol (**9**) gave rise to (*E*)-2-tridecen-4,7-diyn-1-ol (**10**). Partial deuteration of **10** using Lindlar's catalyst was employed to obtain all-*cis* configuration for the C4-C5 and C7-C8 double bonds in the resulting [4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-tridecatrien-1-ol (***d*-11**). The mild and neutral oxidant $MnO_2$ was used to oxidize the allylic alcohol ***d*-11** to the target compound (***d*-6**) without changing the *cis* configuration of the C4-C5 double bond, which is sensitive to acidity.

The MS–CI data *m/z* 197, 214 of ***d*-6** compared to *m/z* 193, 210 of **6** (12) indicated incorporation of four deuterium atoms in ***d*-6**. Ions *m/z* 197 and 214 represent the species $[M + H]^+$ and $[M + NH_4]^+$, respectively. The fragments *m/z* 196, 167, and 125 in the EI mass spectrum (Fig. 1) confirmed the presence of four deuterium atoms as compared to *m/z* 192, 163, and 121 in **6** (12).

Signals of the $^1H$ and $^{13}C$ NMR spectral data of ***d*-6** (Table 2) were assigned by comparison with the spectral data of **6** (12). The proton spectrum of ***d*-6** was almost identical to that of **6**, when the changes due to the deuteration were taken into account. The spectrum indicated approximately 95% deuterium substitution at each of the expected positions C4, C5, C7, and C8, and also that either two deuterium atoms or one




**SCHEME 1**

**TABLE 2**
**$^1$H and $^{13}$C NMR Data of [4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-Tridecatrienal (*d*-6) in $C^2HCl_3$[a]**

| Group | $^1$H NMR[b] | $^{13}$C NMR[c] |
|---|---|---|
| 1-CHO | 9.62, *d*, 1 H, $J_{1,2}$ = 8.0 Hz | 193.9, *d* |
| 2-CH | 6.17, *d d*, ≤ 1 H, $J_{2,3}$ = 15.3 Hz, $J_{1,2}$ = 8.0 Hz | 132.0, *d* |
| 3-CH | 7.476, *d* (~1:1:1), ~0.95 H, $J_{2,3}$ = 15.2 Hz, $^3J_{3,4\text{-}^2H}$ ~1.6 Hz, and | |
| | 7.479, *d d*, ~0.05 H[d], $J_{2,3}$ = 15.2 Hz, $J_{3,4}$ = 11.5 Hz | 146.5, *d* |
| 4-CH | 6.27, *d, sl. br.*, ~0.05H[e], $J_{3,4}$ ~11.3 Hz | 126.4, *d*[f] |
| 4-C$^2$H | — | 126.1, *s* (1:1:1), $^1J_{C^2H}$ = 24.2 Hz |
| 5-CH | 5.96, *"t", sl. br.*, ~0.06 H[e], $J_{5,6}$ ~7.6 Hz | 141.5, *d*[f] |
| 5-C$^2$H | — | 141.1, *s* (1:1:1), $^1J_{C^2H}$ = 24.0 Hz |
| 6-$CH_2$ | 3.08, *s, sl. br.*, ~1.92 H[g] | 26.5, *t* |
| 7-CH | 5.35, *t* "quintet," *sl. br.*, ~0.04 H[e], $J_{6,7}$ = 7.2 Hz, $J_{\text{long range avg.}}$ ~1.4 Hz | 125.1, *d*[f] |
| 7-C$^2$H | — | 124.8, *s* (1:1:1), $^1J_{C^2H}$ = 24.0 Hz |
| 8-CH | 5.50, *t* "quintet," *sl. br.*, ~0.04 H[e], $J_{8,9}$ = 7.3 Hz, $J_{\text{long range avg.}}$ ~1.5 Hz | 132.0, *d*[f] |
| 8-C$^2$H | — | 131.7, *s* (1:1:1), $^1J_{C^2H}$ = 23.4 Hz |
| 9-$CH_2$ | 2.08, *t, sl. br.*, ≥2 H, $J_{avg.}$ = 7.2 Hz | 27.2, *t* |
| 10-$CH_2$ | 1.38, *m*, ≥2 H, $J_{avg.}$ ~6.9 Hz | 29.2, *t* |
| 11-$CH_2$ | 1.36–1.24, *m*, ≥4 H[h] | 31.5, *t* |
| 12-$CH_2$ | | 22.6, *t* |
| 13-$CH_3$ | 0.89, *t*, ≥3 H, $J_{12,13}$ ~6.9 Hz | 14.1, *q* |

[a]Chemical shifts (δ) in ppm from internal trimethylsilane.
[b]$^1$H nuclear magnetic resonance (NMR) multiplicity abbreviations: *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *m* = multiplet, *d t* = doublet of triplets (with decreasing values of coupling constants). Quotes ("…") mean approximate description of the multiplet, *sl. br.* = slightly broadened by unresolved long-range couplings. The coupling constants *J* are directly extracted from the spectrum, after moderate Gaussian resolution enhancement, without equalizing the constants belonging to the respective coupling partners. The coupling partners are indicated by subscripts where there is no ambiguity. Since the $T_1$ relaxation times may be long in this richly deuterated molecule, minor integration errors are not excluded.
[c]$^{13}$C NMR multiplicity abbreviations: *s, d, t, q,* denominate quaternary, CH-, $CH_2$- and $CH_3$-carbons, respectively.
[d]Signal due to isotopomer(s) not deuterated at 4-C.
[e]Residual proton signals of threefold (or less) deuterated isotopomers.
[f]Due to residual protonated carbons.
[g]Dominant signal, accompanied by several small slightly broad singlet signals between 3.1 and 2.9 ppm, likely due to threefold (or less) deuterated isotopomers and/or double-bond stereoisomers.
[h]The proton signals of 11-$CH_2$ and 12-$CH_2$ are overlapping; see (12) for a more precise shift determination from a two-dimensional heteronuclear correlation experiment in the nondeuterated analog.

deuterium and one proton are found on a double bond, while the doubly protonated double bonds are negligible. The configuration of the double bonds was deduced from that of **6**. There, coupling constants of *ca.* 15.3, 10.6, and 10.6 Hz had been found for the $C_2$-$C_3$, $C_3$-$C_5$, and $C_7$-$C_8$ double bonds, respectively (12). This, together with the nuclear Overhauser effect results, had clearly indicated the *E,Z,Z* configuration.

For an incompletely deuterated substance, integration is complicated by the various isotopomers that occur. The 3-H signal of ***d*-6**, for example, was a superposition of two components. The dominating component (~95% of the integral area) was a doublet (due to the coupling to 2-H ) of approximate 1:1:1 patterns (the latter caused by the coupling to the quadrupolar deuterium atom replacing 4-H). This signal component represented four different isotopomers, since the majority of the molecules was at least threefold deuterated. The minor 3-H signal component (~5% integral area) was a doublet of doublets without deuterium coupling, standing for one isotopomer with 4-H instead of 4-$^2$H. The respective integral areas were estimated by simulation of the two overlapping components.

As was the case for **6**, the proton spectrum, e.g., of the aldehyde signal, indicated small amounts of various double-bond stereoisomers of ***d*-6** with a distribution similar to that of **6**, resulting in overinteger values of the aliphatic chain integrals (12). In view of these factors, we estimated the ***d*-6** content to be at least 82% of all (*E,Z,Z*)-2,4,7-tridecatrienal occurring in the solution. Integration of the aliphatic tail signals showed that (*E,Z,Z*)-2,4,7-tridecatrienal represented at least 80% of all 2,4,7-tridecatrienal present. GC analysis (data not shown) suggested that the stereoisomer (*E,E,Z*)-2,4,7-tridecatrienal is probably the second-most abundant 2,4,7-tridecatrienal in the product.

In the $^{13}$C NMR spectra of ***d*-6**, the typical isotope shifts with respect to **6** were found, and the minor signals of remaining nondeuterated carbons 4, 5, 7, and 8 were compatible with the corresponding residual proton integrals found in the $^1$H NMR spectrum.

The main reason for the isotopic impurities is the well-known phenomenon of deuterium scattering in catalytic deuteration (24). An accurate calculation of isotopic labeling is possible through correction of the data for the naturally occurring deuterium isotopes (24,25). This procedure indicated the following labeling pattern for ***d*-6**: $^2H_4$-**6** (84%), $^2H_3$-**6** (15.3%), $^2H_6$-**6** (0.5%), $^2H_2$-**6** (0.1%), and $^2H_5$-**6** (0.1%). As the isotopic purity is already considered in the calibration curve, mixtures of isotopomers do not preclude the application of IDA as long as the labeled molecules are stable with respect to deuterium/hydrogen exchange. This is the case for all labeled standards used in this study (19–21).

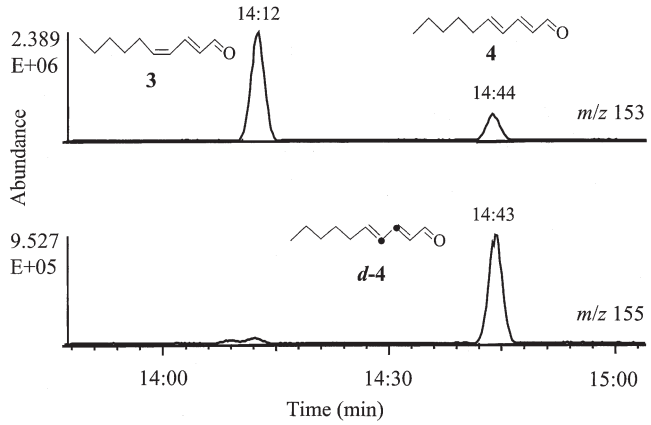



**FIG. 2.** Quantification of (*E,Z*)-2,4-decadienal (**3**) and (*E,E*)-2,4-decadienal (**4**) by isotope dilution assay using ***d*-4** as internal standard. The $[M + 1]^+$ ions *m/z* 153 of **3** and **4** and *m/z* 155 of ***d*-4** were measured by selective ion monitoring (SIM).

*IDA for the quantification of lipid-derived odorants.* Characteristic ion pairs of analyte and labeled IS were selectively monitored as shown in Figure 2 for odorants **3** and **4**. As they have almost identical chemical properties, ***d*-4** was used as IS for the quantification of both odorants. The SIM technique was preferably applied to labile compounds, such as odorant **5** (19), particularly if they occurred at low concentrations.

The calibration graphs obtained after plotting ion area ratio vs. amount area ratio showed typical second-order curves (Fig. 3A), particularly when the amount ratios were extended to values higher than 10. This is due to the natural $^{13}C$ abundance in the analyte, which coincided with the acquired ions of the deuterated IS, i.e., interference of natural isotope enrichment at higher mass or of the isotopic impurity at lower mass (26). To facilitate work within a linear range, we used only the lower part of the calibration curve, which showed excellent linearity (Fig. 3B). Therefore, the amount of IS added to the samples was adjusted to obtain an ion ratio falling in this linear range.

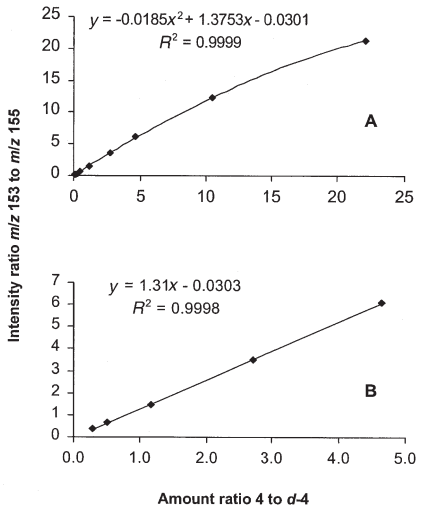



**FIG. 3.** Calibration curves obtained for the quantification of (*E,E*)-2,4-decadienal (**4**) by isotope dilution assay: (A) second-order curve and (B) linear range used in the quantification experiments.

The accuracy of the measured values compared to the theoretical values was checked according to the procedure recently described (26). Knowing the amount of analyte and labeled IS in the mixture, the theoretical enrichment of ***d*-IS** was calculated for each calibration point (data not shown) and expressed in mol% excess. The measured deuterium enrichment was plotted vs. theoretical deuterium enrichment resulting in linear curves as shown for **5** and ***d*-5** (19).

*Quantification of six odorants in autoxidized AA.* To obtain reliable quantitative data, concentrations of the potent odorants **1–6** were determined by IDA in the volatile fraction of autoxidized AA. Compound **1** with a green note was the most abundant odorant with about 7.4 g/kg autoxidized AA, which corresponds to 2.2% yield on a molar basis (Table 3). This is in good agreement with literature data reporting hexanal as the major volatile degradation product of autoxidized AA or methyl arachidonate (7,8,10). The concentrations of the remaining odorants varied from about 50 mg/kg AA for **5** (metallic) to nearly 400 mg/kg AA for **3** (fatty) with molar yields of less than 0.1%. The ratio hexanal to 2,4-decadienal was about 16:1, which is higher than those reported in the literature, i.e., 11:1 (7), 5:1 (10), and 1.4:1 (8).

As the concentrations of all of the volatile compounds identified in this study are far above their odor thresholds, they all contribute to the overall aroma of autoxidized AA. The odor activity values (OAV) were calculated as ratio of concentration to threshold. As summarized in Table 3, odorants **3** and **5** showed the highest OAV based on odor thresholds in air. They can be seen as the character impact odorants of autoxidized AA imparting metallic and fatty notes, which were found to be the main odor characters of the authentic sample (11). Data obtained on the basis of odor thresholds in oil indicated that in addition to **3** and **5**, odorants **1** and **2** significantly contribute with green and mushroom notes, respectively.

The significant contribution of **5** is due to its extremely low odor thresholds of 1.5 pg/L air (30) and 1.3 µg/L oil (14). Therefore, despite the lowest amounts found among the six impact odorants (Table 3), its sensory contribution is pronounced. Because of the low threshold value of **3**, particularly in oil (4 µg/L), and relatively high concentration, the sensory contribution of **3** to the overall aroma of autoxidized AA is of similar importance.

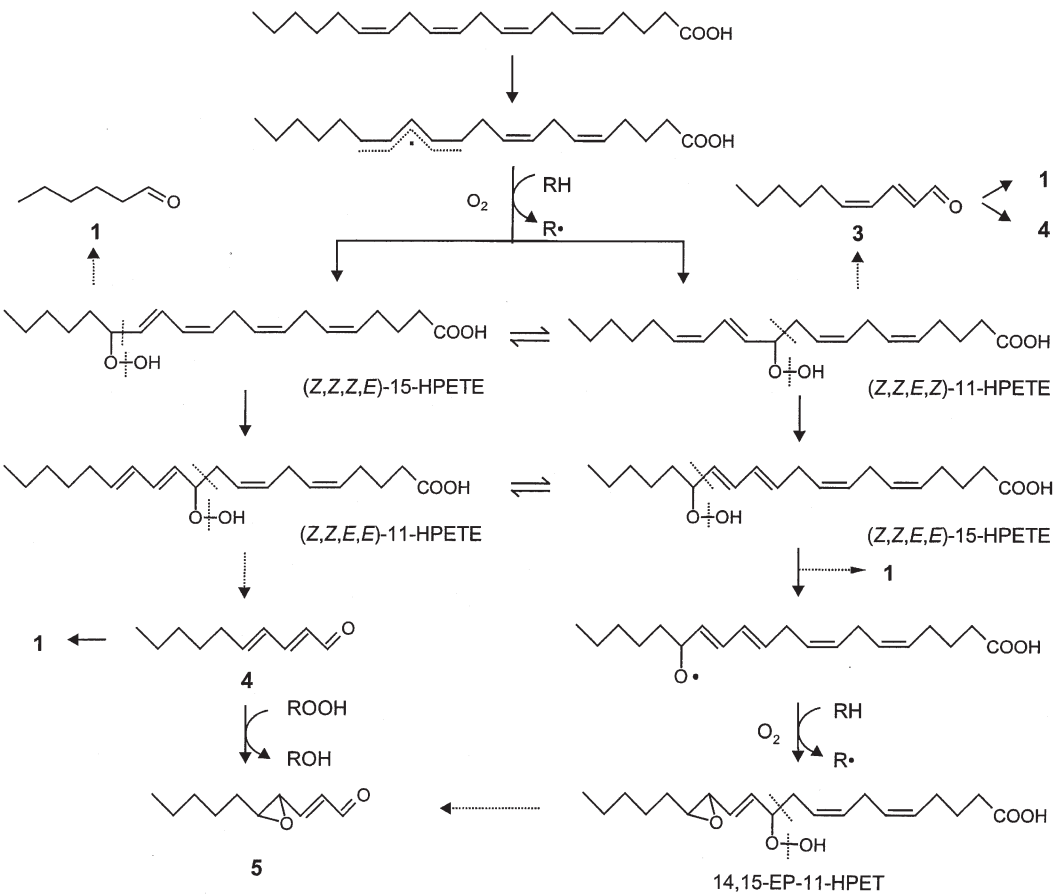
*Formation pathways.* Since AA belongs to the ω-6 fatty acid family, the formation of odorants with 10 carbon atoms and less can be explained in analogy to linoleic acid (6). Hexanal (**1**) is formed by β-cleavage of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) as shown in Scheme 2. It was found to be the major odor-active volatile degradation product in autoxidized AA with 2.2% molar yield (Table 3). Its abundance can be explained on the one hand by the relatively high yields of 15-HPETE (~35%) as reported by Yamagata *et al.* (32). On the other hand, odorant **1** is also known to be a secondary autoxidation product of 2,4-decadienal, compounds **3** and **4,** in Scheme 2 (33,34).

**TABLE 3**
**Odor Activity Values (OAV) of Potent Odorants Found in Autoxidized Arachidonic Acid (AA) Obtained by Calculating the Ratio of Concentration and Odor Thresholds in Air and Oil**

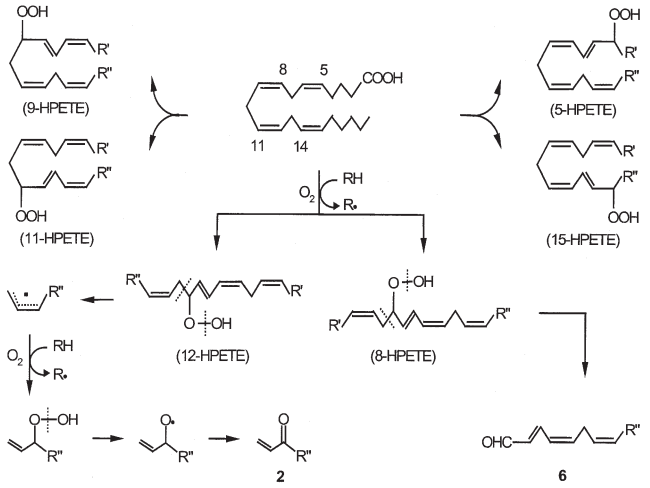
| Odorant | Aroma quality | Concentration (mg/kg AA) | Yield (mol%) | Odor threshold (ng/L air)[a] | OAV (air; × $10^6$) | Odor threshold (µg/L oil)[b] | OAV (oil) |
|---|---|---|---|---|---|---|---|
| **1** | Green | 7370 ± 520 | 2.2 | 30 | 245 | 300 | 24,570 |
| **2** | Mushroom-like | 70 ± 10 | 0.02 | 0.07 | 1000 | 10 | 7,000 |
| **3** | Fatty | 373 ± 7 | 0.07 | 0.01[c] | 37300 | 4 | 93,250 |
| **4** | Fatty | 96 ± 5 | 0.02 | 0.1 | 960 | 180 | 530 |
| **5** | Metallic | 47 ± 6 | 0.008 | 0.0015 | 31330 | 1.3 | 36,150 |
| **6** | Egg white-like | 131 ± 7 | 0.02 | 0.07[c] | 1870 | 180[d] | 730 |

[a]Odor thresholds in air were taken from the literature: **1** (27), **2** (28), **4** (29), **5** (30).
[b]Odor thresholds in oil were taken from the literature: **1** (14), **2** (14), **3** (31), **4** (14), **5** (14).
[c]Odor threshold was determined in this work (30).
[d]Odor threshold of **6** in oil was estimated to be similar to that of odorant **4**.

β-Cleavage of 11-(*Z*,*Z*,*E*,*Z*)-5,8,12,14-HPETE results in compound **3** with the *E*,*Z*-configuration of the double bonds (Scheme 2). In analogy, odorant **4** can be formed from 11-(*Z*,*Z*,*E*,*E*)-5,8,12,14-HPETE. The lower molar yields of **3** (0.07%) and **4** (0.02%) compared to **1** (2.2%) may be due to the lower amounts of the direct precursor 11-HPETE (~10%) formed by autoxidation of AA (32). The higher yields of **3** compared to **4** might be explained by the fact that **3** is directly formed from the primary precursor 11-(*Z*,*Z*,*E*,*Z*)-5,8,12,14-HPETE. In contrast, the precursor of **4**, 11-(*Z*,*Z*,*E*,*E*)-5,8,12,14-HPETE, must first be generated through isomerization of 11-(*Z*,*Z*,*E*,*Z*)-5,8,12,14-HPETE to 15-(*Z*,*Z*,*Z*,*E*)-5,8,11,13-HPETE. Following the mechanistic study of Porter and Wujek (35), the (*E*,*E*)-diene isomerization occurs after β-scission of $O_2$ from the 15-peroxyl radical (not shown in Scheme 2) leading to a pentadiene radical with 13,14 (*E*) and 11,12 (*Z*) conformation. Oxygenation of C-11 gives the desired 11-(*Z*,*Z*,*E*,*E*)-5,8,12,14-HPETE. Furthermore, aldehyde




**SCHEME 2**




**SCHEME 3**

**4** can also originate from **3**. However, **3** is stable at room temperature and in lipophilic media; isomerization to **4** occurs preferably upon heating or in aqueous solutions.

For the formation of **5**, we suggest 14,15-epoxy-11-hydroperoxy-5,8,12-eicosatrienoic acid (14,15-EP-11-HPET) (Scheme 2) as a key intermediate, in analogy to the formation of **5** from autoxidized linoleic acid (36). Alternatively, **5** can also be formed as a secondary oxidation product of 2,4-decadienal as recently reported by Gassenmeier and Schieberle (36). These authors demonstrated that **5** was preferentially formed from 9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD) *via* 2,4-decadienal as the intermediate. Alternatively, **5** may also be formed by oxidation of **4** with peracids (not shown in Scheme 2).

Formation of **6** can be explained by β-cleavage of the corresponding 8-hydroperoxy-5,9,11,14-eicosatetraenoic acid (8-HPETE) as shown in Scheme 3. This is in analogy to the formation of (*E*,*Z*,*Z*)-2,4,7-decatrienal by autoxidation of linolenic acid (8,37). Similar to the formation of **2** in autoxidized linoleic acid from 10-hydroperoxy-8,12-octadecadienoic acid (10-HPOD), the mushroom-smelling odorant can also be generated from AA *via* the alkoxyl radical formed from 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) as shown in Scheme 3, where R′ represents $CH_2$-$CH_2$-$CH_2$-COOH and R″ stands for $CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_3$ (8,37). However, the alkoxyl radical can also lead to 1-octen-3-ol (38) that was detected in autoxidized AA as well (11,12).

*Conclusions*. Quantitative characterization of the aroma composition of autoxidized AA by isotope dilution assay and calculation of the OAV suggest (*E*,*Z*)-2,4-decadienal and *trans*-4,5-epoxy-(*E*)-decenal as the character impact odorants, smelling fatty and metallic, respectively. These aroma notes are representative of the overall aroma of autoxidized AA. Hexanal seems to play an important role, particularly in oily media, due to its low odor threshold in oil. The quantitative data obtained for the six odorants are in good agreement with the possible formation mechanisms and the relative amounts of the corresponding hydroperoxides reported in the literature (32).

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REVIEW

# Genomic and Functional Characterization of Polyunsaturated Fatty Acid Biosynthesis in *Caenorhabditis elegans*

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**ABSTRACT:** The biosynthetic pathway for polyunsaturated fatty acids in the model animal *Caenorhabditis elegans* was examined in the context of the completed genome sequence. The genomic organization and location of seven desaturase genes and one elongase activity, all previously identified by functional characterization, were elucidated. A pathway for the biosynthesis of polyunsaturated fatty acids in *C. elegans* was proposed based on these genes. The role of gene duplication in enzyme evolution and proliferation is discussed.

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The successful completion of the *Caenorhabditis elegans* genome sequencing program (1) has been recognized not only as a major scientific achievement but also as ushering in the era of postgenomic biology for multicellular organisms. The knowledge of an entire genomic sequence provides new opportunities to determine and characterize the number of genes required by that organism to carry out various metabolic processes (1,2). One important and relatively simple metabolic pathway is that for polyunsaturated fatty acid (PUFA) biosynthesis (3). These fatty acids are essential components of the mammalian diet and serve as metabolic precursors for the eicosanoids (a collective group of $C_{20}$ molecules including prostaglandins and leukotrienes), which are important in the regulation of cellular processes (3,4). We examined the PUFA biosynthetic pathway in the model animal *C. elegans* with the aim of furthering our understanding of the genes required for this metabolic process.

The quantity and composition of the lipids (including PUFA) of *C. elegans* are well known (5,6). The total amounts of saturated and unsaturated fatty acids present in *C. elegans* cultured under a range of different temperatures have been determined (7), demonstrating the accumulation of $C_{20}$ PUFA. These include eicosapentanoic acid (EPA; $20{:}5\Delta^{5,8,11,14,17}$ or 20:5n-3), arachidonic acid (AA; $20{:}4\Delta^{5,8,11,14}$ or 20:4n-6), and dihomo-γ-linolenic acid (DHGLA; $20{:}3\Delta^{8,11,14}$ or 20:3n-6), which often occur in higher levels than in other organisms (>25% of total fatty acids). Therefore, PUFA biosynthesis in *C. elegans* (summarized in Fig. 1) serves as a good model system for the study of this biochemical pathway. In *C. elegans,* saturated 18-carbon fatty acids are desaturated to yield oleic acid ($18{:}1\Delta^{9}$; 18:1n-9) by a stearoyl CoA $\Delta^{9}$-desaturase enzyme, as in other animal (8) and fungal (9) systems. Further downstream, linoleic acid (LA; $18{:}2\Delta^{9,12}$; 18:2n-6) is desaturated by a $\Delta^{6}$-fatty acid desaturase to produce γ-linolenic acid (GLA; $18{:}3\Delta^{6,9,12}$; 18:3n-6), which is then $C_2$-elongated and further ($\Delta^{5}$-) desaturated to yield AA. The $\Delta^{6}$- and $\Delta^{5}$-fatty acid desaturases have previously been called "front-end" desaturases because they desaturate between preexisting double bonds and the carboxyl group, most usually between C-3 and C-7 (10). These enzymes appear to differ from "methyl-directed" desaturases (which insert double bonds sequentially toward the methyl end of the molecule) by the presence of an N-terminal domain related to cytochrome $b_5$ (11,12).

Another interesting observation regarding the fatty acid composition of *C. elegans* is that this organism is apparently capable of synthesizing LA (5–7). In the case of vertebrates, LA is classified as an essential fatty acid and must form part of the dietary intake to serve as a substrate for PUFA synthesis *via* endogenous desaturation and elongation (3). However, some biochemical evidence indicates that invertebrates maintain a capacity to synthesize LA from endogenous monounsaturated substrates (13–15).

The above observations allow the prediction of the number of enzymatic reactions required for PUFA biosynthesis in *C. elegans*. This number can then be compared with that determined by whole genome sequencing and functional characterization. The aim of this study is to discuss the various fatty acid desaturases present in *C. elegans* in the context of our current understanding of enzyme evolution and divergence.

## METHODOLOGY FOR GENOMIC CHARACTERIZATION

The complete *C. elegans* genome (1) (available at http://www.sanger.ac.uk/Projects/C_elegans/) was searched for desaturase-like sequences using the BLAST suite of programs (16). Information on genomic organization and location was extracted using the *C. elegans* ACEDB database (http://www.sanger.ac.uk/Projects/C_elegans/webace_front_end.shtml). Data on protein similarities were obtained from the WormPD database (http://www.proteome.com/

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Abbreviations: AA, arachidonic acid; DHGLA, dihomo-γ-linolenic acid; ELO, elongation of fatty acids; EPA, eicosapentanoic acid; ER, endoplasmic reticulum; GLA, γ-linolenic acid; LA, linoleic acid; ORF, open reading frame; OTA, octadecatetraenoic acid; PUFA, polyunsaturated fatty acid.

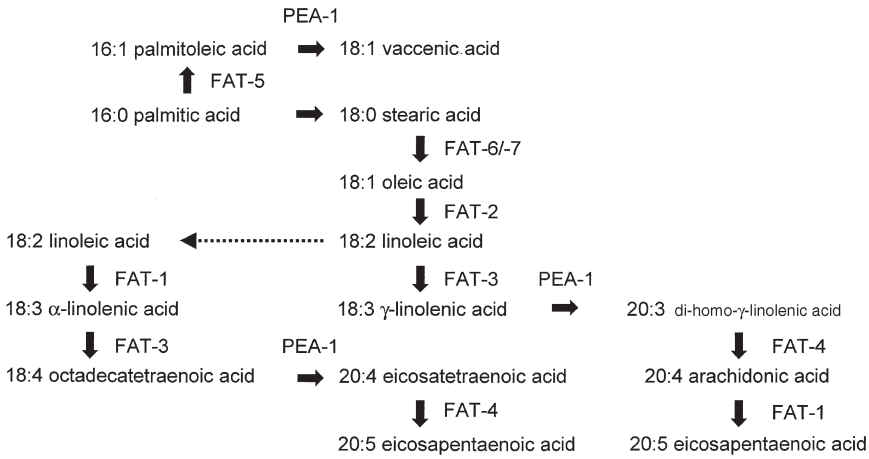


**FIG. 1.** A simplified schematic diagram of steps likely to be involved in the biosynthesis of polyunsaturated fatty acids in *Caenorhabditis elegans*. The names given to each enzyme reaction are shown in Table 1. Two alternative pathways for the synthesis of eicosapentanoic acid are shown, based on functional characterization of the enzymes involved. For clarity, the additional $\Delta^{12}$-desaturation of palmitoleic acid by FAT-2 is not shown. The enzymatic basis for the $C_2$ elongation of palmitic acid is presently unknown.

databases/index.html) (17). Phylogenetic data were produced using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) on protein sequences aligned using GeneDoc (http://www.psc.edu/biomed/genedoc).

The work of Shanklin and others (reviewed in Ref. 18) has indicated that membrane-bound fatty acid desaturases are part of the larger group of class III di-iron-oxo enzymes. Desaturases in this class use either acyl-CoA or glycerolipid (i.e., fatty acids esterified to phosphatidylcholine) substrates. Sequence analysis of membrane-bound fatty acid desaturases from plant, fungal, and animal species (as well as related enzymes such as membrane-associated hydrocarbon hydroxylases) indicated that a number of motifs are absolutely conserved, most notably three short regions termed "histidine boxes," which are essential for the enzyme activity of fatty acid desaturases (19,20). Therefore, the presence of these short histidine box motifs (typically H-X-X-H-H) is indicative of the class of di-iron-oxo enzymes of which fatty acid desaturases are members, facilitating the identification of candidate fatty acid desaturase open reading frames (ORF).

*Functional characterization of fatty acid desaturases in* C. elegans. Our exhaustive searching of the entire *C. elegans* genome yielded (based on these conserved motifs and homology) seven ORF with similarity to fatty acid desaturases from other species. Moreover, recent functional characterization has defined the precise enzymatic role of these ORF, allowing us to classify the desaturases into three distinct groups, and provided a comprehensive description of the *C. elegans* genes involved in the PUFA biosynthetic pathway. Their individual roles in PUFA biosynthesis are summarized in Table 1 and described in detail next. The majority of these ORF have been characterized by Browse and colleagues (21), and we have used their gene nomenclature. The role of each ORF in *C. elegans* PUFA biosynthesis is also shown in Figure 1.

*(i) Group 1 (stearoyl CoA-like desaturases).* The three members of this group encode fatty acid desaturases related

**TABLE 1**
**Classification of *Caenorhabditis elegans* Open Reading Frames Involved in Polyunsaturated Fatty Acid Biosynthesis[a]**

| Enzymatic reaction | Gene ID | Designated name | Group | Locus | Number of introns | Comments |
|---|---|---|---|---|---|---|
| Palmitoyl $\Delta^9$-desaturase | W06D12.3 | FAT-5 | 1 | V, 16.43 cM | 2 | |
| Stearoyl $\Delta^9$-desaturase | VZK822l.1 | FAT-6 | | IV, 5.41 cM | 4 | |
| Stearoyl $\Delta^9$-desaturase | F10D2.9 | FAT-7 | | V, 1.33 cM | 4 | |
| $\Delta^6$-Desaturase | W08D2.4 | FAT-3 | 2 | IV, 4.44 cM | 6 | Tandem gene pair <1 Kb apart |
| $\Delta^5$-Desaturase | T13F2.1 | FAT-4 | | IV, 4.43 cM | 7 | |
| $\Delta^{12}$ $\omega^6$-Desaturase | W02A2.1 | FAT-2 | 3 | IV, 8.79 cM | 2 | Genes <6 Kb apart |
| $\Delta^{15}$ $\omega^3$-Desaturase | Y67H2B.a | FAT-1 | | IV, 8.68 cM | 2 | |
| $\Delta^6$-Fatty acid elongase | F56H11.4 | PEA-1 | | IV, 4.30 cM | 3 | Part of a tandem gene pair <2 Kb apart |

[a]The enzymatic reactions encoded by the seven open reading frames identified as desaturases from the completed *C. elegans* genome. The Sanger Centre's gene identifier code is given, as is the current designated name and our group classification; details of the $C_{18}$-elongating activity are also shown. Chromosomal location, map position in centiMorgans, and intron number are also indicated.

to the stearoyl CoA class of microsomal desaturases (8,9). These three genes, W06D12.3, VZK822l.1, and F10D2.9, have been designated FAT-5, FAT-6, and FAT-7, respectively (21). Functional characterization *via* heterologous expression of the ORF in yeast has indicated that while FAT-6 and FAT-7 are stearoyl $\Delta^9$-desaturases, they also desaturate palmitic acid (21). However, FAT-5 displays a strict substrate preference for palmitic acid and shows negligible activity toward stearate (21). FAT-6 and FAT-7 are 92% identical at the amino acid level and 78–80% identical to FAT-5.

*(ii) Group 2 (front-end desaturases).* This group consists of two genes, W08D2.4 and T13F2.1, which have been designated FAT-3 and FAT-4, respectively. We demonstrated that FAT-3 encodes the $\Delta^6$-fatty acid desaturase responsible for the synthesis of GLA from LA (22). We also showed that FAT-4 encodes the $\Delta^5$-fatty acid desaturase responsible for the final desaturation step in the synthesis of AA (23) (Fig. 1). These enzymes carry out "front-end" desaturation (10) and, like $\Delta^5$- and $\Delta^6$-desaturases from other systems (24–26), differ from the other *C. elegans* desaturases in the presence of an N-terminal cytochrome $b_5$ domain. Both the *C. elegans* $\Delta^5$- and $\Delta^6$-desaturases show sequence homology to higher plant $\Delta^6$-fatty acid desaturases (27) and also to plant sphingolipid long chain base desaturases (28). The similarity between the amino acid sequences of FAT-3 and FAT-4 is 66%.

*(iii) Group 3 ($\omega^{3/6}$-desaturases).* This group consists of two genes, Y67H2B.a and W02A2.1, respectively designated FAT-1 and FAT-2. The encoded enzymes are related to higher plant $\omega^3$ fatty acid desaturases such as the *Arabidopsis thaliana* FAD3 microsomal $\Delta^{15}/\omega^3$ desaturase (29). Spychalla *et al.* (30) reported the functional analysis of FAT-1 in transgenic *Arabidopsis* plants. FAT-1 was shown to encode a $\omega^3$-fatty acid desaturase with specificities for both $C_{18}$ and $C_{20}$ carbon unsaturated substrates (30), though the very small amounts of ALA in *C. elegans* (5,7) may indicate a preference for $C_{20}$ fatty acids. The dual $C_{18/20}$ substrate preference of FAT-1 provides two alternative pathways for the order of desaturation required for EPA synthesis (Fig. 1). FAT-2 displays a high degree of sequence identity (72%) to FAT-1 and has been shown to encode a $\Delta^{12}/\omega^6$ fatty acid desaturase that desaturates both 16:1 and 18:1 substrates. This latter reaction is therefore responsible for the synthesis of LA (31), confirming the predicted presence of this enzyme in invertebrates (15,31). No overlap in enzymatic function of the two desaturases was observed (30,31).

*Identification of other enzymes involved in PUFA biosynthesis.* The synthesis of PUFA such as AA and EPA requires the $C_2$ elongation of $C_{18}$ PUFA. This reaction is catalyzed by a multifunctional elongase complex that consists of four distinct enzymatic reactions (condensation, keto-reduction, dehydration, and enoyl-reduction). Of these four reactions, the (first) condensation step is considered to be rate-limiting. We recently functionally identified a *C. elegans* ORF (F56H11.4) that directs $C_{20}$ PUFA production when expressed in yeast (32). This elongating activity shows specificity for $\Delta^6$-desaturated $C_{18}$ PUFA [e.g., GLA and octadecatetraenoic acid ($18{:}4\Delta^{6,9,12,15}$ or 18:4n-3)], although it has some activity toward palmitoleic acid (the product of FAT-5), resulting in the production of vaccenic acid. Since vaccenic acid is a relatively large component of *C. elegans* total fatty acids (7), our observations may help to explain its abundance. The precise biochemical function within the elongase of F56H11.4 has not yet been elucidated, though this ORF shows some limited homology to the yeast ELO (elongation of fatty acids) gene family, required for medium and very long chain saturated fatty acid elongation (33). F56H11.4 is a member of a small gene family that appears to have undergone gene duplication, as determined by the presence of conserved intron-exon junctions within tandem gene pairs (32). In view of our functional characterization of F56H11.4 (32), we have designated this ORF PEA-1 for PUFA-elongating activity. Moreover, we have also co-expressed PEA-1 with FAT-3 and FAT-4 in yeast, successfully reconstituting the PUFA biosynthetic pathway (32).

*Common motifs and domain structures.* A comparison of the primary sequences of the *C. elegans* desaturases that comprise Groups 1–3 indicates only very limited sequence homology (restricted to the histidine box domains), as represented by the phylogenetic tree shown in Figure 2. However, the amino acid sequences of the seven fatty acid desaturase still share a number of similarities. First, the predicted ORF encode membrane proteins that have a similar topology (as judged by hydrophobicity plots; data not shown). This topology includes an even number of putative transmembrane spans, thereby placing the N- and C-termini of the proteins on the same membrane face; most probably the cytosolic face of the endoplasmic reticulum (ER). Thus, the three highly conserved histidine boxes present in all the desaturases are likely to be exposed to the cytosol, as opposed to the ER lumen. This predicted topology is in good agreement with models for desaturases from other species (18). It is also likely that the cytochrome $b_5$ domain of the Group 2

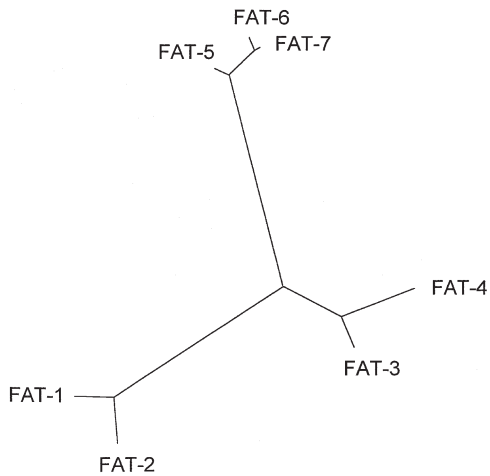


**FIG. 2.** A phylogenetic tree of the similarity between the seven *C. elegans* desaturase open reading frames. Deduced amino acid sequences were aligned and subjected to phylogenetic analysis *via* PHYLIP and TreeView. The degree of similarity between sequences is proportional to their distance apart.

front-end desaturases (FAT-3 and FAT-4) is present on the cytosolic side of the ER membrane. This would allow this domain to interact with other components of the electron transport chain (i.e., cytochrome $b_5$ reductase), which are predicted to be required for full enzymatic function. The proposed topology of the *C. elegans* desaturases is given further support by the presence of C-terminal ER-recycling/retention motifs in their amino acid sequences. This is most obvious in FAT-4 (motif K-K-I-A-stop) and FAT-7 (K-K-S-I-M-stop). Although these examples are from different groups of fatty acid desaturases, it is clear that they conform well to the consensus retention sequence for ER membrane proteins (K-K-$X_{2-3}$-stop) (34). Other studies in yeast have shown that the di-lysine C-terminal signal must be located on the cytoplasmic face of the ER in order to function (35), thus agreeing with the proposed topology. Interestingly, the PEA-1 elongating activity also contains an even number of transmembrane spans and a di-lysine retention motif (32). Perhaps more intriguingly, this ORF also contains a histidine box motif, though the precise function of this sequence is as yet unclear.

*Genomic organization of* C. elegans *desaturase genes.* Comparison between the locations of the genes encoding the PUFA biosynthetic enzymes revealed that several are in close proximity to each other (Table I). We initially observed this with the two front-end desaturases of Group 2 (22,23), where the stop codon of the $\Delta^5$-desaturase (FAT-4) is 990 bp upstream of the initiating methionine of the $\Delta^6$-desaturase (FAT-3). Although such proximity is consistent with polycistronic transcription (which accounts for 25% of all transcripts in *C. elegans*) (36), Watts and Browse demonstrated that the mRNA for these two fatty acid desaturases are transcribed from distinct operons (37). A similar level of proximity was observed for the two desaturases of Group 3, in which the FAT-1 $\omega^3$-desaturase is <6 Kb upstream of the $\Delta^{12}$-desaturase (FAT-2) (31). Furthermore, these desaturases (together with FAT-6 and PEA-1) map to a single region (4.3–8.79 cM) at the top of chromosome IV. Whether there is any evolutionary significance to the localization of all the enzymatic activities (i.e., stearoyl CoA-desaturase, $\Delta^{12}$-desaturase, $\Delta^6$-desaturase, elongating activity, $\Delta^5$-desaturase, and $\omega^3$-desaturase) required for PUFA biosynthesis to a region of <4 Mb in a genome of 97 Mb remains to be determined.

C. elegans *fatty acid desaturases display conserved intron-exon junctions*. It is likely that the number of desaturases in *C. elegans* has increased as a result of gene duplication, and certainly gene duplication in general was observed at an early stage in the *C. elegans* genome sequencing program (exemplified in Ref. 38). However, although there are many annotated examples of presumptive gene duplications present in the databases, there are considerably fewer examples of functional characterization of these tandem gene products. In that respect, our work showing that tandemly arranged genes have precise and distinct enzyme activities (e.g., $\Delta^5$- and $\Delta^6$-desaturases) is relevant. The conserved intron-exon junctions in these genes indicate that they may have resulted from a duplication event, as reflected by a level of identity between the two (spliced) gene transcripts of 60% (23,37). The last two intron-exon junctions of these two desaturases are perfectly conserved, even though the sizes of the respective introns differ between the two genes. It may also be significant that the last intron-exon junction is within the third histidine box and that both these desaturases have a glutamine for histidine substitution in this domain. Thus, these two front-end desaturases have not only an N-terminal cytochrome $b_5$ domain but also a variant third histidine box, which itself is bisected by a conserved intron. However, within these two genes we did not detect the presence of common elements in either the promoters or the untranslated regions of transcripts.

Similarly, the two $\omega^{3/6}$-desaturases in close proximity to each other (FAT-1 and FAT-2) also contained conserved junctions between the penultimate and last exons, despite variations in intron size (31). However, the position of this conserved intron is distinct from that present in the front-end desaturases in that it does not span the third histidine box. It is likely that the two $\omega^{3/6}$-desaturases have also arisen as a result of gene duplication (31). Thus, for both front-end and methyl-directed desaturases, duplications have likely resulted in the evolution of distinct and, more importantly, nonoverlapping enzyme activities.

Although the three genes of the stearoyl CoA-desaturase Group 1 are not tandemly located or in close proximity, conserved intron-exon junctions are also observed. For example, FAT-6 and FAT-7 contain four identical intron-exon junctions, even though they are located on different chromosomes. This identity may indicate that these two genes resulted from a gene-duplication event followed by subsequent chromosomal translocation. In addition, there are conserved intron-exon junctions between FAT-6/-7 and FAT-5 (one between exons 2 and 3 of FAT-6/-7 corresponding to one between exons 1 and 2 of FAT-5 and another between exons 3 and 4 of FAT-6/-7 homologous to one between exons 2 and 3 of FAT-5).

*Gene duplication: functional redundancy or enzyme divergence?* One obvious perspective on gene duplication events is that they are an evolutionary mechanism for generating discrete new enzymatic activities. In the case of essential genes, duplication must be considered a prerequisite to any alteration in function (39). Interestingly, in the cases of the *C. elegans* desaturases described previously, there appears to be no overlap or functional redundancy between enzyme activities contained within the two presumptive gene duplicatations (FAT-1/2 and FAT-3/4). It is an interesting yet open question as to whether the ancestral progenitors of these pairs encoded a discrete single enzyme activity similar to that of one of the current tandem pair enzymes, or whether the activity of the ancestors broadly encompassed the current activities of both members. Such questions also have implications in terms of the evolution of the PUFA-derived eicosanoids, and their roles in a wide range of important cellular processes (3). Another open question is why functional redundancy exists in the stearoyl CoA desaturases and whether there is any selective advantage in this. The fact that these enzymes catalyze the primary desaturation reaction in the biosynthesis of unsaturated fatty acids may also be a relevant consideration.

The recent advances toward the completion of the human genome sequence have also provided further insights into the genomic organization of fatty acid desaturases involved in PUFA biosynthesis. Remarkably, the *Homo sapiens* $\Delta^5$- and $\Delta^6$-fatty acid desaturases are also in close proximity, even in this much more complex genome (40). However, in the case of these two human front-end desaturases, the genes are in opposite 5′ to 3′ orientations and are separated by ~63 Kb (40). Perhaps a more intriguing question is why these desaturase genes (from worm to human) have remained in proximity to each other, and it will therefore be important to determine if this apparent synteny is related to coordinated transcriptional regulation. Tandem clusters of membrane proteins have been observed in a number of (prokaryote) genome sequences (41), though the reasons for these phenomena are not clear.

## CONCLUSION

Genome sequencing programs, as exemplified by the completion of the *C. elegans* genome project, provide a very powerful tool for the investigation of metabolic diversity and evolution. However, our studies on *C. elegans* PUFA desaturases indicate that, in the absence of functional characterization, assignment of enzyme activity *via* homology alone is potentially misleading. Moreover, the fact that gene duplication does not necessarily result in partial or complete functional redundancy, but instead can generate distinct enzyme activities (even in a pathway of primary metabolism) indicates the need for comprehensive functional characterizations of predicted gene products. At a more applied level, the investigation of important metabolic processes such as PUFA synthesis will help elucidate appropriate targets for the improvement of human health and nutrition. It will also be important to determine the role discrete *in vivo* changes in PUFA metabolism have on the whole organism, and, in that respect, *C. elegans* provides an excellent experimental system for study. In particular, RNA-mediated interference provides a powerful tool for the confirmation and dissection of enzymatic functions within the nematode (42).

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ARTICLE

# Conjugated Linoleic Acid Supplementation in Humans: Effects on Fatty Acid and Glycerol Kinetics

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**ABSTRACT:** Recent studies with mouse adipocytes have shown that dietary conjugated linoleic acid (CLA) may reduce body fat by increasing lipolysis. The present study examined the effect of CLA supplementation on fatty acid and glycerol kinetics in six healthy, adult women who were participating in a controlled metabolic ward study. These women were fed six CLA capsules per day (3.9 g/d) for 64 d following a baseline period of 30 d. The subjects were confined to a metabolic suite for the entire 94-d study, where diet and activity were controlled and held constant. The rate of appearance (Ra) of glycerol, which indicates lipolytic rates, was similar at baseline and after 4 wk of CLA supplementation at rest (1.87 ± 0.21 and 2.00 ± 0.39 μmol/kg/min, respectively) and during exercise (7.12 ± 0.74 and 6.40 ± 0.99 μmol/kg/min, respectively). Likewise, the Ra of free fatty acids (FFA) was not significantly different after 4 wk of dietary CLA at rest (2.72 ± 0.06 and 2.74 ± 0.12 μmol/kg/min, respectively) or during exercise (6.99 ± 0.40 and 5.88 ± 0.29 μmol/kg/min, respectively). CLA supplementation also had no effect on the percentage of FFA released from lipolysis that were re-esterified. The apparent rate of FFA re-esterification was 65.2 ± 4.2% at rest and 32.1 ± 3.44% during exercise. Four weeks of CLA supplementation had no significant effect on fatty acid or glycerol metabolism in healthy, weight-stable, adult women.

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Conjugated linoleic acid (CLA) is a group of linoleic acid isomers found naturally in foods such as grilled ground beef (1) and some dairy products (2). CLA is reported to have anticarcinogenic properties (3–5) and to be protective against atherosclerosis (6) in animal models fed supplemental CLA. Recently, dietary CLA has been shown to reduce body fat in mice (7–11).

Studies with adipocytes have been performed to determine the mechanism by which CLA reduces body fat in mice. Adipocytes (3T3-L1) exposed to exogenous CLA showed reduced lipoprotein lipase activity, reduced levels of triglyceride and glycerol inside the cells, and increased glycerol levels outside the cells (8,10). These data suggest that CLA may reduce body fat by affecting key enzymes involved in lipid mobilization and storage. However, lipolysis has not been measured directly.

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Abbreviations: CLA, conjugated linoleic acid; FFA, free fatty acids; GC, gas chromatography; MS, mass spectrometry; Ra, rate of appearance; RQ, respiratory quotient; $VCO_2$, carbon dioxide production; $VO_2$, oxygen consumption.

Evidence on the effect of CLA supplementation on body fat in humans is contradictory. In a recent study, adults taking CLA supplements for 12 wk had reduced body fat mass (12). In contrast, we reported that body fat did not decrease significantly when adult women took CLA supplements for 9 wk (13). One factor that might have contributed to these discordant findings is the magnitude of the error associated with the measurement of human body composition. Generally, with the methods used in these studies, the standard error of estimate for body fat mass is about 1.5 kg (14,15). Thus, changes in fat mass on the order of 1–2 kg are difficult to measure with confidence. For this reason, longer periods of supplementation are needed to determine the impact of CLA on body composition in humans. Further, it is important to identify metabolic changes attributable to CLA that occur sooner and could serve as the basis of a long-term change in body fat. Accordingly, as part of our study, we measured the effect of CLA supplementation (3.9 g/d/person) on fatty acid and glycerol kinetics in a subset of six healthy women taking the CLA supplements. The dosage of CLA used was approximately fourfold higher than the average amount consumed in a typical diet containing meat and dairy products (2). However, this amount is similar to that used in previous animal and human studies, and it is within the reasonable range for a healthy, nonvegan adult. Stable isotopes of palmitate and glycerol were used to measure the rate of appearance (Ra) of free fatty acids (FFA) and glycerol as well as whole body lipolysis and apparent re-esterification in the resting state and during moderate activity.

## MATERIALS AND METHODS

*Subjects.* Seventeen women, 24–41 yr of age, completed the entire 94-d study. Subject selection criteria included being a healthy nonsmoker, premenopausal with normal menstrual cycles, and free of any abnormal physiological conditions or diseases. Prior to being selected for the study, all subjects completed medical and dietary histories, physical examinations, urinary tests for pregnancy, resting electrocardiograms, and standard batteries of blood tests. A subset of six women from the CLA supplement group participated in the stable isotope tracer studies. This subset was similar to the original group of 10 women in the CLA group (Table 1). Participation was by informed consent. The study protocol was approved by the Human Subject Committees of the U.S. Department of Agriculture and the University of California, Davis.

**TABLE 1**
**Comparison of Subject Characteristics in the CLA Subset and CLA Total Group**

| Subject | Age (yr) | BMI[a] (kg/m$^2$) | Body fat (%) | Change in body fat with CLA[b] (kg) |
|---|---|---|---|---|
| #29*[c] | 31 | 24.2 | 33.3 | –1.49 |
| #30 | 20 | 23.1 | 28.9 | –0.01 |
| #31* | 24 | 24.1 | 31.8 | 0.36 |
| #32 | 23 | 22.9 | 22.7 | –3.16 |
| #34* | 27 | 24.4 | 36.4 | 0.94 |
| #38* | 28 | 23.8 | 31.7 | 0.35 |
| #40* | 25 | 24.8 | 35.6 | –0.26 |
| #41 | 24 | 20.3 | 27.7 | 0.38 |
| #43 | 29 | 21.9 | 25.9 | 0.38 |
| #47* | 41 | 23.4 | 36.1 | 0.50 |
| Group mean ± SEM | 27.2 ± 1.8 | 23.2 ± 0.5 | 31.0 ± 1.5 | –0.20 ± 0.39 |
| Subgroup ± SEM | 29.3 ± 2.5 | 24.1 ± 0.2 | 34.2 ± 0.9 | 0.07 ± 0.35 |

[a]BMI, body mass index; CLA, conjugated linoleic acid.
[b]Data from Reference 13.
[c,*]Denotes those subjects in the stable isotope infusion protocol subset.

Subjects lived in the metabolic suite at the Western Human Nutrition Research Center, 24 h/d, 7 d/wk for 94 d of the study. Times for meals and daily outdoor walks were standardized. While in the metabolic suite, the lifestyle was predominantly sedentary, but activity was provided in the form of walking 2 mi twice a day.

*Experimental design.* This study was part of a randomized, blind, and placebo-controlled study conducted with two cohorts totaling 17 subjects and has been described previously (13). Six of the 17 subjects participated in the stable isotope infusion study. The six were not randomly selected, but rather chosen because venous access was good, enabling the placement of the catheters, essential for the infusion of isotope and blood sampling. On day 31, following 30 d of baseline, subjects were randomly assigned to either the group receiving the sunflower oil placebo or the group receiving supplemental CLA (3.9 g/d) for the final 64 d of the study.

CLA capsules were obtained from Pharmanutrients, Inc. (Lake Bluff, IL). CLA constituted approximately 65% of the total fatty acids in the capsule (~3.9 g), with the remainder consisting mainly of oleic acid. Isomer composition of the CLA was determined by gas chromatography and found to be 22.6% *trans*-10, *cis*-12; 23.6% *cis*-11, *trans*-13; 17.6% *cis*-9, *trans*-11; 16.6% *trans*-8, *cis*-10; 7.7% *trans*-9, *trans*-11 and *trans*-10, *trans*-12; and 11.9% other isomers. The placebo capsule contained 72.6% linoleic acid, with the remainder consisting mainly of palmitic, stearic, and oleic acids with no detectable CLA isomers. The capsules used were identical in appearance and were packaged in the same manner.

*Dietary intake.* The subjects' diets were equivalent to the American Heart Association's Step II Diet containing the Recommended Dietary Allowance for all known nutrients and 30% of calories from fat. The energy intake of each subject was estimated using the Harris-Benedict equation. During the baseline period, the energy intake was adjusted if body weight changed by ±3% over time. Dietary fat consisted of saturated, monounsaturated, and polyunsaturated at 10% each for both placebo and intervention groups with saturated fat, linoleic acid, and other n-6 polyunsaturated fats held constant among the two groups. The cholesterol content of the diets was 250–300 mg/d.

*Infusion protocol.* The stable isotope infusion protocol was performed at the end of the baseline period and during the fourth week of intervention. These timepoints for testing were chosen so that the subjects would be in the same phase of their menstrual cycles for both tests. In order to measure palmitate and glycerol kinetics, a Teflon catheter was placed into an antecubital vein for [1-$^{13}$C]palmitate and glycerol-$d_5$ infusion. A second sampling catheter was inserted in a dorsal hand vein of the contralateral arm. The heated hand technique was used to obtain arterialized blood samples (16). The subjects rested for 1 h after catheter placement. After a blood sample was drawn to determine background enrichment, primed constant infusions of glycerol-$d_5$ and [1-$^{13}$C]palmitate were started according to Romijn *et al.* (17). In conjunction with the infusion protocol, indirect calorimetry was used to estimate fat oxidation rates. The gas exchange measurements were made with an automated respiratory gas exchange system (model 2900; SensorMedics, Anaheim, CA) and collected between 30 and 50 min of rest and exercise. The system was calibrated with standard gas mixtures, and the calibration was verified at intervals throughout the collection periods. Subjects wore inflatable facemasks that were connected to the gas analyzers *via* a tubing assembly.

The glycerol and palmitate tracers (99% enriched) were purchased from Cambridge Isotope Inc. (Andover, MA), and the infusates were prepared by the Parenteral Solutions Laboratory, University of California, San Francisco. The palmitate infusate contained 200 mg [1-$^{13}$C]palmitate and 100 mL 25% human albumin mixed with 0.9% sterile saline in a 500-mL bag and was infused at 92 and 184 mL/h at rest and during exercise, respectively. The glycerol infusate contained 128 mg glycerol-$d_5$ in 100 mL 0.9% sterile saline and was infused at 15 and 30 mL/h at rest and during exercise, respectively. The glycerol bolus injection contained 72 gm glycerol-$d_5$ in 15 mL 0.9% saline. The exact infusion rate in each experiment was determined by measuring the concentrations in the infusates. After 65 min of infusion at rest, treadmill walking was initiated, and the isotope administration was doubled for both palmitate and glycerol to minimize changes in substrate isotope enrichment. The infusion was terminated after 50 min of walking.

*Blood sampling.* Blood was taken at 50, 55, 60, and 65 min after the beginning of the infusion to measure resting kinetics. During walking at 60% of the maximal oxygen consumption ($VO_2$max), blood was taken after 5, 15, 25, 35, 40, 45, and 50 min of exercise. All samples were placed in 3-mL vacutainers containing lithium heparin and placed on ice. Plasma was separated by centrifugation shortly after sampling

and subsequently frozen for future analysis of palmitate and glycerol enrichment.

*Sample analysis.* FFA were extracted from plasma using heptane/isopropanol (30:70) and isolated by thin-layer chromatography (20 × 20 cm silica gel GF; Alltech, Deerfield, IL) and derivatized to their methyl esters. Palmitate and total FFA concentrations were measured by gas chromatography (GC)/flame-ionization detection (model 6890; Hewlett-Packard, Fullerton, CA) using pentadecanoic acid as an internal standard. A 100-m SP 2380 capillary column (Supelco, Bellefonte, PA) was used with the following temperature program: initial temperature of 150°C for 0 min, increase 2°C/min up to 170°C and hold for 5 min, increase 4°C/min up to 190°C and hold for 30 min.

GC/mass spectrometry (MS) (Hewlett-Packard 6890) was used for analysis of isotopic enrichments of palmitate. A 20-m fused DB-1 silica column (Fisher Scientific, Pittsburgh, PA) was used with electron ionization and the following temperature program: initial temperature of 70°C for 0 min, increase 25°C/min up to 250°C, hold for 2.8 min. Ions of *m/z* 270 (M + 0) and 271 (M + 1) were selectively monitored. Plasma samples were analyzed for glycerol concentration and enrichment by Metabolic Solutions, Inc. (Nashua, NH). A known amount of [2-$^{13}$C]glycerol was added as an internal standard to the samples analyzed for glycerol concentration. The trimethylsilyl derivative of glycerol was formed according to Beylot *et al.* (18) and analyzed in a Hewlett-Packard 5890 gas chromatograph coupled to a 5898A mass spectrometer using electron ionization and the following temperature program: initial temperature of 100°C for 0 min, increase at 5°C/min up to 120°C, then 30°C/min up to 300°C. Ions of *m/z* 205 (M + 0), 206 (M + 1, internal standard) and 208 (M + 5) were selectively monitored. Glycerol analysis was not performed on the plasma samples taken at 50 min of resting or at 5, 15, and 25 min of walking.

*Calculations.* Fat oxidation was calculated from oxygen consumption ($VO_2$), carbon dioxide production ($VCO_2$), and nitrogen excretion using the equation of Frayn (19). Nitrogen excretion rate was assumed to be 0.01 g/min. This estimate was based on the assumption that subjects were in nitrogen balance and included a value for dietary nitrogen intake adjusted for typical fecal and insensible nitrogen loss.

The palmitate Ra and glycerol Ra were calculated by the dilution technique (20). The Ra (μmol/kg/min) is equal to the isotope infusion rate (μmol/kg/min) divided by the molar excess followed by subtraction of the isotope infusion rate. FFA Ra was calculated by dividing the palmitate Ra by the fractional contribution of palmitate to the total FFA concentration, as determined by GC.

Triglyceride-fatty acid cycling was calculated using the equation of Wolfe *et al.* (21). The difference between three times the glycerol Ra (total fatty acids released) and the rate of total fatty acid oxidation (determined by indirect calorimetry) will give the apparent rate of re-esterification, since re-esterification is the only other fate of fatty acids released by lipolysis. Re-esterification is described as apparent because re-esterification may be underestimated due to intracellular FA oxidation.

*Statistical methods.* Values are reported as means ± SEM. The probability level for significance was set at $P < 0.05$. All statistical analyses were performed using the Statistical Analysis System (Version 6.12, SAS Institute Inc., Cary, NC), using one-way analysis of variance to estimate the effect of time (baseline vs. intervention) on the outcome variables.

## RESULTS

*Substrate concentrations.* The average plasma FFA concentrations during rest were not significantly different after 4 wk of CLA supplementation compared to baseline (0.23 ± 0.01 and 0.25 ± 0.01 mmol/L, respectively; Fig. 1). During exercise, plasma FFA concentrations increased relative to resting values. The average plasma FFA concentration during exercise was not significantly different after 4 wk of CLA supplementation (0.29 ± 0.03 mmol/L) from the baseline (0.35 ± 0.04 mmol/L). Four weeks of CLA supplementation had no significant effect on glycerol concentrations at rest (0.04 ± 0.01 and 0.04 ± 0.01 mmol/L, respectively) or during exercise (0.24 ± 0.03 and 0.21 ± 0.03 mmol/L; Fig. 1).

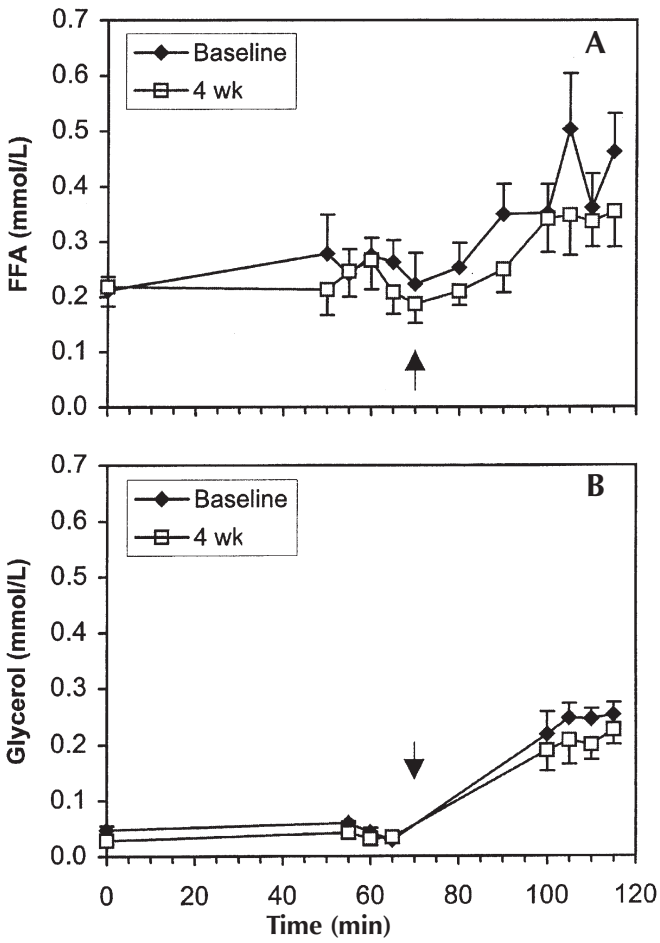


**FIG. 1.** Plasma free fatty acids (FFA) (A) and glycerol (B) concentrations at baseline and week 4. The arrow indicates the onset of treadmill walking at 60% maximal oxygen consumption ($VO_2$max). No significant differences were detected between baseline and week 4 in the average resting or average walking values for either FFA or glycerol concentrations. Error bars represent SEM ($n$ = 6).

*Rate of appearance.* After 4 k of CLA supplementation, the resting FFA Ra was unchanged compared to baseline (2.72 ± 0.06 and 2.74 ± 0.11 μmol/kg/min, respectively; Fig. 2). With exercise, the FFA Ra increased twofold over resting values; however, there was no significant difference between baseline and week 4 values (7.11 ± 0.40 and 5.89 ± 0.29 μmol/kg/min, respectively). The glycerol Ra was similar at baseline and after 4 wk of CLA supplementation during rest (1.87 ± 0.21 and 2.00 ± 0.39 μmol/kg/min, respectively) and during exercise (7.12 ± 0.74 and 6.40 ± 0.99 μmol/kg/min; Fig. 2).

*Triglyceride-fatty acid cycling.* Four weeks of CLA supplementation had no significant effect on apparent fatty acid re-esterification rates at rest or during exercise (Fig. 3). With the onset of exercise, the rate of re-esterification declined at both timepoints.

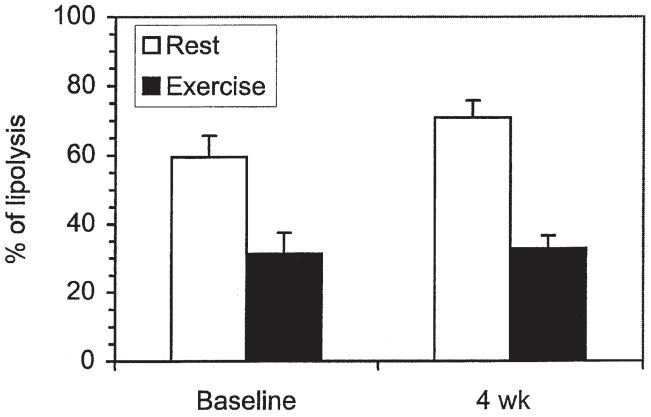


**FIG. 3.** Apparent re-esterification rates as a percentage of lipolysis at baseline and week 4. Error bars represent SEM ($n$ = 6).

## DISCUSSION

Four weeks of daily CLA supplementation in six healthy, adult women had no significant effect on lipolytic rates (glycerol Ra), FFA release from adipose tissue (FFA Ra), or apparent FFA re-esterification rates under conditions of rest or during exercise at 60% $VO_2$max. This is the first report of fatty acid and glycerol kinetics in CLA-supplemented subjects. Earlier work with adipocytes suggested that CLA reduced body fat by increasing lipolysis and decreasing fat deposition (8,10). When 3T3-L1 adipocytes were exposed to exogenous CLA, decreased levels of esterified and free glycerol were observed inside the cell while glycerol release from the cell was increased up to twofold. These data are in contrast with the present data in which the glycerol Ra was similar during the baseline and after 4 wk of CLA supplementation.

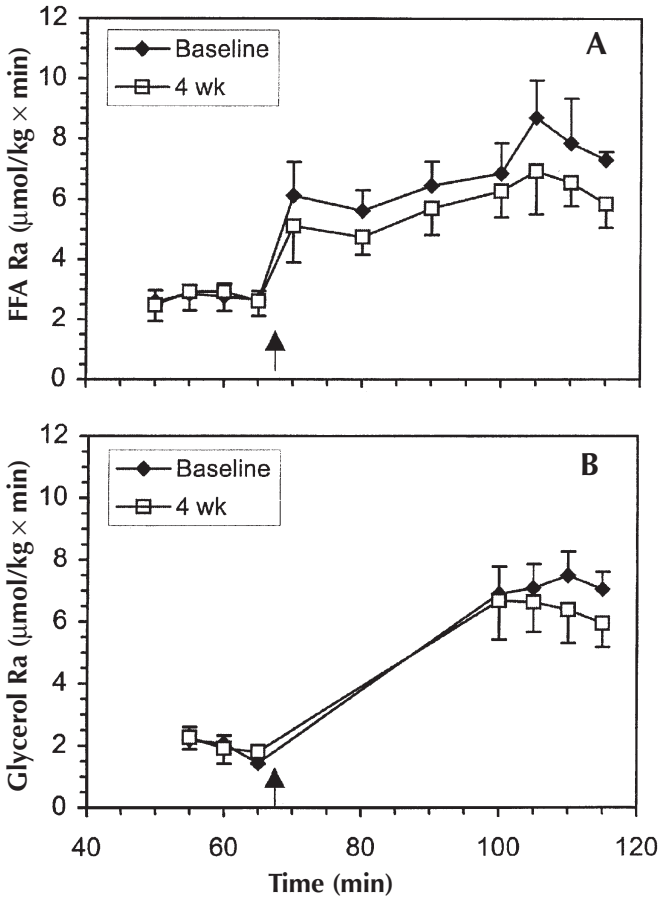


**FIG. 2.** FFA rate of appearance (Ra) (A) and glycerol Ra (B) values at baseline and week 4 . The arrow indicates the onset of treadmill walking at 60% $VO_2$max. No significant differences were detected between baseline and week 4 in the average resting or average walking values for either FFA Ra or glycerol Ra. For other abbreviations see Figure 1. Error bars represent SEM ($n$ = 6).

After 6 wk of dietary CLA supplementation in mice, West *et al.* (11) found that CLA appeared to block the normal day–night difference in the respiratory quotient (RQ). In untreated animals, the nighttime RQ was significantly greater than the daytime RQ, whereas in the CLA-supplemented animals there was no significant difference between the day and night values. The authors attributed the decrease in nighttime RQ to the promotion of fat oxidation by CLA *via* an increase in lipolysis and/or a decrease in fat deposition. The current study did not show any effect of CLA on lipolytic rates, but fat deposition (lipoprotein lipase activity) was not measured. Results from our indirect calorimetry studies indicated that the resting and exercising RQ did not decrease and the fat oxidation rates did not increase after 4 wk of CLA supplementation in the six women who participated in the infusion protocol. Similarly, there was no indication of increased fat oxidation after 8 wk of supplementation in all 10 subjects receiving CLA or in the 7 subjects receiving the placebo capsules (13).

Previous work examining the effect of dietary CLA on body composition, energy expenditure, and fat oxidation in mice was performed using weanling or adolescent animals that were still growing. There are data to suggest that CLA may affect the adipocytes of growing animals differently from those of adults (22,23). It is possible that CLA only affects lipid metabolism in young animals and exerts no effect in adults. Unfortunately, no studies are currently available reporting the effect of dietary CLA on fatty acid and glycerol kinetics in an adult animal model.

CLA supplementation also had no significant effect on the release of FFA from adipose tissue at rest or during exercise. The FFA Ra is not a good indicator of lipolysis since FFA can be re-esterified into triglycerides without ever leaving the adipocyte. FFA that are released into the plasma also may be re-esterified in the liver. Any FFA released by lipolysis that is not oxidized will be re-esterified in either the liver or the adipose tissue. Four weeks of CLA supplementation had no effect on apparent FFA re-esterification rates under any condition tested. At rest, 60–70% of the released FFA were re-esterified in the CLA group. The increase in fat oxidation associated with the onset of exercise resulted in a decline in the apparent re-esterification rate to approximately 30%. These rates of FFA re-esterification agree well with previous results in males where re-esterification rates were approximately 70% at rest and 25% during the first hour of exercise at 40% $VO_2$max (21). This combination of a decrease in FFA re-esterification and an increase in FFA Ra in response to exercise allows for an increase in plasma FFA for oxidation. However, dietary CLA had no significant effect on the FFA Ra or apparent re-esterification rates during rest or exercise.

It should be noted that the CLA supplement used in the present experiment was not pure and included a number of isomers along with the presumed biologically active form for body composition, energy expenditure and lipolytic changes, *trans*-10, *cis*-12 (9,10). In the present study, the *trans*-10, *cis*-12 isomer was the most abundant isomer, along with the *cis*-11, *trans*-13 (~23% of total CLA isomers for each), in the CLA supplement. However, we failed to show any effect of CLA on fatty acid and glycerol kinetics after 28 d of supplementation or on body composition after 64 d of supplementation in normal-weight women (13). In a recent study (12), 12 wk of CLA supplementation in overweight and obese adults resulted in a significant reduction in body fat mass. Although the authors stated that the CLA supplement contained equal parts of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers, they failed to report what percentage of the total CLA isomers were *trans*-10, *cis*-12. Therefore, it is difficult to compare our data with those of the study mentioned above. In addition, CLA may affect overweight or obese individuals differently from normal-weight humans.

In conclusion, 4 wk of supplemental CLA had no effect on lipolytic rates, FFA release by lipolysis, or apparent FFA re-esterification rates in six healthy, adult women. These findings are consistent with our observation that body fat did not change in response to the CLA supplementation period of 64 d. A higher dose of the *trans*-10, *cis*-12 isomer of CLA may be needed to alter lipid metabolism in humans.

## ACKNOWLEDGMENTS

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# Conjugated Linoleic Acid Supplementation in Humans—Metabolic Effects

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**ABSTRACT:** Supplementation with conjugated linoleic acid (CLA) induces a number of physiological effects in experimental animals, including reduced body fat content, decreased aortic lipid deposition, and improved serum lipid profile. Controlled trials on the effects of CLA in humans have hitherto been scarce. The aim of this study was to evaluate the effects of supplementation with CLA in healthy humans on anthropometric and metabolic variables and on the fatty acid composition of serum lipids and thrombocytes. Fifty-three healthy men and women, aged 23–63 yr, were randomly assigned to supplementation with CLA (4.2 g/d) or the same amount of olive oil during 12 wk in a double-blind fashion. The proportion of body fat decreased (–3.8%, $P < 0.001$) in the CLA-treated group, with a significant difference from the control group ($P = 0.050$). Body weight, body mass index, and sagittal abdominal diameter were unchanged. There were no major differences between the groups in serum lipoproteins, nonesterified fatty acids, plasma insulin, blood glucose, or plasminogen activator inhibitor 1 (PAI-1). In the CLA group the proportions of stearic, docosatetraenoic, and docosapentaenoic acids increased in serum lipids and thrombocytes, while proportions of palmitic, oleic, and dihomo-γ-linolenic acids decreased, causing a decrease of the estimated Δ-6 and Δ-9 and an increase in the Δ-5 desaturase activities. These results suggest that supplementation with CLA may reduce the proportion of body fat in humans and that CLA affects fatty acid metabolism. No effects on body weight, serum lipids, glucose metabolism, or PAI-1 were seen.

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Conjugated linoleic acid (CLA) is the common name of a group of fatty acids found in dairy products and meat from ruminants (1). CLA is an octadecadienoic acid (18:2) with two conjugated double bonds, predominantly found in the 9 and 11 or 10 and 12 positions. Each of the double bonds can be in *cis* or *trans* conformation. Lately, CLA has received considerable attention due to its metabolic and chemoprotective properties in experimental animals. These effects include reduced body fat content, improved serum lipid profile, decreased aortic lipid deposition, enhanced glucose metabolism and inhibited tumorigenesis (2,3). Whereas there are a large number of data from animal experiments, there are hitherto few reports on the effects of CLA in humans with inconclusive results (4–6).

The aim of the present study was to investigate the effects of CLA supplementation in humans under doubly-blinded placebo-controlled conditions and evaluate the effects on anthropometric variables, body composition, serum lipids, and fatty acid composition of serum lipids and thrombocytes. We previously reported the effects of CLA on lipid peroxidation in humans (7), which was also evaluated in this trial.

## MATERIALS AND METHODS

*Subjects.* Fifty-three healthy subjects, 27 men and 26 women, between 23 and 63 yr of age were included and randomly assigned to either a CLA-treated group or a control group before entering the study. Table 1 shows baseline characteristics of the participants. At the baseline there were no statistical differences between the groups with regard to the variables in Table 1. All subjects gave their informed consent, and the study was approved by the Ethical Committee of the Faculty of Medicine at Uppsala University.

*Study design.* During the initial 2 wk all subjects were given control capsules containing olive oil. For the following 12 wk, in a double-blind design, the subjects in the CLA group were given capsules containing 4.2 g/d of CLA while the control group continued taking capsules containing the corresponding amount of the control oil consisting of olive oil. The CLA capsules contained 75.9% CLA with equal amounts of the CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12, respectively, and only minor amounts of other isomers. In addition to CLA, the capsules contained small amounts of oleic acid (18:1n-9) (14.0%), palmitic acid (16:0) (4.4%), stearic acid (18:0) (1.5%), and linoleic acid (18:2n-6) (0.4%). All capsules were provided by Natural Ltd. A/S (Oslo, Norway). The examinations and blood samplings were done in the morning after an overnight fast. The main investigations, on which the calculations were based, were on the first and the last days of the trial. Minor examinations were performed during the fourth and eighth weeks of the trial. The participants were requested not to change their habits regarding diet and physical activity and to abstain from any dietary supplementation with vitamins, minerals, or fatty acids prior to and during the study.

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Abbreviations: Apo(a), apolipoprotein(a); Apo A-I, apolipoprotein A-I; Apo B, apolipoprotein B; BMI, body mass index; CLA, conjugated linoleic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; PAI-1, plasminogen activator inhibitor 1; PPAR, peroxisome proliferator activator receptor; VLDL, very low density lipoprotein.

**TABLE 1**
**Baseline Characteristics of the Participants[a]**

| | Control group ($n$ = 24) | | CLA group ($n$ = 26) | |
|---|---|---|---|---|
| | Mean (SD) | Range | Mean (SD) | Range |
| Sex (men/women) | 10/14 | — | 15/11 | — |
| Age (yr) | 47.6 (10.2) | 27.4–59.9 | 42.8 (13.1) | 23.0–63.4 |
| TG (mmol/L) | 1.3 (0.6) | 0.7–3.4 | 1.4 (0.8) | 0.3–3.2 |
| Serum cholesterol (mmol/L) | 5.9 (1.1) | 3.9–7.4 | 5.4 (1.0) | 3.4–6.9 |
| VLDL cholesterol (mmol/L) | 0.32 (0.24) | 0.06–0.94 | 0.36 (0.25) | 0.04–0.93 |
| LDL cholesterol (mmol/L) | 4.0 (1.1) | 2.0–5.9 | 3.6 (1.0) | 1.6–4.8 |
| HDL cholesterol (mmol/L) | 1.3 (0.3) | 0.8–2.1 | 1.2 (0.3) | 0.9–2.2 |
| VLDL-TG (mmol/L) | 0.66 (0.51) | 0.14–2.34 | 0.85 (0.66) | 0.08–2.51 |
| LDL-TG (mmol/L) | 0.41 (0.14) | 0.18–0.75 | 0.37 (0.11) | 0.16–0.59 |
| HDL-TG (mmol/L) | 0.13 (0.08) | 0.05–0.38 | 0.12 (0.06) | 0.05–0.24 |
| NEFA (mmol/L) | 0.47 (0.21) | 0.19–1.00 | 0.38 (0.16) | 0.13–0.69 |
| LDL/HDL | 3.2 (1.2) | 0.9–5.5 | 3.1 (1.0) | 0.7–4.5 |
| Apo A1 (g/L) | 1.39 (0.24) | 1.11–2.12 | 133 (17) | 108–175 |
| Apo B (g/L) | 0.95 (0.26) | 0.57–1.47 | 92 (26) | 41–133 |
| Apo(a) (U/L) | 404 (366)$^{n=22}$ | 39–1192 | 234 (246) | 31–924 |
| Waist (cm) | 83 (12) | 66–111 | 85 (14)$^{n=24}$ | 64–114 |
| Hip (cm) | 101 (7) | 93–116 | 101 (8)$^{n=24}$ | 89–122 |
| SAD (cm) | 22 (3) | 18–28 | 22 (3)$^{n=24}$ | 18–31 |
| BMI (kg/m$^2$) | 24.5 (4.3) | 19.1–34.5 | 25.5 (3.9) | 19.5–33.5 |
| Weight (kg) | 73.8 (15.5) | 54.0–109.0 | 77.1 (15.1) | 53.0–105.0 |
| Body fat (%) | 29.6 (6.9) | 15.9–46.2 | 29.3 (7.1) | 11.4–46.7 |
| WHR | 0.8 (0.1) | 0.7–1.0 | 0.8 (0.1)$^{n=24}$ | 0.7–1.0 |
| Fasting insulin (mU/L) | 8.3 (14.5) | 2.7–75.4 | 8.4 (8.0) | 2.4–40.0 |
| Fasting blood glucose (mmol/L) | 4.7 (0.5) | 3.7–5.6 | 4.4 (0.8) | 3.1–7.6 |
| SBP (mm Hg) | 122 (12) | 94–146 | 121 (14) | 106–170 |
| DBP (mm Hg) | 72 (10) | 54–92 | 72 (7) | 60–84 |
| PAI-1 (U/mL) | 8.5 (11.5) | 0.1–37.2 | 15.7 (17.2) | 0.1–48.4 |

[a]Apo, apolipoprotein; BMI, body mass index; HDL, high density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; NEFA, nonesterified fatty acids; PAI-1, plasminogen activator inhibitor 1; SAD, sagittal abdominal diameter; SD, standard deviation; TG, triglycerides; VLDL, very low density lipoprotein cholesterol; WHR, waist to hip.

*Dietary assessment.* On three occasions, before start and during the fifth and ninth weeks of the test period, the subjects were asked to perform 3-d weighed dietary records, including two weekdays and one weekend day. Dietary data from the registrations were computerized using the personal computer software MATs (MATs program version 4_03e, Rudans Lättdata, Västerås, Sweden). Calculations were made using the Swedish National Food Administration Food Database 2_97, PC version (Swedish National Food Administration, Uppsala, Sweden). The mean dietary intake at baseline is shown in Table 2. The fat provided by the CLA and control capsules was not included in the calculations of intakes.

*Anthropometric measurements.* Body weight was determined to the nearest kilogram and height to the nearest centimeter, wearing light indoor clothing and no shoes. The body mass index (BMI) was calculated as the body weight in kilograms divided by the square of the height in meters. The waist circumference was measured midway between the lowest rib and the iliac crest and the hip circumference at the widest part of the hip; and from these the waist-to-hip ratio was calculated. The sagittal abdominal diameter was measured as the height of the stomach when lying on the back on a firm bed with the knees bent. The percentage body fat was calculated from the three-compartment model based on measurement of skin fold thickness, measured with Harpenden skin fold calipers (John Bull, British Indicators Ltd., St Albans, Great Britain), and body water volume was estimated by a multifrequency bioelectric impedance analyzer Hydra 4200 (Xitron Technologies Inc., San Diego, CA) as described by Forslund *et al.* (8).

*Fatty acid composition of serum lipids.* The extraction, separation, and methylation of the plasma lipids were performed as described in detail by Boberg *et al.* (9). In short, plasma lipids were extracted with chloroform. Butylated hydroxytoluene and $NaH_2PO_4$ were added prior to evaporation under nitrogen. Phospholipids, triglycerides, and cholesterol esters were separated by thin-layer chromatography. The lipids esters were transmethylated with methanol and $H_2SO_4$. The fatty acid methyl esters were separated with gas chromatography using a Hewlett-Packard GC system (Avondale, PA) consisting of an HP 5890 Series II GC apparatus, HP 7673 automatic sampler, HP 3365A Series II Chemstation integrator software, and a 50 m × 0.25 mm CP-Sil 88 Chrompack capillary column, with helium as carrying gas. Standards from Nu-Chek-Prep (Elysian, MN) were used for identification of the individual fatty acids and as a control of the GC system. The technique used is not optimal for resolving different CLA isomers. Thus, the peaks identified as CLA when using a reference standard (Sigma Chemical, St. Louis, MO) were added, and the sum of the total CLA is presented here as CLA. The proportions of fatty acids are given as the relative percentage of the sum of the fatty acids analyzed. The desaturase

**TABLE 2**
**Dietary Intake Before Start and During the Fifth and Ninth Weeks, $n = 50$[a]**

| | | Control group ($n$ = 24) | | CLA group ($n$ = 26) | |
|---|---|---|---|---|---|
| | Week | Mean (SD) | Range | Mean (SD) | Range |
| Energy (kJ) | 0 | 10,110 (2115) | 5,430–14000 | 9,710 (1810) | 6,860–13360 |
| | 5 | 10,270 (2153) | 6,250–15240 | 9,990 (2400) | 5,910–15990 |
| | 9 | 9,480 (1609) | 5,580–12030 | 9,870 (2240) | 5,300–15470 |
| Energy (kCal) | 0 | 2,420 (506) | 1,300–3350 | 2,320 (430) | 1,640–3200 |
| | 5 | 2,460 (515) | 1,500–3650 | 2,390 (580) | 1,410–3830 |
| | 9 | 2,270 (390) | 1,340–2880 | 2,360 (540) | 1,270–3700 |
| Fat (g) | 0 | 87 (27) | 48–153 | 81 (27) | 39–134 |
| | 5 | 88 (24) | 50–160 | 89 (32) | 28–161 |
| | 9 | 81 (17) | 38–115 | 87 (28) | 26–150 |
| Carbohydrate (g) | 0 | 299 (70) | 137–435 | 282 (44) | 183–358 |
| | 5 | 303 (82) | 176–555 | 282 (55) | 180–407 |
| | 9 | 277 (63) | 144–378 | 271 (56) | 173–412 |
| Protein (g) | 0 | 93 (21) | 48–137 | 88 (17) | 58–114 |
| | 5 | 91 (20) | 59–148 | 88 (20) | 46–124 |
| | 9 | 89 (16) | 52–116 | 86 (21) | 41–123 |
| Fiber (g) | 0 | 25 (6) | 13–37 | 25 (9) | 10–44 |
| | 5 | 24 (6) | 16–36 | 24 (9) | 9–44 |
| | 9 | 23 (7) | 10–33 | 22 (8) | 11–41 |
| Alcohol (g) | 0 | 9 (10) | 0–27 | 15 (15) | 0–47 |
| | 5 | 11 (13) | 0–37 | 15 (19) | 0–83 |
| | 9 | 9 (10) | 0–30 | 20 (20) | 0–84 |
| Fat (E%) | 0 | 31 (5) | 22–43 | 30 (6) | 18–45 |
| | 5 | 31 (4) | 21–43 | 32 (6) | 18–41 |
| | 9 | 31 (4) | 25–39 | 32 (6) | 18–42 |
| Carbohydrate (E%) | 0 | 50 (7) | 36–63 | 50 (7) | 39–67 |
| | 5 | 50 (6) | 37–62 | 49 (8) | 36–68 |
| | 9 | 50 (6) | 32–58 | 48 (8) | 32–68 |
| Protein (E%) | 0 | 16 (3) | 12–24 | 16 (2) | 11–20 |
| | 5 | 15 (3) | 10–20 | 15 (2) | 11–18 |
| | 9 | 16 (3) | 11–23 | 15 (2) | 10–19 |
| Saturated fat (E%) | 0 | 13 (3) | 9–20 | 12 (3) | 4–20 |
| | 5 | 13 (3) | 7–21 | 13 (4) | 3–19 |
| | 9 | 13 (2) | 9–19 | 13 (3) | 4–18 |
| Monounsaturated fat (E%) | 0 | 12 (2) | 8–17 | 11 (2) | 8–17 |
| | 5 | 11 (2) | 8–16 | 12 (2) | 7–17 |
| | 9 | 12 (2) | 9–15 | 12 (2) | 6–17 |
| Polyunsaturated fat (E%) | 0 | 4 (1) | 3–9 | 4 (1) | 3–7 |
| | 5 | 5 (2) | 3–11 | 5 (1) | 3–10 |
| | 9 | 5 (1) | 3–9 | 5 (1) | 3–9 |

[a]Assessed using a 3-d weighed dietary record. SD, standard deviation; E%, energy percentage; CLA, conjugated linoleic acid.

activities were assessed by calculating product to precursor ratios as follows: 20:4n-6/20:3n-6 for Δ-5 desaturase, 18:3n-6/18:2n-6 for Δ-6 desaturase in triglycerides and cholesterol esters, 20:3n-6/18:2n-6 for Δ-6 desaturase in phospholipids and thrombocytes, and 18:1n-9/18:0 for Δ-9 desaturase activities.

*Fatty acid composition of the thrombocytes.* Blood was drawn using minimal venous pressure. A $16 \times g$ butterfly cannula was used to collect blood drop by drop. Thrombocyte clot was received through gentle centrifugation $120 \times g$ for 20 min, washed in saline, and dissolved with a Polytron mixer. From the dissolved clot the total platelet lipids were extracted with chloroform/methanol, including butylated hydroxytoluene. The fatty acid composition was analyzed as in the serum lipids.

*Serum lipoprotein analyses.* Serum lipoproteins (very low density lipoprotein, VLDL; low density lipoprotein, LDL; and high density lipoprotein, HDL) were isolated by a combination of preparative ultracentrifugation (10) and precipitation with a sodium phosphotungstate and magnesium chloride solution (11). Triglyceride and cholesterol concentrations in serum and in the isolated lipoprotein fractions were measured by enzymatic methods in a Monarch 2000 centrifugal analyzer. The concentrations of apolipoprotein A-I (Apo A-I) and apolipoprotein B (Apo B) were measured by immunoturbidimetry in a Monarch apparatus. Apolipoprotein(a) [Apo(a)] was determined by a Coda Automated EIA automatic enzyme-linked immunosorbent assay analyzer (Bio-Rad Laboratories, Hercules, CA) using Mercodia Apo(a) reagents (Mercodia AB, Uppsala, Sweden). Serum free fatty acids were measured by an enzymatic colorimetric method (Wako Chemicals, Neuss, Germany).

*Glucose and insulin.* Blood glucose was measured by the glucose dehydrogenase method (Gluc-DH; Merck,

Darmstadt, Germany). Plasma insulin was analyzed using an enzymatic-immunological assay (Enzymmun; Boehringer Mannheim) in an ES300 automatic analyzer (Boehringer Mannheim, Germany).

*Plasminogen activator inhibitor-1 (PAI-1) activity.* The amount of active PAI-1 was analyzed in citrate plasma using a commercially available bioimmunoassay (Chromolize PAI-1 kits; Biopool AB, Umeå, Sweden).

*Statistical analyses*. Two groups of subjects including 25 persons each was calculated to give a power of 80% to detect a difference in serum or LDL cholesterol of 9% if $P < 0.05$. For analyses of differences between the changes in the two groups, unpaired *t*-tests were used. Changes within each group were analyzed using paired *t*-tests. Variables with a skewed distribution ($W < 0.95$ in Shapiro-Wilk's *W* test for normality) were logarithmically transformed prior to the *t*-test. Variables that were not normally distributed after logarithmic transformation were analyzed using Wilcoxon Mann-Whitney two-sample test. Results with a *P*-value less than or equal to 0.05 were considered as significant. Percent change was calculated as [(mean value after − mean value before)/mean value before] × 100. The main statistical analyses were performed according to protocol, i.e., excluding participants with a low compliance (who had taken less than 80% of the prescribed number of capsules). Additional analyses were also made according to intention to treat, i.e. all subjects were included irrespective of their degree of compliance. Results from the latter analyses are shown in the text when differing from analyses according to the protocol. The statistical analyses were performed using the software systems Statistical Analysis System and STATA (Stata Corporation, College Station, TX).

## RESULTS

*Compliance and tolerance.* All participants fulfilled the trial. The compliance, counted as percentage of eaten capsules out of those prescribed, was more than 91% in 46 of the participants, between 81 and 90% in three, and 80% or less of the capsules in three of the participants. The participants with a compliance of 80% or less were excluded from the main statistical analyses. The capsules were well tolerated, and only a few subjects reported mild diarrhea at some occasions. No effect on serum levels of the liver enzymes aspartate amino transferase and alanine amino transferase was found. Fifty out of the 53 subjects completed the three 3-d weighed dietary registrations. The participants did not statistically change their dietary intake during the study with regard to energy, fat, carbohydrates, or protein (data not shown).

*Anthropometry.* As shown in Table 3 there was no significant change in body weight, BMI, waist-to-hip ratio, and sagittal abdominal diameter. The proportion of body fat was reduced by 3.8% in the CLA group, $P = 0.0006$ ($P = 0.05$ for difference between the groups) (Table 3). When analyzing according to intention to treat (including all participants), reduction of body fat in the CLA group was 3.7%, $P < 0.001$ ($P = 0.07$ for difference between the groups). There was no relation between change in body fat and the proportion of body fat at start (data not shown).

**TABLE 3**
**Change of Anthropometric Variables[a]**

| | Absolute change (range) | Percentage change[b] | *P* for difference within group | *P* for difference between groups |
|---|---|---|---|---|
| Weight (kg) | | | | |
| Control | 0.21 (–2–3) | 0.28 | 0.487 | 0.664 |
| CLA | 0.4 (–6–4) | 0.55 | 0.282 | |
| BMI ($kg/m_2$)[c] | | | | |
| Control | 0.06 (–0.7–1.1) | 0.25 | 0.409 | 0.655 |
| CLA | 0.14 (–1.9–1.5) | 0.53 | 0.181 | |
| WHR[(n=48)] | | | | |
| Control | 0.0 (–0.1–0.1) | 1.04 | 0.311 | 0.560 |
| CLA | 0.0 (–0.1–0.1) | 0.23 | 0.811 | |
| SAD (cm)[d(n=48)] | | | | |
| Control | 0.1 (–2.5–2.0) | 0.57 | 0.493 | 0.423 |
| CLA | 0.0 (–2.0–2.5) | –0.19 | 0.674 | |
| Body fat (%) | | | | |
| Control | –0.4 (–3.8–1.9) | –1.23 | 0.150 | 0.050 |
| CLA | –1.1 (–3.9–1.7) | –3.84 | 0.0006 | |

[a]$n = 50$ unless otherwise stated. BMI, body mass index; SAD, sagittal abdominal diameter; WHR, waist-to-hip ratio.
[b]Percentage change is calculated as: [(value after – value before)/value before] × 100, using mean values.
[c]Logarithmically transformed prior to *t*-test.
[d]Analyzed using Wilcoxon Mann-Whitney two-sample test.

*Serum lipids and apolipoproteins.* As shown in Table 4, an increase in Apo B was observed in the CLA-treated group ($P = 0.009$) with a significant difference between the two groups ($P = 0.044$). Within the control group we observed a decreased LDL triglyceride concentration ($P = 0.033$) and a significant difference between the groups ($P = 0.039$). When including all participants in the statistical analyses, there were no significant differences between the changes in serum lipid and apolipoprotein concentrations in the two groups.

*Plasma variables.* There was a borderline significant difference between the changes of fasting blood glucose in the groups ($P = 0.053$), as shown in Table 4. When including all participants, there were no significant differences between the two groups with regard to changes in fasting glucose, insulin, nonesterified fatty acids, or PAI-1.

*Fatty acid composition of serum lipids and thrombocytes.* In the serum phospholipids the proportion of stearic acid (18:0), docosatetraenoic acid (22:4n-6), and docosapentaenoic acid (22:5n-3) increased, and palmitic acid (16:0), oleic acid (18:1n-9), γ-linolenic acid (18:3n-6) ($P = 0.065$), and dihomo-γ-linolenic acid (20:3n-6) decreased in the CLA group, as compared to the control group (Table 5). The fatty acid composition in the thrombocytes changed with an increase of docosapentaenoic acid (22:5n-3) and a decrease of oleic acid (18:1n-9) and dihomo-γ-linolenic acid (20:3n-6) as shown in Table 5. The changes of fatty acid composition of serum cholesterol esters and triglycerides were similar to the changes of the serum phospholipids (data not shown). The changed proportions of the fatty acid composition in the serum lipids correspond to an increase in the estimated Δ-5 desaturase activity (20:4n-6/20:3n-6) in the CLA group as com-

**TABLE 4**
**Changes of Serum Lipids, Blood Glucose, Plasma Insulin, and PAI-1[a]**

| | Absolute change (range) | Percentage change[b] | *P* for difference within group | *P* for difference between groups |
|---|---|---|---|---|
| Total cholesterol (mmol/L) | | | | |
| Control | 0.17 | 2.82 | 0.197 | 0.432 |
| CLA | 0.29 | 5.35 | 0.006 | |
| HDL cholesterol[c] (mmol/L) | | | | |
| Control | 0.16 | 12.1 | <0.0001 | 0.204 |
| CLA | 0.09 | 7.35 | 0.004 | |
| LDL cholesterol (mmol/L) | | | | |
| Control | 0.09 | 2.25 | 0.386 | 0.415 |
| CLA | 0.20 | 5.46 | 0.022 | |
| LDL/HDL | | | | |
| Control | –0.30 | –9.25 | 0.018 | 0.168 |
| CLA | –0.10 | –3.40 | 0.193 | |
| TG[c] (mmol/L) | | | | |
| Control | –0.23 | –17.8 | 0.006 | 0.445 |
| CLA | –0.07 | –4.82 | 0.184 | |
| HDL-TG[c] (mmol/L) | | | | |
| Control | –0.02 | –16.1 | 0.186 | 0.869 |
| CLA | –0.01 | –7.69 | 0.401 | |
| LDL-TG (mmol/L) | | | | |
| Control | -0.04 | -8.84 | 0.033 | 0.039 |
| CLA | 0.01 | 2.06 | 0.566 | |
| VLDL cholesterol[c] (mmol/L) | | | | |
| Control | 0.01 | 1.19 | <0.0001 | 0.577 |
| CLA | 0.11 | 13.0 | <0.0001 | |
| VLDL-TG[c] (mmol/L) | | | | |
| Control | –0.13 | –20.0 | 0.224 | 0.785 |
| CLA | –0.01 | –0.45 | 0.479 | |
| NEFA[c] (mmol/L) | | | | |
| Control | 0.02 | 3.40 | 0.926 | 0.941 |
| CLA | 0.01 | 3.02 | 0.980 | |
| Apo A1[d] (g/L) | | | | |
| Control | –0.62 | –0.45 | 0.303 | 0.472 |
| CLA | –2.88 | –2.16 | 0.109 | |
| Apo B (g/L) | | | | |
| Control | –1.0 | –1.06 | 0.702 | 0.044 |
| CLA | 5.77 | 6.24 | 0.009 | |
| Apo(a)[d] (U/L)[(n=48)] | | | | |
| Control | 16.9 | 4.20 | 0.833 | 0.482 |
| CLA | 12.8 | 5.47 | 0.208 | |
| Blood glucose[c] (mmol/L)[(n=49)] | | | | |
| Control | –0.06 | –1.30 | 0.159 | 0.053 |
| CLA | 0.11 | 2.45 | 0.174 | |
| Plasma insulin[d] (mU/L) | | | | |
| Control | –2.30 | –27.81 | 0.875 | 0.600 |
| CLA | 1.16 | –13.7 | 0.424 | |
| PAI-1[c] (U/mL)[n=50] | | | | |
| Control | 3.69 | 43.2 | 0.184 | 0.575 |
| CLA | 5.08 | 32.4 | 0.385 | |

[a]*n* = 50 unless otherwise stated. For abbreviations see Table 1.
[b]Percentage change is calculated as: [(value after – value before)/value before] × 100, using mean values.
[c]Logarithmically transformed prior to *t*-test.
[d]Analyzed using Wilcoxon Mann-Whitney two-sample test.

pared to the control group, whereas the estimated activities of Δ-6 desaturase (18:3n-6/18:2n-6 in triglycerides and cholesterol esters and 20:3n-6/18:2n-6 in phospholipids) and Δ-9 desaturase (18:1n-9/18:0) decreased as compared to the control group (data not shown). Also in the thrombocytes, there was an increase in Δ-5 (20:4n-6/20:3n-6) and decrease in Δ-6 (20:3n-6/18:2n-6) estimated activities in the CLA group as compared to the control group while Δ-9 (18:1n-9/18:0) was unchanged (data not shown). No changes were seen in the activity of Δ-9 desaturase calculated as the ratio between 16:1n-7 and 16:0 in any of the compartments analyzed.

The effects of CLA and control supplementation on any of the parameters analyzed were not significantly different in men and women and were not changed when including mean fat intake in the statistical analyses as a possible confounder (data not shown)

## DISCUSSION

In this study the proportion of body fat in the subjects given CLA decreased significantly within the CLA-treated group by 3.8% with a borderline significant decrease as compared to the control group ($P = 0.050$). BMI, body weight, waist-to-hip ratio, and sagittal abdominal diameter were unchanged (Table 3).

Hitherto, there are two published studies on the effects of CLA on human body composition (4,6). In the study by Zambell *et al*., where healthy normal weight women were fed 3 g of CLA, consisting of minor amounts of several different isomers, no effect on body weight or composition was found (4). Contrastingly, Blankson *et al.* (6) observed a decreased body fat mass in overweight or moderately obese men and women when supplementing with 3.4 or 6.8 g of a CLA preparation with equal proportions of the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers. The diverging results between the two studies mentioned and the present could possibly be due to the different amounts and isomer compositions of the CLA preparation because different isomers and different study designs have been hypothesized to have different effects (12,13). It could also be due to the different study designs. Another interesting possibility is that there might be diverging effects of CLA in obese compared to normal-weight subjects. There could possibly also be gender and/or genetically determined difference. CLA supplementation to animals has been observed to reduce body fat (14). Little work has been done on the mechanisms of action of CLA on energy metabolism and the explanations of the effects are as yet not known. CLA has been suggested to affect the rate of *de novo* lipogenesis and/or the rate of lipolysis. Increased lipolysis and decreased lipoprotein lipase activity have been observed *in vitro* in adipocytes when CLA was added to the medium (14). An increased carnitine palmitoyltranferase activity giving an increased fatty acid oxidation in adipose and skeletal muscle tissue was found in mice after CLA supplementation (14). However, no effects on energy expenditure or fat oxidation was observed after CLA supplementation in healthy women (4).

In the present study, we observed an increase in Apo B and LDL triglycerides in the CLA group when compared to the control group (Table 4). Within the CLA group there were increases in total and LDL cholesterol, although not different from the changes in the control group, as also observed in a recent study of healthy women (15). In another study, reduc-

**TABLE 5**
**Fatty Acid Composition of Serum Phospholipids and Thrombocytes[a]**

| | Serum phospholipids | | | Thrombocytes | | |
|---|---|---|---|---|---|---|
| | Mean at baseline | Change (%)[b] | *P* for difference between groups | Mean at baseline | Change (%) | *P* for difference between groups |
| 14:0 | | | | | | |
| Control | 0.46 | –8.0 | 0.926 | 0.67[c] | –7.1 | 0.885 |
| CLA | 0.43 | –7.8 | | 0.63[c] | –1.9 | |
| 15:0 | | | | | | |
| Control | 0.22 | –5.0 | 0.669 | 0.23[d] | –8.5 | 0.533($n$=44) |
| CLA | 0.20 | –7.4* | | 0.22[d] | –14.7** | |
| 16:0 | | | | | | |
| Control | 31.3 | 0.3 | 0.045 | 23.3 | –0.4 | 0.920 |
| CLA | 31.2 | –1.2* | | 23.5 | –0.6 | |
| 16:1n-7 | | | | | | |
| Control | 0.52[c] | –8.4 | 0.160($n$=46) | 0.42[c] | 7.0 | 0.507($n$=48) |
| CLA | 0.55[c] | –14.8** | | 0.42[c] | 14.3 | |
| 17:0 | | | | | | |
| Control | 0.43[d] | –6.8 | 0.595 | 0.38[c] | –3.9 | 0.829($n$=48) |
| CLA | 0.39[d] | 4.9 | | 0.38[c] | –5.3 | |
| 18:0 | | | | | | |
| Control | 14.2 | –0.3 | <0.0001 | 15.7 | –0.9 | 0.467 |
| CLA | 13.8 | 6.3**** | | 16.2 | –2.5 | |
| 18:1n-9 | | | | | | |
| Control | 12.1 | 1.7 | <0.0001 | 19.1 | 0.7 | 0.015 |
| CLA | 12.8 | –8.9**** | | 19.5 | –3.1* | |
| 18:2n-6 | | | | | | |
| Control | 20.5 | –1.0 | 0.461 | 6.8 | -2.1 | 0.112 |
| CLA | 21.0 | 1.2 | | 6.7 | 3.4 | |
| 18:3n-6 | | | | | | |
| Control | 0.08[c] | –9.3 | 0.065($n$=27) | ND | ND | ND |
| CLA | 0.09[c] | –24.2** | | ND | ND | |
| 18:3n-3 | | | | | | |
| Control | 0.49[c] | 2.2 | 0.778 | ND | ND | ND |
| CLA | 0.55[c] | 0.9 | | ND | ND | |
| 20:3n-6 | | | | | | |
| Control | 2.91 | –1.5 | 0.0001 | 1.45 | 0.7 | <0.0001 |
| CLA | 3.25 | –16.0**** | | 1.64 | –11.5**** | |
| 20:4n-6 | | | | | | |
| Control | 8.26 | –0.8 | 0.645 | 22.1 | –0.6 | 0.441 |
| CLA | 7.95 | –2.1 | | 21.5 | –2.3 | |
| 20:5n-3 | | | | | | |
| Control | 1.87[c] | 2.3 | 0.746 | 1.14[c] | 7.9 | 0.663 |
| CLA | 1.60[c] | 8.3 | | 1.00[c] | 17.8* | |
| 22:4n-6 | | | | | | |
| Control | 0.25 | –3.3 | 0.039 | 2.08 | –6.0* | 0.476 |
| CLA | 0.25 | 6.0* | | 2.08 | –3.5 | |
| 22:5n-3 | | | | | | |
| Control | 1.05 | –1.0 | <0.001 | 1.87 | 1.1 | 0.002 |
| CLA | 0.98 | 16.3**** | | 1.78 | 12.3**** | |
| 22:6n-3 | | | | | | |
| Control | 4.85[c] | 4.4 | 0.983 | 2.65 | 3.9 | 0.143 |
| CLA | 4.31[c] | 6.8 | | 2.48 | 11.3** | |
| CLA | | | | | | |
| Control | 0.07 | 4.4 | <0.0001($n$=32) | ND | ND | ND |
| CLA | 0.07 | 609**** | | ND | | |

[a]$n$ = 49 unless otherwise stated. *$P$ < 0.05 for a change within the group, **$P$ < 0.01 for a change within the group, ***$P$ < 0.001 for a change within the group, ****$P$ < 0.0001 for a change within the group.
[b]Percentage change is calculated as: [(mean value after – mean value before)/mean value before] × 100.
[c]Logarithmically transformed prior to t-test.
[d]Analyzed using Wilcoxon Mann-Whitney two-sample test.

tions of total, HDL, and LDL cholesterol, but no changes in triglycerides and lipoprotein(a), were seen within the treatment groups after CLA treatment (6). CLA has, in studies of experimental animals, been suggested to affect serum lipoprotein concentrations. In a recent study in hamsters, the levels of total, LDL, and HDL cholesterol decreased and triglycerides increased after treatment with a mixture of CLA isomers (16). Other animal studies have shown decreased plasma LDL cholesterol (17), decreased total and non-HDL cholesterol and triglycerides (18), and decreased levels of total cholesterol and triglycerides (19) after CLA supplementation. The diverging results between the present study in humans and earlier studies in experimental animals could be due to species differences, but should be further investigated.

Insulin and nonesterified fatty acid levels were unchanged after CLA supplementation in the present study, while there was a tendency to an increased glucose concentration in the CLA group as compared to the controls (Table 4). In another human study, no changes in insulin or glucose concentrations were observed (5). Belury and coworkers (20), on the other hand, have found that dietary CLA normalizes glucose tolerance and prevents the progression to hyperglycemia and diabetes in diabetic fatty fa/fa Zucker rat, with an effect similar to that of the insulin-sensitizing drug thiazolidindione. The insulin-sensitizing effects are suggested to be mediated *via* activation of peroxisome proliferator activator receptor (PPAR) γ and a subsequent stimulation of adipocyte differentiation. In the same study, they also found a reduction of plasma nonesterified fatty acid concentration after CLA supplementation. This effect was assumed to be caused by an increased β-oxidation, *via* an activation of hepatic PPAR γ.

The PAI-1 activity was not significantly changed in any of the groups. PAI-1 has been found to be elevated in obesity, hypertension, glucose intolerance, insulin resistance, and type 2 diabetes, all included in the metabolic syndrome (21). In the adipose tissue of humans, mice, and rats, relatively high levels of PAI-1 have been detected, with increasing amounts in obese subjects (22). One could anticipate a decreased PAI-1 activity in the CLA supplemented group as body fat was reduced in this group and as PAI-1 levels have been observed to decrease when reducing body fat or body weight (21,23), but no such effect was seen in the present study.

In contrast to our findings (Table 5), no changes in the fatty acid composition of plasma lipids, thrombocytes, or adipose tissue were observed in another study of the effects of CLA in humans (15,24). The authors suggested that this could be due to a low number of participants ($n = 17$), but it could also be due to the lower amounts of several different isomers. In two studies of CLA feeding to rats, different tissues showed decreased proportions of dihomo-γ-linolenic acid (20:3n-6) and increased docosatetraenoic (22:4n-6) and docosapentaenoic acid (22:5n-3) (25,26), as also observed in the present study. However, in the rats the proportions of γ-linolenic (18:3n-6) and arachidonic acid (20:4n-6) decreased, which was not observed in the present study. Decreased proportions of linoleic (27) and arachidonic acid (25,27) were found in mouse liver and rat mammary gland after supplementation with CLA. In the present study the proportions of linoleic acid (18:2n-6) were unchanged, possibly indicating that CLA is not displacing linoleic acid to any larger extent or is not metabolized in the same way, as suggested by Banni *et al.* (25). The decrease in oleic acid (18:1n-9) can be interpreted as a decreased Δ-9 activity, as observed previously after CLA supplementation in experimental animals (28,29). A decrease in the activity of Δ-9 desaturase has been suggested to be due to an inhibitory effect of CLA on the mRNA expression of stearoyl-CoA desaturase, an enzyme catalyzing the Δ-9 desaturation (28,29). The estimated Δ-5 desaturase activity increased in the CLA group compared to the control group. The Δ-5 desaturase activity in humans has been suggested to be inversely related to the proportion of body fat (30), insulin levels (31), and risk for myocardial infarction (32). The Δ-6 desaturase activity (18:3n-6/18:2n-6) decreased after CLA treatment, as observed earlier in *in vitro* rat liver microsomes (29). The increased proportion of docosapentaenoic acid (22:5n-3) and the unchanged docosahexaenoic acid (22:6n-3) supports the indications of decreased Δ-6 desaturase activity.

Although the method used here was not optimal for analysis of the different CLA isomers, the proportions of total CLA increased in serum phospholipids (Table 5), cholesterol esters, and triglycerides (data not shown) in the CLA-treated group as compared to the control group. This supports earlier findings in humans (15) and animals (25). The fact that the proportions of CLA increased only in the CLA-treated group could be regarded as a verification of compliance.

The altered fatty acid profile of the serum lipids and thrombocytes, especially the decreased proportions of dihomo-γ-linolenic acid (20:3n-6) and increased activity of Δ-5 desaturase may lead to an altered eicosanoid metabolism. We have elsewhere reported on increased urinary levels of 8-iso-prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 15-keto-dihydro-$PGF_{2\alpha}$ in humans after CLA supplementation (7). In some contrast to this Kavanaugh *et al.* (33) reported reduced $PGE_2$ levels in CLA-treated mice.

The intake of CLA in 123 Swedish men and women was estimated to be on average 160 mg/d (*cis*-9,*trans*-11) (34). CLA intakes in German men and women was estimated to 430 and 350 mg/d (*cis*-9,*trans*-11), respectively (35), and in 12 American men and women CLA intake was 127 mg/d (*cis*-9,*trans*-11) (36).

Of the known isomers of CLA, *cis*-9,*trans*-11 is the most abundant in natural food products (1). The two isomers considered most biologically active are *cis*-9,*trans*-11 and *trans*-10,*cis*-12. For example, *trans*-10,*cis*-12 has been suggested to be responsible for body composition changes in mice (37) and affect lipid metabolism in hamsters (16), and it has been observed to reduce milk fat synthesis in dairy cows (38). In the present study we used a mix of equal proportions of *cis*-9,*trans*-11 and *trans*-10,*cis*-12. It would be interesting to investigate the metabolic effects of the individual isomers.

The participants in the present study consisted of a rather heterogeneous group of healthy, nonobese, normolipidemic

men and women aged 23 to 63 yr. It is possible that the moderate changes seen after supplementation with CLA might be due to the heterogeneity of the group and to difficulties in changing physiological parameters within the normal range. The magnitude of a possible effect of CLA on, for example, body composition was unknown when designing the study. Thus the number of participants needed was estimated by a calculation of statistical power based on a change of serum lipid concentrations comparable to what has been seen when changing the fatty acid quality of the diet. Possibly, a larger number of participants is needed to detect changes in nonobese subjects. It would be interesting to study the effects of CLA supplementation in subjects with, for example, hyperlipidemia or abdominal obesity. An interesting view of this study is that the participants were weight-stable adult humans, as opposed to several studies where CLA has been given to growing animals. Judging from the effect on body composition in the present study and from the effects observed in growing animals, it could be hypothesized that CLA may be useful in inhibition of weight gain rather than in weight reduction *per se*.

These results indicate that supplementation with CLA for a limited period of time may cause reduction of the proportions of body fat and alter the fatty acid metabolism in healthy humans. However, CLA appeared to have no major effects on body weight, serum lipids, glucose metabolism, or PAI-1 in this group of subjects, in apparent contrast to results of earlier studies in animals. Whether the low concentrations of CLA naturally occurring in milk fat could have metabolic effects when eaten habitually for a long time still remains to be investigated.

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# Serum Cholesterol Predictive Equations with Special Emphasis on *Trans* and Saturated Fatty Acids. An Analysis from Designed Controlled Studies

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**ABSTRACT:** The effects of dietary *trans* fatty acids on serum total and low density lipoprotein (LDL) cholesterol have been evaluated by incorporating *trans* fatty acids into predictive equations and comparing their effects with the effects of the individual saturated fatty acids 12:0, 14:0, and 16:0. *Trans* fatty acids from partially hydrogenated soybean oil (*TRANS* V) and fish oil (*TRANS* F) were included in previously published equations by constrained regression analysis, allowing slight adjustments of existing coefficients. Prior knowledge about the signs and ordering of the regression coefficients was explicitly incorporated into the regression modeling by adding lower and upper bounds to the coefficients. The amounts of oleic acid (18:1) and polyunsaturated fatty acids (18:2, 18:3) were not sufficiently varied in the studies, and the respective regression coefficients were therefore set equal to those found by Yu *et al.* [Yu, S., Derr, J., Etherton, T.D., and Kris-Etherton, P.M. (1995) Plasma Cholesterol-Predictive Equations Demonstrate That Stearic Acid Is Neutral and Monounsaturated Fatty Acids Are Hypocholesterolemic, *Am. J. Clin. Nutr. 61*, 1129–1139]. Stearic acid (18:0), considered to be neutral, was not included in the equations. The regression analyses were based on results from four controlled dietary studies with a total of 95 participants and including 10 diets differing in fatty acid composition and with 30–38% of energy (E%) as fat. The analyses resulted in the following equations, where the change in cholesterol is expressed in mmol/L and the change in intake of fatty acids is expressed in E%: Δ Total cholesterol = 0.01 Δ(12:0) + 0.12 Δ(14:0) + 0.057 Δ(16:0) + 0.039 Δ(*TRANS* F) + 0.031 Δ(*TRANS* V) – 0.0044 Δ(18:1) – 0.017 Δ(18:2,18:3) and ΔLDL cholesterol = 0.01 Δ(12:0) + 0.071 Δ(14:0) + 0.047 Δ(16:0) + 0.043 Δ(*TRANS* F) + 0.025 Δ(*TRANS* V) – 0.0044 Δ(18:1) – 0.017 Δ(18:2,18:3). The regression analyses confirm previous findings that 14:0 is the most hypercholesterolemic fatty acid and indicate that *trans* fatty acids are less hypercholesterolemic than the saturated fatty acids 14:0 and 16:0. *TRANS* F may be slightly more hypercholesterolemic than *TRANS* V or there may be other hypercholesterolemic fatty acids in partially hydrogenated fish oil than those included in the equations. The test set used for validation consisted of 22 data points from seven recently published dietary studies. The equation for total cholesterol showed good prediction ability with a correlation coefficient of 0.981 between observed and predicted values. The equation has been used by the Norwegian food industry in reformulating margarines into more healthful products with reduced content of cholesterol-raising fatty acids.

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The effects of dietary fatty acids on serum cholesterol are an important criterion when nutritional properties of fat products are evaluated. Since the 1950s, several equations have been

**TABLE 1**
**Some Published Predictive Equations for Estimating Changes in Plasma Cholesterol (TC) and Low-Density Lipoprotein Cholesterol (LDL-C) (mmol/L)[a] in Response to Changes in Dietary Fatty Acids (in percent of energy)**

Keys *et al.* (2)
ΔTC = 0.0621ΔS – 0.0310ΔP[a]

Hegsted *et al.* (6)
ΔTC = 0.0543ΔS – 0.03115ΔP – 0.00318ΔM[b]
ΔLDL-C = 0.0449ΔS – 0.0198ΔP

Mensink and Katan (7)
ΔTC = 0.0556[c]ΔS – 0.0031ΔM – 0.015ΔP
ΔLDL-C = 0.033ΔS – 0.006ΔM[b] – 0.014ΔP

Yu *et al.* (8)
ΔTC = 0.0522Δ(12:0–16:0) – 0.0008Δ18:0 – 0.0124ΔM – 0.0248ΔP
ΔLDL-C = 0.0378Δ(12:0-16:0) + 0.0018Δ18:0 – 0.0178ΔMUFA – 0.0248ΔPUFA
ΔTC = 0.0248Δ12:0 + 0.1443Δ14:0 +0.0277Δ16:0 + 0.01442Δ18:0 – 0.0044ΔMUFA – 0.017ΔPUFA

Clarke *et al.* (9)
ΔTC= 0.052 ΔS – 0.026 ΔP + 0.005 ΔM
ΔLDL-C = 0.036 ΔS – 0.022P – 0.008 ΔM

Howell *et al.* (10)
ΔTC = 0.0496ΔS – 0.0233ΔP
ΔLDL-C = 0.0468ΔS – 0.0128ΔP

[a]To convert mmol/L into mg/dL, multiply the regression coefficients by 38.67.
[b]ΔM is not statistically significant.
[c]0.0556ΔMS assumed that 18:0 has the same effect as carbohydrates; otherwise a coefficient for total saturated fatty acids of 0.039 mmol% of energy. S = saturated fatty acids, including 12:0, 14:0, and 16:0; M = MUFA = monounsaturated fatty acid; P = PUFA = polyunsaturated fatty acid.

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Abbreviations: COCO, coconut oil; E%, energy percentage; HDL, high density lipoprotein; LDL, low density lipoprotein; MUFA, monounsaturated fatty acids; PALM, palm oil; PHFO, partially hydrogenated fish oil; PHSO, partially hydrogenated soybean oil; PUFA, polyunsaturated fatty acids; RMSEP, root mean square error of prediction; SAFA, saturated fatty acids; *TRANS*, *trans* fatty acids; *TRANS F*, *trans* fatty acids from PHFO; *TRANS V*, *trans* fatty acids from PHSO.

published that predict the effects of saturated fatty acids (SAFA), polyunsaturated fatty acids (PUFA), and monounsaturated fatty acids (MUFA) on serum total and low density lipoproteins (LDL) cholesterol levels in groups of individuals (1–10). The equations are fairly similar (Table 1) and indicate that SAFA increase serum cholesterol levels whereas PUFA have a lowering effect.

It should be noted that most of these equations predict changes in response to groups of fatty acids. Furthermore, they do not include *trans* fatty acids. To be of practical use to predict changes in response to commercial fat blends it is desirable to incorporate regression coefficients for individual dietary fatty acids including *trans* fatty acids. *Trans* fatty acids are formed during partial hydrogenation of liquid oils, a process that has been used by the food industry for many years to obtain edible fat blends with desired melting point behavior. During the last few years the European margarine industry has moved rapidly toward development of products virtually free of *trans* fatty acids. To avoid ending up with less healthful products by simply replacing *trans* fatty acids with cholesterol-raising SAFA one has to consider the effect of all individual fatty acids present in the products and choose the blends of raw materials with the most favorable fatty acid composition.

The aim of the present study was therefore to establish the cholesterolemic effects of *trans* fatty acids and compare them with the hypercholesterolemic effects of the SAFA 12:0, 14:0, and 16:0. Based on four strictly controlled dietary studies, *trans* fatty acids were incorporated into predictive equations by constrained linear regression analysis, utilizing prior knowledge about the regression coefficients for individual saturated and unsaturated fatty acids (11,12). We introduced *trans* fatty acids from two commonly used raw materials in Norway, partially hydrogenated soybean oil (PHSO) and partially hydrogenated fish oil (PHFO). *Trans* fatty acids from these raw materials differ with regard to both chain length and number of *trans* bonds. Hence, regression equations were developed that consider the effects of individual SAFA and *trans* fatty acids as well as MUFA and PUFA. Such equations are potentially useful for the food industry to optimize the fatty acid composition in edible fat products.

## MATERIALS AND METHODS

*Dietary studies.* The equations were based on results from four strictly controlled dietary studies conducted at the University College of Akershus, Norway, during the period 1993–1998 (13–15; Müller H., Lindman, S.A., Brantsæter, A.L., and Pedersen, J.I., unpublished results). They were Latin-square designed studies, or crossover studies where each individual consumed the diets in random order. Thus, each individual acted as his or her own control, and the variability among the individuals could be eliminated. The number of individuals was sufficient to assess significant differences in total and LDL cholesterol (13–15). As shown in Table 2 all participants had similar characteristics. However, in the first study (13) only young men participated, whereas in the last three studies only women participated.

All four studies were carried out following the same procedure. The meals were prepared at the college and the diets in each separate study differed only in the source of test margarine used for spreading, baking, and cooking. The test fats were incorporated into the menus in several foods including bread, buns, porridge, and sauces for dinner. The fat from the background diet was calculated to supply 6–8% of energy (E%), while the test fat was planned to provide the remaining fat energy, resulting in a total fat intake of about 30–38 E% (Table 3). The fatty acid composition of the diets and the content of dietary cholesterol were analyzed in duplicate portions by gas–liquid chromatography (Table 3).

A wide range of raw materials was used to produce the test fats: PHSO, PHFO, butter, palm oil (PALM), coconut oil (COCO), soybean oil, rapeseed oil, and sunflower oil. Since the main goal was to assess the effects of *trans* fatty acids and compare them with the effects of individual SAFA, diets enriched in PHSO and PHFO were tested twice with two separate groups of individuals (Table 3). In Study 1, PHSO and PHFO were tested against each other and butter (high in 14:0 and 16:0); in Study 2, PHFO was tested against PALM (high in 16:0); and in Study 3, PHSO was tested against PALM. Diets high in PUFA were tested against PHSO and PALM in Study 3, and against COCO (high in 12:0 and 14:0) in Study 4.

Blood samples were taken at the end of each test period of 3 wk duration in Studies 1, 3, and 4, and 2 wk in Study 2. Serum cholesterol was measured by enzymatic methods (16). Serum high density lipoprotein (HDL) cholesterol was measured by an enzymatic technique after precipitation of the LDL fraction with dextran sulfate and magnesium (16). LDL cholesterol concentrations were calculated using the equation of Friedewald *et al.* (17).

The four study protocols were approved by the Regional Committee for Ethics in Biomedical Research of Norway.

**TABLE 2**
**Characteristics of the Participants in Studies 1–4 at Baseline[a]**

| Study | Number | Sex | Age (yr) | Body mass index ($kg/m^2$) | Habitual dietary cholesterol (mg/d) | Baseline serum cholesterol (mmol/L) |
|---|---|---|---|---|---|---|
| Study 1 | 31 | Male | 28 | 26 | 535 | 5.35 |
| Study 2 | 16 | Female | 22 | 23 | 220 | 4.44 |
| Study 3 | 23[b] | Female | 27 | 25 | 248 | 5.30 |
| Study 4 | 25 | Female | 31 | 25 | 271 | 4.95 |

[a]From references 13–15; Müller H., Lindman, S.A., Brantsæter, A.L., and Pedersen, J.I., unpublished data.
[b]Number remaining after exclusion of four individuals; see text.

**TABLE 3**
**Fatty Acid Composition (% of total fatty acids), Content of Cholesterol (mg per 10 MJ) and Fat (E%) of Duplicate Portions of Experimental Diets in Studies 1–4**

| | Study 1[a] | | | Study 2[b] | | Study 3[c] | | | Study 4[d] | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acids | Butter | PHSO | PHFO | PHFO | PALM | PALM | PHSO | PUFA | COCO | PUFA |
| 4:0 | 0.9 | | | | | | | | | |
| 6:0 | 1.0 | | | 0.1 | 0.2 | | | | 0.6 | |
| 8:0 | 0.8 | 0.2 | 0.4 | 0.8 | 1.2 | | | | 6.4 | 0.2 |
| 10:0 | 1.7 | 0.3 | 0.6 | 0.8 | 1.2 | | | | 4.7 | 0.2 |
| 12:0 | 2.1 | 0.4 | 0.5 | 4.8 | 6.5 | 0.5 | 0.5 | 2.6 | 34.3 | 2.0 |
| 14:0 | 6.6 | 1.0 | 5.3 | 6.8 | 4.2 | 1.6 | 1.0 | 1.8 | 13.9 | 0.7 |
| 14:1 | 0.5 | 0.2 | 0.2 | | | | | | | |
| 15:0 | 0.6 | 0.3 | 0.4 | 0.5 | 0.2 | | | | | |
| 15:1 | 0.8 | 0.1 | 0.2 | 0.1 | — | | | | | |
| 16:0 | 22.7 | 14.1 | 16.3 | 18.2 | 30.4 | 33.9 | 11.0 | 15.8 | 10.8 | 9.4 |
| 16:1*t* | 0.7 | 0.4 | 3.8 | 4.0 | 0.2 | | | | | |
| 16:1*c* | 1.6 | 0.7 | 1.7 | 2.5 | 0.4 | 0.4 | 0.4 | 0.4 | 0.25 | 0.1 |
| 17:0 | 0.8 | 0.3 | 0.3 | 0.5 | 0.2 | 0.1 | 0.1 | 0.1 | | |
| 17:1 | 0.8 | 0.2 | | 0.2 | | | — | | | |
| 18:0 | 8.7 | 12.7 | 6.0 | 6.8 | 5.9 | 5.3 | 8.8 | 4.4 | 3.6 | 8.0 |
| 18:1*t* | 1.5 | 22.8 | 6.5 | 6.6 | 2.9 | 0.1 | 22.6 | 0.2 | 0.5 | |
| 18:1*c* | 23 | 25 | 11.7 | 12.2 | 27.5 | 37.9 | 35.2 | 38.6 | 14.0 | 36.7 |
| 18:2*t*[e] | | 0.3 | 0.6 | 1.0 | 0.3 | 0.1 | 0.4 | 0.3 | | |
| 18:2*c* | 15.4 | 15.0 | 15.7 | 10.2 | 13.5 | 16.0 | 13.5 | 27.3 | 8.6 | 36.2 |
| 18:3*c* | 2.3 | 3.0 | 2.5 | 1.2 | 1.7 | 2.4 | 4.7 | 6.1 | 1.6 | 4.6 |
| 20:0 | 0.6 | 0.4 | 2.2 | 1.5 | 0.4 | 0.4 | 0.3 | 0.2 | 0.1 | 0.4 |
| 20:1*t* | 0.4 | | 5.4 | 3.0 | — | 0.1 | 0.1 | | 0.2 | |
| 20:1*c* | 0.5 | 0.3 | 1.3 | 2.2 | 0.2 | | | | 0.1 | 0.6 |
| 20:2*t* | | | 2.4 | 5.2 | — | | | | | |
| 20:2*c,c* | | | 2.3 | 1.2 | — | | | | | |
| 22:0 | 0.5 | 0.6 | 1.5 | 0.8 | 0.2 | 0.2 | 0.3 | 0.3 | <0.1 | 0.2 |
| 22:1*t* | | | 3.5 | 1.6 | — | | | | | |
| 22:1*c* | 0.3 | | 2.4 | 1.6 | | | — | | | 0.1 |
| 22:2*t* | | | 1.4 | 3.6 | — | | | | | |
| 22:2*c,c* | | | 1.3 | 0.9 | — | | | | | |
| 24:0 | | | 0.2 | 0.4 | 0.3 | | | | | |
| 24:1*t* | | | 0.2 | 0.1 | — | | | | | |
| 24:1*c* | | | 0.2 | 0.2 | — | | | | | |
| Cholesterol | 420 | 430 | 420 | 246 | 98 | 102 | 67 | 94 | 59 | 65 |
| Fat | 34.8 | 36.2 | 33.5 | 30.7 | 32.4 | 31 | 30.1 | 30.4 | 38.4 | 38.2 |

[a]From Reference 13.
[b]From Reference 14.
[c]From Reference 15.
[d]From Müller, H., Lindman, S.A., Brantsæter, A.L., and Pedersen, J.I., unpublished data.
[e]Includes *trans,cis* and *cis,trans*. Abbreviations: PHSO, diets using margarines with a high content of *trans* fatty acids from partially hydrogenated soybean oil; PHFO, diets using a hard margarine with a high content of *trans* fatty acids from partially hydrogenated fish oil; PALM, diets using hard margarines containing vegetable oil, mainly palm oil; PUFA, diet using a soft highly polyunsaturated margarine; COCO, diet using a hard zero-*trans* margarine containing coconut oil.

*Regression analysis.* To be able to incorporate *trans* fatty acids into existing predictive equations that relate changes in intake of dietary fatty acids to changes in serum total and LDL cholesterol, we made use of constrained linear least-square regression (Optimization Toolbox in MATLAB, version 5.2; The Mathworks Inc., Natick, MA). This technique can be applied to regression modeling and optimization problems when the analyst possesses some knowledge about the regression coefficients, such as the signs and the ordering of the coefficients (11,12). Adequate constraints were added to the coefficients by using lower and upper bounds according to previous results in the literature. We assumed that the introduction of *trans* fatty acids did not severely influence the other coefficients. In other words, we allowed a slight adjustment of existing coefficients described in the literature while introducing *trans* fatty acids into the equations.

The data set with corresponding bounds is shown in Table 4. The table shows the differences in intake of the different fatty acids when changing from one diet to another (predictor variables, X). The corresponding difference in serum total and LDL cholesterol (response variables, Y) have been corrected for the effect of dietary cholesterol intake according to the the formulas of Hopkins (18) and Clarke *et al.* (9), respectively. However, except for Study 2 (14) the intake of cholesterol was similar for all diets within the same study and omission of the effect of dietary cholesterol had only a minor effect on the regression coefficients. The dietary studies with two diets yielded one data point, whereas the studies

**TABLE 4**
**Data Set Used for Constrained Linear Regression, Differences in Intake of Various Fatty Acids in E% (X1–X7) and the Corresponding Difference in Total (Y1) and LDL-C (Y2) in mmol/L. Upper and Lower Bounds for the Various Regression Coefficients Are Shown[a]**

| Study (number) | Y1 ΔTotal | Y2 ΔLDL | X1 Δ12:0 | X2 Δ14:0 | X3 Δ16:0 | X4 Δ*TRANS* V | X5 Δ*TRANS* F | X6 Δ(18:2 + 18:3) | X7 Δ18:1 |
|---|---|---|---|---|---|---|---|---|---|
| Butter-PHFO (1) | –0.10 | –0.13 | 0.56 | 0.52 | 2.44 | 1.04 | –8.04 | 0.17 | 4.08 |
| Butter-PHSO (1) | 0.21 | 0.23 | 0.59 | 1.93 | 2.80 | –7.28 | 0.00 | –0.25 | –1.05 |
| PHSO-PHFO (1) | –0.30 | –0.36 | –0.02 | –1.41 | –0.36 | 8.33 | –8.04 | 0.42 | 5.13 |
| PHFO-PALM (2) | 0.12 | 0.18 | –0.63 | 0.73 | –4.17 | –1.10 | 7.71 | –1.43 | –5.16 |
| PALM-PUFA (3) | 0.40 | 0.38 | –0.63 | –0.04 | 5.77 | 0.00 | 0.00 | –4.32 | 0.17 |
| PHSO-PUFA (3) | 0.21 | 0.29 | –0.63 | –0.24 | –1.44 | 6.90 | 0.00 | –4.56 | –1.02 |
| PHSO-PALM (3) | –0.18 | –0.09 | 0.00 | –0.20 | –7.21 | 6.90 | 0.00 | –0.24 | –1.18 |
| COCO-PUFA (4) | 0.97 | 0.68 | 12.37 | 5.07 | 0.54 | 0.00 | 0.00 | –11.69 | –8.00 |
| Lower bound | | | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.017 | –0.0044 |
| Upper bound | | | 0.02 | 0.2 | 0.2 | 0.2 | 0.2 | –0.017 | –0.0044 |

[a]*TRANS* V, *trans* fatty acids from PHSO; *TRANS* F, *trans* fatty acids from PHFO. See Tables 1 and 3 for other abbreviations.

with three diets yielded three data points that represented the difference between any two groups. Hence, a total of eight data points were included in the analyses in order to estimate the coefficients for Δ12:0, Δ14:0, Δ16:0, Δ*TRANS* V, and Δ*TRANS* F. Because the amounts of MUFA and PUFA were not sufficiently and independently varied, their respective regression coefficients were set equal to those found by Yu *et al.* (8). Stearic acid (18:0) was not included in the regressions because it is considered to be neutral (1,2,5,6,9,10).

Estimates of the uncertainty variance of the regression coefficients were obtained using the "Jack-knife" (19). The method consists of leaving out one data point at a time and performing the regression analysis with the remaining data, resulting in eight single values of each coefficient for the calculation of the uncertainty variance. If a segment model differs greatly from the common model based on all data, it means that the data point left out seriously affects the common model by pressing some unique pattern of variation into the model parameters. Thus, the result shows the robustness of the common model against peculiarities in individual data points.

The predictive accuracy of the models is described by the root mean square error of prediction (RMSEP):

$$\text{RMSEP} = \left[ \frac{1}{N} \sum_{i=1}^{N} (y_{\text{obs}} - y_{\text{pred}})^2 \right]^{1/2} \quad [1]$$

which is a measure of the average difference between predicted ($y_{\text{pred}}$) and observed ($y_{\text{obs}}$) values. RMSEP can be interpreted as the average prediction error expressed in the same units as the original response (Y) variable.

The statistical packages SPSS Advanced Statistics, version 8.0 (SPSS Inc., Chicago, IL), were used for data analyses of the individual studies (Studies 1–4) and calculation of the Pearson correlation coefficient.

*Validation based on test set.* The serum cholesterol predictive equation was validated using a test set consisting of results from recently published Western studies (20–26) that fulfilled the requirements of well-controlled randomized cross-over or Latin-square design studies with normolipidemic or slightly hypercholesterolemic individuals (summarized in Table 5). The fat intake varied from 22 to 40 E%. All studies reported the fatty acid composition of the experimental diets, including *trans* fatty acids. The studies yielded 22 unique data points, each representing the difference between two diets within a study, i.e., changes in serum cholesterol that could be derived from other observed changes were omitted.

## RESULTS

All participants complied well. In Study 3, four overweight participants lost between 1.7–7 kg during the study and were therefore omitted from the regression analyses (Table 2).

*Serum total cholesterol.* Figure 1 presents the regression coefficients for the relation between variations in serum total cholesterol and variations in fatty acid intake, expressed as mmol/L per 1 E% change in fatty acid. The standard deviations were estimated by Jack-knifing. The coefficients were as follows: lauric acid (12:0) 0.01 ± 0.166, myristic acid (14:0) 0.12 ± 0.026, palmitic acid (16:0) 0.057 ± 0.019, *TRANS* V (18:1*t*, 16:1*t*) 0.031 ± 0.025, *TRANS* F (16:1*t*, 18:1*t*, 18:2*t*, 20:1*t*, 20:2*t*, 22:2*t*, 24:1*t*) 0.039 ± 0.022. The coefficients for oleic acid (18:1) and linoleic acid (18:2)/(α-linolenic acid (18:3) were set equal to the coefficients given by Yu *et al.* (8). Thus, the following equation was developed: Total cholesterol = 0.01 Δ(12:0) + 0.12 Δ(14:0) + 0.057 Δ(16:0) + 0.039 Δ(*TRANS* F) + 0.031 Δ(*TRANS* V) – 0.0044 Δ(18:1) – 0.017 Δ(18:2,18:3). The correlation coefficient between observed and predicted values was 0.996 and the RMSEP was 0.026 mmol/L (Fig. 2A).

*Serum LDL cholesterol.* There was a significant correlation (Pearson coefficient $r = 0.922$, $P < 0.01$) between serum total and LDL cholesterol in the four studies (Fig. 3). The regression coefficients for LDL cholesterol and their respective standard deviations were as follows: lauric acid (12:0) 0.01 ± 0.17, myristic acid (14:0) 0.071 ± 0.011, palmitic acid (16:0) 0.047 ± 0.032, *TRANS* V (18:1*t*, 16:1*t*) 0.025 ± 0.042, and

**TABLE 5**
**Summary of Studies Used for Validation**

| | Men | Women | Age (yr) | Test diets |
|---|---|---|---|---|
| Wood *et al.* (20) | 40 | | 30–60 | Butter<br>Butter-olive oil<br>Butter-sunflower oil<br>Hard margarine<br>Soft margarine |
| Mensink and Katan (21) | 25 | 34 | 25–26 | Saturated fat<br>*Trans*<br>Oleic acid-rich |
| Zock and Katan (22) | 26 | 30 | 24–25 | *Trans*<br>Stearate-rich<br>Linoleate-rich |
| Judd *et al.* (23) | 29 | 29 | 25–65 | High *trans*<br>Moderate *trans*<br>Oleic acid-rich<br>Saturated fatty acid-rich |
| Nelson *et al.* (24) | 11 | | 20–35 | High fat, low fat |
| Judd *et al.* (25) | 23 | 23 | 28–65 | Butter<br>Polyunsaturated fatty acid-rich margarine<br>*Trans*-rich margarine |
| Lichtenstein *et al.* (26) | 18 | 18 | >50 | Soybean oil<br>Semiliquid margarine<br>Soft margarine<br>Shortening<br>Stick margarine<br>Butter |

*TRANS* F (16:1*t*, 18:1*t*, 18:2*t*, 20:1*t*, 20:2*t*, 22:2*t*, 24:1*t*) 0.043 ± 0.028. The coefficients for oleic acid (18:1) and linoleic acid (18:2)/α-linolenic acid (18:3) were set equal to the coefficients given by Yu *et al.* (8). Thus, the following equation was developed: ΔLDL cholesterol = 0.01 Δ(12:0) + 0.071 Δ(14:0) + 0.047 Δ(16:0) + 0.043 Δ(*TRANS* F) + 0.025 Δ(*TRANS* V) – 0.0044 Δ(18:1) – 0.017 Δ(18:2,18:3). The correlation coefficient between observed and predicted values was 0.969, and the RMSEP was 0.079 mmol/L (Fig. 2B).

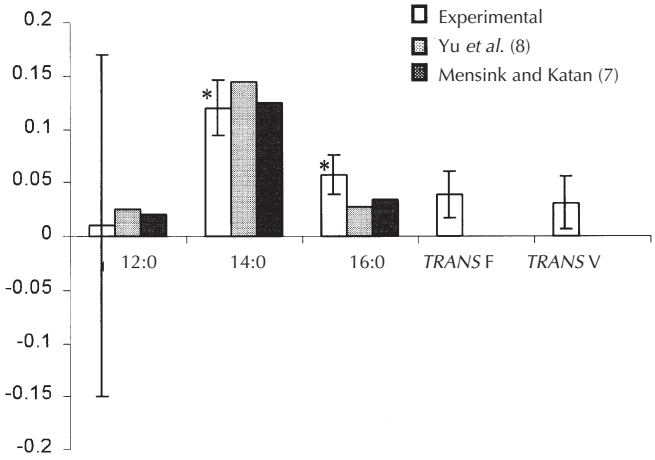


**FIG. 1.** Bar graph showing the coefficients of individual saturated and *trans* fatty acids obtained by constrained linear regression and expressed as mmol/L per energy% change in fatty acid intake. The standard deviations are calculated by Jack-knifing (*, regression coefficients are significantly different from zero, $P < 0.05$). Regression coefficients for individual saturated fatty acids published by Yu *et al.* (8) and Mensink and Katan (7) are shown for comparison.

*Test set validation.* Results from seven published dietary studies including diets with *trans* fatty acids (20–26) were used to test the prediction ability of the equation for total cholesterol. Based on the reported intake of individual fatty acids, the expected change in serum cholesterol was calculated and compared to the observed changes (Fig. 4). A significant correlation (Pearson coefficient $r = 0.981$, $P < 0.01$) between predicted and observed values was found (Fig. 4) with a RMSEP of 0.076 mmol/L, which shows that the equation gives good predictions not only of our dietary studies but also of a considerable number of studies performed in other laboratories.

## DISCUSSION

Recently, new interest is focused on the cholesterolemic effect of individual fatty acids rather than classes of fatty acids (7,8). The outcome of the regression analyses performed in the present study is in good agreement with previous studies (7,8), as demonstrated in Figure 1, and confirms that myristic acid (14:0) is more hypercholesterolemic than lauric acid (12:0) and palmitic acid (16:0). The present results also indicate that *trans* fatty acids are slightly less hypercholesterolemic than 16:0.

In most published equations (2,5–7,10) a regression coefficient is given only for the sum of 12:0, 14:0, and 16:0, and

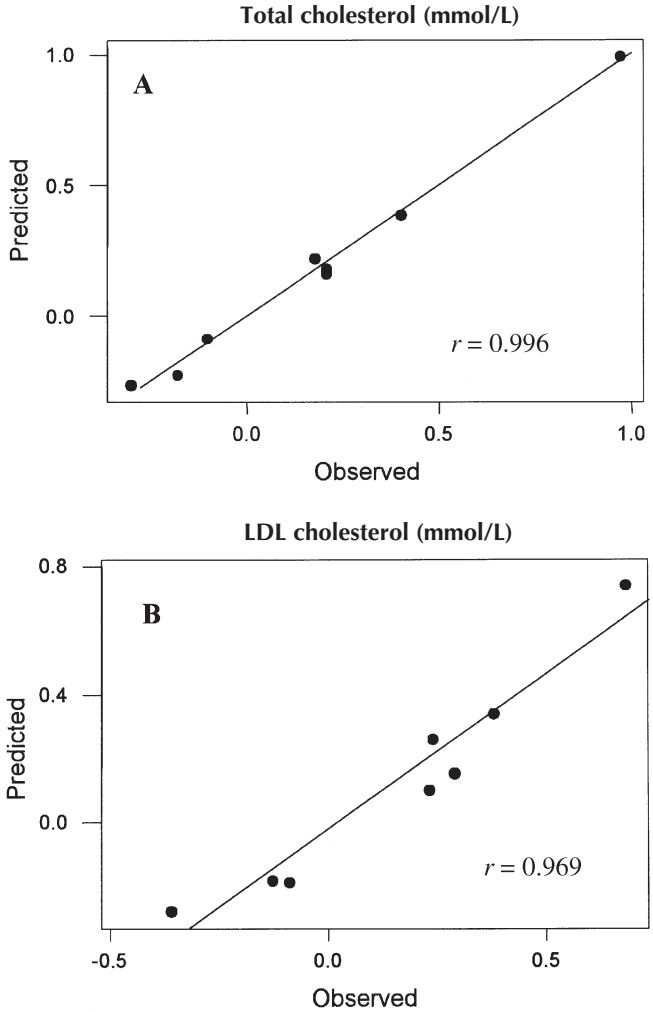


**FIG. 2.** Correlation between observed and predicted change in serum total cholesterol (A) and low-density lipoprotein (LDL) cholesterol (B) for data points included in the constrained regression analyses (Table 4). Pearson correlation coefficient between predicted and observed changes in total cholesterol is 0.996 ($P < 0.01$) and root means square error of prediction (RMSEP) = 0.026 mmol/L. Pearson correlation coefficient between predicted and observed changes in LDL cholesterol is 0.969 ($P < 0.01$) and RMSEP = 0.079 mmol/L.

*trans* fatty acids are not included. Since individual SAFA and *trans* isomers occur in different amounts in edible fats, those equations will not be applicable to every data set and may give erroneous predictions. The importance of splitting up the SAFA was also clearly demonstrated when we performed the constrained regression analyses with only one coefficient for total saturated fat. This resulted in much poorer models for both serum total cholesterol (RMSEP = 0.069 vs. 0.026 mmol/L) and LDL cholesterol (RMSEP = 0.112 vs. 0.079 mmol/L).

Our main goal was to incorporate *trans* fatty acids from PHSO and PHFO into predictive equations that would offer a valuable tool for the Norwegian food industry, particularly in the manufacture of margarine. We used constrained linear regression, a technique where lower and upper bounds can be added to the regression coefficients. The bounds were chosen

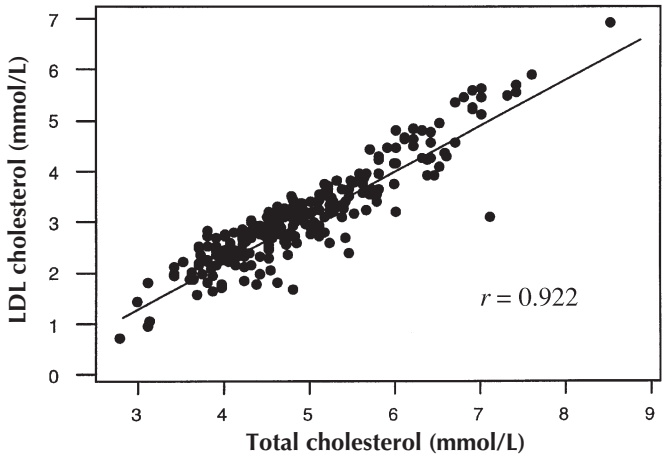


**FIG. 3.** Correlation between serum total cholesterol and low-density lipoprotein (LDL) cholesterol. Results from Studies 1–4 are included. The Pearson correlation coefficient between observed and predicted values is 0.922 ($P < 0.01$).

according to previously reported coefficients in the literature (7,8). The use of constrained regression is motivated by the need to utilize existing knowledge about the signs and ordering of the regression coefficients and the realization that regression estimates can be misleading when the X-variables are correlated.

As seen in Figure 1, there is a large uncertainty in the coefficient for lauric acid (12:0). The value of the coefficient is similar to the lower bound, which is lower than coefficients reported in the literature. This is due to the influence of the data point from Study 4 (COCO–PUFA), which seems to pull down the effect of 12:0. However, Study 4 is the only study where 12:0 is sufficiently varied and therefore it should be included. Caution must, of course, be taken when interpreting the standard deviations of coefficients that touch the bounds.

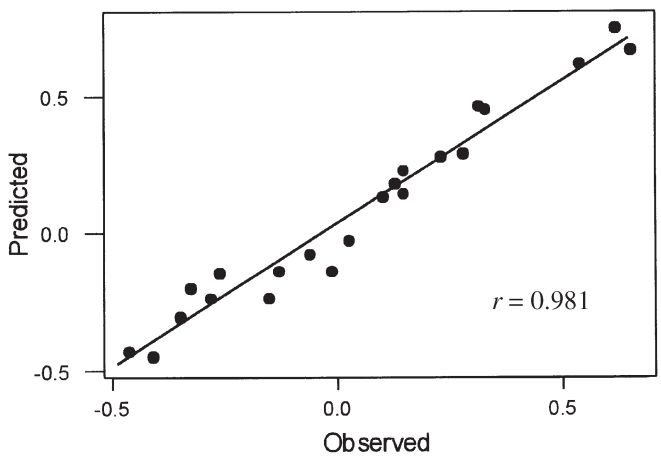


**FIG. 4.** Correlation between observed and predicted change in serum total cholesterol from seven studies (20–26) included in the test set (summarized in Table 5). The predicted values were calculated with Equation 1 given in the text. The Pearson correlation coefficient between observed and predicted values is 0.981 ($P < 0.01$). Root mean square error of prediction = 0.076 mmol/L.

Except for the coefficient for 12:0, this was not a problem as Jack-knifing showed that all estimated coefficients in the submodels were far from touching the upper and lower bounds. We therefore conclude that the coefficient for 12:0, although not significant, indicates that 12:0 does not raise serum total cholesterol as much as 14:0 and 16:0. This is in agreement with previously published data (7,8,27,28).

Cases with small data sets are often difficult to handle because there is little information for estimating a model, and each sample is unique. Furthermore, the fatty acids investigated should be varied independently of each other in order to assess coefficients that reflect a physiological role. This is difficult to do with natural oils where the different fatty acids are correlated to a greater or lesser extent. However, although our data are limited, they are precise, resulting from strictly controlled randomized cross-over studies reporting the fatty acid composition in duplicate portions of the experimental diets. Fat from the background diet supplied only 6–8%, and the observed effects were therefore due almost entirely to the test fat. In order to assess the effects of *trans* fatty acids, diets enriched in PHSO and PHFO were tested twice with two separate groups of individuals (Table 3), and this strengthens the results.

In the end it is the validation step that shows how good the regression model is. A nonrepresentative calibration set may introduce erroneous predictions of future data sets. In order to test predictive capability and validate our model for total serum cholesterol, we used a test set consisting of results from studies published from different laboratories in Western countries. We selected seven recently published papers from the literature that fulfilled the requirements of well-controlled studies reporting the complete fatty acid composition of the experimental diets. These were mainly studies including diets with *trans* fatty acids. Figure 4 demonstrates how well the equation predicts the results from these studies ($r = 0.981$, RMSEP = 0.076 mmol/L), suggesting that it may have a broader application. On the other hand, the equation gave rather poor predictions of the results from a study of Sundram *et al.* (29) where healthy Malaysians were exposed to *trans* fatty acids (PHSO). The authors report that the observed response to *trans* fatty acids differed from other studies and suggest that different ethnic background and habitual diet may explain the Malaysians' exceptional sensitivity to *trans*. Other contributing factors may be differences in the source and type of *trans* isomers and the way the *trans* content is measured. Some authors have also discussed the possibility of interactions, in particular between SAFA or *trans* and 18:2. In the present experimental diets the content of 18:2 varied from 8.6 to 36.2% (Table 3). Our data set is too small for incorporating interactions in the regression analyses. However, the precise predictions of the 11 studies (30 data points) in the calibration and test sets indicate that, if there are interaction effects, these are small compared with the main effects.

Possible differences between men and women in the response to dietary fatty acids would also affect the prediction ability. Both men and women participated in our studies (Table 2). Keys *et al.* (1,2) and Hegsted *et al.* (5,6) used data from middle-aged men in their regression analyses while others have used data from both men and women (7–10). Predictive equations from Yu *et al.* (8) indicate that the response to fatty acids are similar for women and men. However, some difference in HDL cholesterol response to diet between women and men cannot be excluded.

There are several reports showing that myristic acid (14:0) increases total cholesterol more than palmitic acid (16:0) (6,8,27,30). It should also be noted that in one study no difference was found in the cholesterol-raising effect of 14:0 and 16:0 (31), except that 14:0 resulted in an 8% higher HDL cholesterol level. In another study, the effect of 14:0 on serum total cholesterol was 1.5 higher than that of 16:0, and half of this increase was due to an increase in HDL cholesterol (30). The HDL cholesterol-increasing effect of 14:0 may explain why our regression coefficient for 14:0 is much larger for serum total than for LDL cholesterol. Constrained regression analysis of the present data failed to obtain a credible predictive equation for HDL. It is also apparent from Figure 2 that the developed equations predict serum total cholesterol (RMSEP = 0.026 mmol/L) better than LDL cholesterol (RMSEP = 0.079 mmol/L). There may be several reasons for this. Total serum cholesterol may simply be better described by a linear relationship between dietary intake of individual fatty acids than LDL and HDL cholesterol. Also, some fatty acids may have different effects on LDL and HDL cholesterol. Thus, as discussed above, 14:0 increases HDL cholesterol, while *trans* fatty acids have a decreasing effect (13,21, 23,26). Furthermore, unlike total cholesterol, LDL cholesterol is a calculated, not a measured, value and the methodological error is therefore probably greater. However, the regression coefficients for total and LDL cholesterol are in relatively good agreement with each other, and the correlation between total and LDL cholesterol is high (Fig. 3), indicating that changes in the level of total serum cholesterol were mainly due to changes in LDL cholesterol.

The effect of *trans* fatty acids on serum cholesterol has not been included in previously published predictive equations (2,5–10). However, Clarke *et al.* (9) give a regression coefficient of 0.038 mmol/L for 18:1 *trans* isomers, similar to our regression coefficient of *TRANS* F. It is now well established that *trans* fatty acids are hypercholesterolemic even if the exact increasing effect on serum total cholesterol is uncertain (3,21–23,32). Some have proposed that *trans* acids increase total and LDL cholesterol to a lesser extent than $C_{12}$, $C_{14}$, and $C_{16}$ saturated acids (22,32), while others have proposed that *trans* fatty acids and SAFA have similar effects on LDL cholesterol (33). The results of our study indicate that the effect of *TRANS* V is slightly lower than that of 16:0 on serum total and LDL cholesterol.

Very little is known about the effects of the very long chain saturated and *trans* fatty acids ($C_{20}$–$C_{24}$) in PHFO on serum lipoproteins (14). Several of these may be cholesterol increasing. The possible cholesterol-increasing fatty acids 14:1 *cis*, 16:1 *cis* (34), or 22:0 (35) were not included in our equation. Such fatty acids are present in hydrogenated fish oils, and

their effects will be reflected in the coefficient for *trans* fatty acids. This may explain why our regression coefficients for *TRANS* F from fish oil are higher than for *TRANS* V. Our model also indicates that *TRANS* F, unlike *TRANS* V increase LDL cholesterol more than total cholesterol. The explanation may be that *TRANS* F appear to decrease HDL cholesterol more than *TRANS* V (13).

Predictive equations such as those previously published (Table 2) or the one we have developed may certainly have their weaknesses and limited theoretical utility. This does not mean that they may be of no practical use. The equation for total serum cholesterol presented here has been used by a Norwegian food company to formulate a cholesterol-reducing margarine. When the margarine was tested against butter in an open clinical trial (36), the observed cholesterol-lowering effect corresponded reasonably well to the predicted effect (0.77 vs. 0.64 mmol/L, respectively). This study was not strictly controlled and Hopkins' formula (18) was used to correct for the difference in cholesterol content of margarine and butter. Together with the result from the test set validation, this confirms that the equation has practical applicability and can be used to formulate and nutritionally optimize fat products as well as evaluate already existing products on the market.

## ACKNOWLEDGMENTS

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# Comparative Bioavailability of Dietary α-Linolenic and Docosahexaenoic Acids in the Growing Rat

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**ABSTRACT:** Animal and human studies have indicated that developing mammals fed only α-linolenic acid (18:3n-3) have lower docosahexaenoic acid (22:6n-3) content in brain and tissue phospholipids when compared with mammals fed 18:3n-3 plus 22:6n-3. The aim of this study was to test the hypothesis that low bioavailability of dietary 18:3n-3 to be converted to 22:6n-3 could partly explain this difference in fatty acid accretion. For that purpose, we determined the partitioning of dietary 18:3n-3 and 22:6n-3 between total n-3 fatty acid body accumulation, excretion, and disappearance (difference between the intake and the sum of total n-3 fatty acids accumulated and excreted). This was assessed using the quantitative method of whole-body fatty acid balance in growing rats fed the same amount of a 5% fat diet supplying either 18:3n-3 or 22:6n-3 at a level of 0.45% of dietary energy (i.e., 200 mg/100 g diet). We found that 58.9% of the total amount of 18:3n-3 ingested disappeared, 0.4% was excreted in feces, 21.2% accumulated as 18:3n-3 (50% in total fats and 46% in the carcass-skin compartment), and 17.2% accumulated as long-chain derivatives (14% as 22:6n-3 and 3.2% as 20:5n-3 + 22:5n-3). Similar results were obtained from the docosahexaenoate balance (as % of the total amount ingested): disappearance, 64.5%; excretion, 0.5%; total accumulation, 35% with 30.1% as 22:6n-3. Thus, rats fed docosahexaenoate accumulated a twofold higher amount of 22:6n-3, which was mainly deposited in the carcass-skin compartment (68%). Similar proportions of disappearance of dietary 18:3n-3 and 22:6n-3 lead us to speculate that these two n-3 polyunsaturated fatty acids were β-oxidized in the same amount.

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Docosahexaenoic acid (DHA, 22:6n-3) is the main polyunsaturated fatty acid (PUFA) found in excitable membrane phospholipids of the mammalian brain and retina and is intensively deposited in these tissues during the brain growth spurt to ensure optimal visual and cognitive functions (reviewed in Ref. 1). In normal nutritional conditions, the 22:6n-3 requirement is covered by the dietary supply of both its essential precursor α-linolenic acid (ALA, 18:3n-3) and preformed 22:6n-3 (1). Several data suggest that this requirement is not fulfilled when only dietary 18:3n-3 is supplied. Indeed, infants fed formulas containing only 18:3n-3 have lower 22:6n-3 concentrations in their blood, liver, and brain phospholipids than breast-fed infants receiving both n-3 PUFA, even if formulas have a low ratio of linoleic acid (18:2n-6) to 18:3n-3 and/or a high 18:3n-3 content (reviewed in Refs. 2–4). Recent data obtained from artificially reared rat pups and young adult rats also indicate that a high dietary intake of 18:3n-3 is unable to sustain adequate neural and other tissue 22:6n-3 accretion (5,6). All these results suggest that the growing mammal has an insufficient metabolic capacity to biosynthesize an adequate amount of 22:6n-3 from its essential precursor, a hypothesis that has been confirmed in newborn infants by their extremely low *in vivo* conversion of deuterated 18:3n-3 to 22:6n-3 (7).

Limiting capacity to biosynthesize 22:6n-3 from 18:3n-3 might result from (i) a competitive inhibitory effect of 18:3n-3 on Δ6-desaturation of 24:5n-3 to 24:6n-3 in the new alternative pathway of Δ4-desaturation as some authors suggested (8), and/or (ii) a low bioavailability of dietary 18:3n-3 owing to its high *in vivo* rate of β-oxidation (9), recently confirmed by the indirect quantitative method of the whole-body fatty acid balance showing that 85% of the dietary 18:3n-3 consumed disappeared in the growing rat (10). In contrast, dietary 22:6n-3 is considered to be more bioavailable because it is a poor substrate for peroxisomal and mitochondrial β-oxidation pathways (11) and is preferentially acylated in membrane phospholipids. But to our knowledge, there are no *in vivo* quantitative data on the metabolic partitioning of dietary 22:6n-3 between its disappearance and body accumulation as 22:6n-3 and its derivatives.

Therefore, this study compared the overall partitioning of dietary 18:3n-3 and 22:6n-3 in the growing rat by quantifying (i) the amount of dietary 18:3n-3 and 22:6n-3 that disappeared, and (ii) the amount of 18:3n-3 and 22:6n-3 and their derivatives accumulated in the whole body and in different organ compartments. The whole-body fatty acid balance methodology was chosen, as it is relevant to determine these parameters specifically for PUFA and over a long period of time of dietary utilization. Moreover, it estimates indirectly the overall *in vivo* β-oxidation of PUFA as it is supposed that their disappearance is mainly attributable to this catabolic pathway, inasmuch as it is accepted that essential fatty acids are not synthesized *de novo* and that other catabolic processes

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Abbreviations: ALA, α-linolenic acid 18:3n-3; ANOVA, analysis of variance; DHA, docosahexaenoic acid 22:6n-3; FAME, fatty acid methyl ester; LC, long-chain; PUFA, polyunsaturated fatty acid.

of PUFA, such as eicosanoid synthesis, are quantitatively minor (10,12). The dietary supply of 18:3n-3 and 22:6n-3 was chosen in order to satisfy the n-3 fatty acid requirement of the growing rat (200 mg/100 g diet, corresponding to 0.45% of energy) (13).

## MATERIALS AND METHODS

*Animals and diets.* All procedures involving animals followed official French regulations for the care and use of laboratory animals (nos. 87 848 and 03 056). Twelve 2-mon-old female rats were the offspring of Wistar female rats rendered deficient in n-3 fatty acids (second generation) by consuming a semisynthetic diet very low in 18:3n-3 (5 wt% of peanut oil providing 9 mg 18:3n-3/100 g diet) (14). They were mated over 4 d and then housed individually in a polycarbonate cage. One week before parturition, they were randomly divided into two dietary groups (6 females per group). The first group received an α-linolenate (ALA) diet containing about 200 mg 18:3n-3/100 g diet (0.45% of total dietary energy), which is sufficient to meet the n-3 requirements of the growing rat (13). This diet was obtained by mixing peanut and flaxseed oils (Table 1). The second group received a DHA diet that provided the same supply of n-3 fatty acids (200 mg/100 g diet), but only as 22:6n-3, by using a microalgae oil containing about 42 wt% 22:6n-3 and <0.1% 18:3n-3, 20:5n-3, and 22:5n-3 (Neuromins™ DHA oils, Martek Biosciences Corporation, Columbia, MD). As the fat blend included peanut oil, the DHA diet supplied a low 18:3n-3 content (0.2 wt%, i.e. 9 mg/100 g diet) (Table 1). This experimental rearing procedure was used to obtain female then male weaned young rats in the DHA group with low 18:3n-3 body stores, which is crucial for studying the 22:6n-3 balance. Both diets contained 5% fat (weight %) and satisfied the 18:2n-6 requirement of the growing rat (about 1.1 g 18:2n-6/100 g diet, i.e., 2.5% of total dietary energy) (13). They were prepared weekly and stored at 4°C to avoid oxidative damage of n-3 fatty acids. Fatty acid composition was monitored during storage of each preparation. During lactation, litter sizes were equalized to 8 pups.

At weaning, in each dietary group and for each litter, male pups of similar body weight were paired up. One rat was housed in a metabolic cage for a 5-wk balance period and continued to receive the same diet (6 rats per group), and the other was sacrificed by decapitation to quantify whole-body fatty acid partitioning (6 rats per group). Then, to avoid unbalanced dietary intake and different body weight gain between rats, all animals daily received the same amount of diet during the balance period, from 12 g on day 1 to 24 g on day 35. They were weighed and their food intake was recorded every day. Feces were also collected daily, freeze-dried and stored at –80˚C until analysis. At the end of the balance period, 8-wk-old rats were sacrificed by decapitation.

*Dissection and lipid analysis.* Before sacrifice, rats were fasted overnight. The major organs were carefully dissected as described by Cunnane and Anderson (10) for comparison purposes. Except for brain and liver, they were pooled as follows: viscera (gut, pancreas, spleen, testes, bladder, kidneys, heart, thymus, and lungs), internal fats (epididymal, mesenteric, omental, and perirenal adipose tissues), subcutaneous fats (all the subcutaneous adipose tissues that could possibly be removed), and remaining carcass (skin, muscles, the whole skeleton, and the head without the brain). Organ compartments were weighed, freeze-dried, reduced to powder with dry ice and stored at –80˚C until analysis.

Total lipids of the lyophilized organs, organ compartments, and feces were extracted by the procedure of Folch *et al.* (15) in the presence of 0.02% (wt/vol) butylated hydroxytoluene. To assess the effectiveness of this extraction procedure and quantify total fatty acids in each organ compartment, known amounts of diheptadecanoyl (17:0) phosphatidylcholine and trinonadecanoyl (19:0) glycerol (Sigma, St. Quentin Fallavier, France) were added as internal standards during extraction and represented approximately 10% of estimated total fatty acids. Total lipids were then transmethylated with 10% boron trifluoride (Fluka, Socolab, Paris, France) at 90°C for 40 min (16). Fatty acid methyl esters (FAME) were injected into a Carlo Erba gas

**TABLE 1**
**Oil Content and Fatty Acid Composition of α-Linolenate (ALA) and Docosahexaenoate (DHA) Diets[a]**

| | Diets | |
|---|---|---|
| | ALA | DHA |
| Oil content | g/kg diet | |
| Peanut | 46.3 | 44.9 |
| Flaxseed[b] | 3.7 | — |
| Neuromins™ DHA[b] | — | 5.1 |
| Total | 50.0 | 50.0 |
| Fatty acids | % Total fatty acids | |
| 16:0 | 9.2 | 10.1 |
| 18:0 | 3.1 | 2.9 |
| 16:1n-7 | 0.2 | 0.3 |
| 18:1n-9 | 47.3 | 47.8 |
| 18:2n-6 | 24.4 | 23.3 |
| 18:3n-3 | 4.6 | 0.2 |
| 20:0 | 1.2 | 1.2 |
| 20:1n-9 | 0.9 | 1.0 |
| 20:5n-3 | ND[c] | <0.05 |
| 22:0 | 2.2 | 2.3 |
| 22:5n-3 | ND | <0.05 |
| 24:0 | 1.0 | 1.0 |
| 22:6n-3 | ND | 4.3 |
| | mg/100 g diet | |
| 18:2n-6 | 1159 | 1107 |
| 18:3n-3 | 218 | 9 |
| 22:6n-3 | — | 204 |

[a]The experimental diets contained (g/kg diet): lipids, 50; casein, 220; DL-methionine, 1.6; cellulose, 20; starch, 438.4; saccharose, 220; vitamin mixture, 10; mineral mixture, 40 (caloric density: 3970 kcal/kg or 16.61 MJ/kg). The vitamin mixture was a total vitamin supplement (United States Biochemical Corp., Cleveland, OH), and the mineral mixture was as follows (g/100 g): $CaHPO_4 \cdot 2H_2O$, 38.0; $K_2HPO_4$, 24.0; $CaCO_3$, 18.0; NaCl, 6.9; MgO, 2.0; $MgSO_4 \cdot 7H_2O$, 9.0; $FeSO_4 \cdot 7H_2O$, 0.86; $ZnSO_4 \cdot H_2O$, 0.5; $MnSO_4 \cdot H_20$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.1; NaF, 0.08; $CrK(SO_4)_2 \cdot 12H_2O$, 0.05; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.002; KI, 0.004; $CoCO_3$, 0.002; $Na_2SeO_3$, 0.002.
[b]Flaxseed and Neuromins™ DHA oils were purchased from Novance (Compiègne, France) and Martek Biosciences Corporation (Columbia, MD), respectively.
[c]ND, not detected.

chromatograph (HRGC 5300; Fisons Instruments, Arcueil-Cachan, France) equipped with an on-column injector, a flame-ionization detector, and a CP WAX 52 CB bonded fused-silica capillary column (50 m × 0.2 mm i.d. with 0.20 µm film thickness) purchased from Chrompack (Les Ulis, France). Hydrogen was used as carrier gas at a 1–2 mL/min flow rate. FAME with a C8 to C24 chain length and dimethyl acetals were identified by comparing equivalent chain lengths with those of commercial standards (Nu-Check-Prep Inc., Coger, Paris, France) and quantified by a computing integrator using the Nelson Analytical Program System (SRA, Gagny, France). The content of total fatty acids was corrected by the proportion of recovered internal standards. The fatty acid composition of the diets was determined by the same experimental procedure.

The whole-body fatty acid balance analysis was performed using the mode of expression of Cunnane and Anderson (10). The amount of dietary 18:3n-3 and 22:6n-3 that disappeared during the balance period was calculated as the difference between the total intake and the total amount of n-3 fatty acids excreted in feces and accumulated in the various organ compartments. The total amount of long-chain (LC) n-3 PUFA (20:5n-3, 22:5n-3, and 22:6n-3) accumulated in the whole body was used to determine the proportion of 18:3n-3 converted and accumulated in the ALA group, and the amount of both 20:5n-3 and 22:5n-3 was used to determine the proportions of 22:6n-3 retroconverted and accumulated in the DHA group. The same calculation method was used for the balance analysis of 18:2n-6. The sum of LC n-6 fatty acids (20:3n-6, 20:4n-6, 22:4n-6, and 22:5n-6) was used to determine the total proportion of linoleate converted.

*Statistics.* Results are means ± standard deviation for $n = 6$ individual animals analyzed independently. Differences in body weights, organ compartment weights, and fatty acid content in the various organ compartments between the two dietary groups were established by one-way analysis of variance. They were compared by the Scheffé test, with the level of significance set at $P < 0.05$ and $P < 0.01$ (StatView SE + Graphics™, Abacus Concepts Inc., ASD Meylan, France).

## RESULTS

*Dietary intake and growth.* The total amount of diet and fatty acids ingested during the 5-wk balance period and the body weights of the rats at the beginning and at the end of the balance period were similar in both groups (Table 2). No significant differences in weight of any of the organ compartments were found between the diet groups.

*n-3 Fatty acid balance.* The comparative effects of dietary 18:3n-3 and 22:6n-3 on the n-3 fatty acid balance are presented in Table 3. During the 5-wk balance period, the amounts of n-3 fatty acids ingested and accumulated in the whole body were similar between the two groups (about 1150 mg and 410–470 mg, respectively). Thus, taking into account the amount of n-3 fatty acid excreted, the disappearance of both dietary 18:3n-3 and 22:6n-3 was similar and represented about 700 mg. When expressed as a percentage of n-3 fatty acid intake, balance analysis revealed no significant difference between the groups: 0.5 to 0.7% of n-3 fatty acids consumed were excreted, 35 to 40% accumulated, and 58.9 to 64.5% disappeared. The DHA diet contained 18:3n-3 but in amounts too low (i.e., 9 mg per 100 g diet) to interfere with the 22:6n-3 metabolism. Indeed, rats fed this diet ingested only 49 mg of this fatty acid, of which 30 mg were deposited in their whole body (Fig. 1). Moreover, this diet supplied preformed 22:6n-3 and a high ratio of 18:2n-6 to 18:3n-3, which are known to strongly inhibit the desaturation–elongation rate

**TABLE 2**
**Ingestion, Body and Organ Weights of Rats Fed Either ALA or DHA[a]**

| | Dietary group | | | |
|---|---|---|---|---|
| | ALA | | DHA | |
| Amount ingested (g) | | | | |
| Diet | 534 ± 22 | | 548 ± 28 | |
| Total fatty acids | 25.4 ± 1.1 | | 26.0 ± 1.3 | |
| | Age (wk) | | | |
| | 3 | 8 | 3 | 8 |
| Body weight (g) | 52.9 ± 1.8 | 235.8 ± 19.0 | 51.9 ± 3.0 | 245.9 ± 17.8 |
| Organ compartments (g) | | | | |
| Brain | 1.4 ± 0.1 | 1.7 ± 0.3 | 1.5 ± 0.0 | 1.8 ± 0.1 |
| Liver | 1.8 ± 0.1 | 8.0 ± 1.1 | 1.8 ± 0.1 | 8.9 ± 1.1 |
| Viscera[b] | 5.0 ± 0.3 | 19.2 ± 3.4 | 5.2 ± 0.5 | 21.5 ± 2.1 |
| Internal fats[b] | 1.0 ± 0.3 | 8.3 ± 3.0 | 1.0 ± 0.2 | 10.1 ± 2.3 |
| Subcutaneous fats[b] | 2.2 ± 0.8 | 7.1 ± 2.1 | 2.1 ± 0.9 | 9.5 ± 3.2 |
| Remaining carcass[b] | 36.7 ± 1.3 | 168.8 ± 13.7 | 35.5 ± 1.6 | 173.1 ± 14.4 |

[a]Values (means ± standard deviation, $n = 6$). In both diets, the n-3 fatty acid amount was 200 mg/100 g diet. No significant effect of the diet ($P < 0.05$) by analysis of variance was noted for any of the parameters studied.
[b]Viscera was the pool of gut, pancreas, spleen, testes, bladder, kidneys, heart, thymus, and lungs. Internal fats were the pool of epididymal, mesenteric, omental, and perirenal adipose tissues. Subcutaneous fats corresponded to all the subcutaneous adipose tissues that were possible to collect. The remaining carcass corresponded to the pool of the skin, muscles, the whole skeleton, and the head minus the brain. For abbreviations see Table 1.

**TABLE 3**
**Intake, Excretion, Accumulation, and Disappearance of n-3 Fatty Acids in the Growing Rat Fed Either ALA or DHA[a]**

| | Dietary group | | | | | |
|---|---|---|---|---|---|---|
| | ALA | | | DHA | | |
| | 18:3n-3 | 22:6n-3 | Σ LC-PUFA[b] | 18:3n-3 | 22:6n-3 | Σ LC-PUFA |
| | Amount of fatty acids (mg) | | | | | |
| Dietary intake | 1168 ± 48 | ND[c] | ND | 49 ± 2* | 1094 ± 55* | 1100 ± 55* |
| | | Σ = 1168 ± 48 | | | Σ = 1149 ± 54 | |
| Excretion | 5 ± 1 | ND | 4 ± 2 | ND | 5 ± 1* | 5 ± 1 |
| Whole-body content[d] | | | | | | |
| Beginning (3-wk-old) | 45 ± 6 | 29 ± 2 | 41 ± 3 | 4 ± 1* | 91 ± 15* | 97 ± 14* |
| End (8-wk-old) | 293 ± 64 | 193 ± 14 | 242 ± 9 | 34 ± 9* | 420 ± 61* | 480 ± 41* |
| Whole-body accumulation[e] | 248 ± 65 | 164 ± 13 | 201 ± 14 | 30 ± 8* | 329 ± 63* | 383 ± 70* |
| | | Σ = 471 ± 60[f] | | | Σ = 413 ± 74 | |
| Disappearance[g] | 688 ± 63 | — | — | 19 ± 3 | 706 ± 99 | — |
| % of fatty acid intake[h] | | | | | | |
| Excretion | 0.4 ± 0.1 | — | 0.3 ± 0.2 | — | 0.5 ± 01* | 0.5 ± 01 |
| Whole-body accumulation | 21.2 ± 4.9 | 14.0 ± 1.3 | 17.2 ± 1.2 | — | 30.1 ± 5.6* | 35.0 ± 5.2* |
| | | Σ = 40.3 ± 5.1 | | | Σ = 35.0 ± 5.1 | |
| Disappearance | 58.9 ± 5.0 | — | — | — | 64.5 ± 7.4 | — |

[a]Values are means ± standard deviation ($n$ = 6); in both diets, the n-3 fatty acid amount was 200 mg/100 g diet; * indicates a significant effect of the diet ($P < 0.01$) by analysis variation.
[b]LC-PUFA, long-chain polyunsaturated fatty acid: sum of 20:5n-3, 22:5n-3, and 22:6n-3.
[c]ND, not detected. For other abbreviations see Table 1.
[d]The whole-body content was the sum of the fatty acid amounts in the six organ compartments (brain, liver, viscera, internal and subcutaneous fats, remaining carcass).
[e]The whole-body accumulation was calculated as the difference between 8 and 3 wk of age in whole body content.
[f]In the ALA group the whole-body accumulation of total n-3 fatty acids included 18:4n-3 (22 mg mostly deposited in adipose tissues).
[g]18:3n-3 and 22:6n-3 disappearance was calculated as the difference between the dietary intake and the amount excreted in feces and accumulated in the whole body.
[h]In the DHA group, the amount of 18:3n-3 deposited in the whole body was not taken into account for the analysis of the 22:6n-3 fatty acid balance expressed as percentage of fatty acid intake.

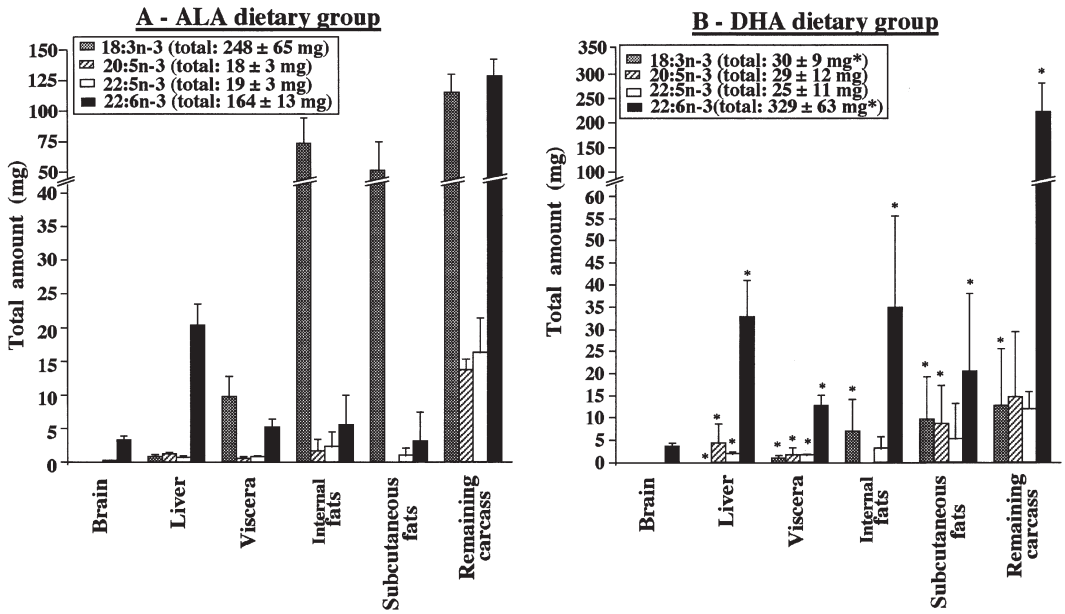


**FIG. 1.** Organ distribution of n-3 fatty acids deposited in the growing rat fed either α-linolenate (ALA) or docosahexaenoate (DHA). Values are means ± standard deviation ($n$ = 6). In both diets, the n-3 fatty acid amount was 200 mg/100 g diet. For each n-3 fatty acid, * indicates a significant effect of the diet, $P < 0.05$.

of 18:3n-3. So we can estimate that less than 1% of the total long-chain n-3 fatty acids deposited in rats fed DHA diet was biosynthesized from 18:3n-3.

In the ALA group, the amounts of both 18:3n-3 and the sum of its LC derivatives deposited in the whole body were similar (248 and 201 mg, respectively, i.e., 21 and 17% of the total intake) (Table 3), with 22:6n-3 as the main LC n-3 fatty acid derivative deposited (164 mg, i.e., 81.6%). However, their distribution between organ compartments differed (Fig. 1), 50% of accumulated 18:3n-3 was found in adipose tissues (29% in internal fats and 21% in subcutaneous fats) and 46% in the remaining carcass, whereas for 22:6n-3, 78% was deposited in the remaining carcass, 12% in the liver and only 5% in adipose tissues. In the DHA group, the total amount of 22:6n-3 accumulated in the whole body was twofold higher than in the ALA group (329 ± 63 mg vs. 164 ± 13 mg, $P < 0.01$), which corresponded to 86% of total deposited n-3 fatty acids and 30% of the total intake of 22:6n-3. This higher deposition was observed in all organ compartments. The distribution of 22:6n-3 among the six organ compartments was similar for the two groups except that the proportion found in adipose tissues was significantly higher in the DHA group (17%). The type of diet had no significant effect on the total amounts of 20:5n-3 and 22:5n-3 or on their organ distribution (Fig. 1). These fatty acids were found in equal amounts and accounted for 14 to 18% of total deposited n-3 LC-PUFA. They were mainly accumulated in the remaining carcass (81%) for the ALA group and in the remaining carcass (50%) and adipose tissues (34%) for the DHA group.

*n-6 Fatty acid balance.* The n-6 fatty acid balance was similar for the two groups, except that the amount of n-6 LC-PUFA deposited in the whole body was 22% lower for the DHA diet group (359 ± 60 mg vs. 459 ± 43 mg, $P < 0.01$) (Table 4). The percentage of dietary 18:2n-6 excreted was 0.5%, 46 to 51% was accumulated, and, by subtraction, 48 to 53% had disappeared. The main n-6 fatty acid deposited was 18:2n-6 (2407 to 2692 mg, i.e., 84 to 88% of total n-6 PUFA) and was principally found in the adipose tissues (47–51% with 28–30% in internal fats and 19–21% in subcutaneous fats) and the remaining carcass (44–48%) (Fig. 2). Of the n-6 LC-PUFA, 20:4n-6 was the main component deposited (324 mg in the ALA group and 243 mg in the DHA group) and represented 11% of total n-6 fatty acids deposited in the ALA group and significantly less in the DHA group (8%, $P < 0.01$) (Table 4). In both groups, the remaining carcass (50–61%, significant difference, $P < 0.01$), the liver (20–25%), and the viscera (11–13%) were the three major organs that accumulated 20:4n-6, contrary to brain, in which only 0.3 to 0.5% of total 20:4n-6 was deposited (Fig. 2). On the other hand, a significantly higher deposition of 20:3n-6 was observed in the whole body, mainly in the liver and viscera, in animals of the DHA group.

## DISCUSSION

*Whole-body balance of n-3 fatty acids.* During the 5-wk balance period, both n-3 fatty acids were readily absorbed (>99%). The high absorption of 22:6n-3 may have resulted from the predominant acylation of this fatty acid in the internal *sn*-2

**TABLE 4**
**Intake, Excretion, Accumulation, and Disappearance of n-6 Fatty Acids in the Growing Rat Fed Either ALA or DHA[a]**

| | Dietary group | | | | | |
|---|---|---|---|---|---|---|
| | ALA | | | DHA | | |
| | 18:6n-6 | 20:4n-6 | Σ LC-PUFA[b] | 18:2n-6 | 20:4n-6 | Σ LC-PUFA |
| | Amount of fatty acids (mg) | | | | | |
| Dietary intake | 6193 ± 255 | — | — | 5928 ± 300 | — | — |
| Excretion | 12 ± 2 | 6 ± 1 | 16 ± 2 | 16 ± 2 | 6 ± 2 | 14 ± 3 |
| Whole-body content[c] | | | | | | |
| Beginning (3-wk-old) | 480 ± 45 | 111 ± 5 | 174 ± 10 | 443 ± 94 | 87 ± 8* | 144 ± 17* |
| End (8-wk-old) | 2887 ± 512 | 435 ± 30 | 633 ± 40 | 3135 ± 503 | 330 ± 41* | 503 ± 58* |
| Whole-body accumulation[e] | 2407 ± 518 | 324 ± 27 | 459 ± 43 | 2692 ± 502 | 243 ± 42* | 359 ± 60* |
| | | Σ = 2866 ± 538 | | | Σ = 3051 ± 519 | |
| Disappearance[g] | 3293 ± 518 | — | — | 2841 ± 626 | — | — |
| % of fatty acid intake[h] | | | | | | |
| Excretion | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.1 ± 0.0 | 0.2 ± 01 |
| Whole-body accumulation | 38.9 ± 7.3 | 5.2 ± 0.5 | 7.4 ± 0.6 | 45.4 ± 9.1 | 4.1 ± 0.7* | 6.1 ± 0.6 |
| | | Σ = 46.3 ± 8.7 | | | Σ = 51.5 ± 8.8 | |
| Disappearance | 53.2 ± 7.7 | — | — | 47.9 ± 9.4 | — | — |

[a]See footnotes in Table 3.
[b]Sum of 20:2n-6, 20:3n-6, 20:4n-6, 22:2n-6, 22:4n-6, and 22:5n-6.
[c]The whole-body content was the sum of the fatty acid amounts in the six organ compartments (brain, liver, viscera, internal and subcutaneous fats, remaining carcass).
[d]The whole-body accumulation was calculated as the difference between 8 wk and 3 wk of age in whole-body content.
[e]18:2n-6 disappearance was calculated as the difference between the dietary intake and the amount excreted in feces and accumulated in the whole body. For abbreviations see Table 1.

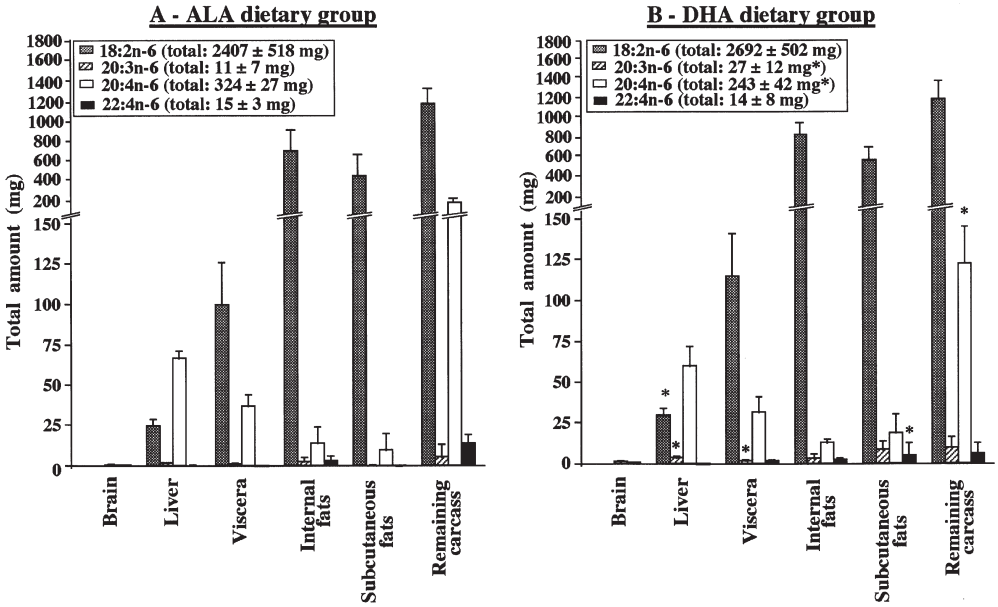


**FIG. 2.** Organ distribution of n-6 fatty acids deposited in the growing rat fed either ALA or DHA. Values are means ± standard deviation ($n = 6$). In both diets, the n-3 fatty acid amount was 200 mg/100 g diet. For each n-6 fatty acid, * indicates a significant effect of the diet, $P < 0.05$. For abbreviations see Figure 1.

position in algae-derived triglycerides (50% of the total 22:6n-3) (17), which seems to favor the efficiency of its digestion (18).

*In vivo* tracer studies have shown that the oxidation rate of 18:3n-3 is efficient in the growing rat (up to 65%) and is comparable to the one observed for the most highly oxidized fatty acid, lauric acid (12:0) (9). Using the fatty acid balance method, Cunnane and Anderson (10) suggested that the β-oxidation pathway is responsible for much of the whole-body utilization of dietary 18:3n-3 in the growing rat, as 85% of 18:3n-3 ingested disappeared. With the same method and a similar growth period in the rat, we confirmed these data but found here that only 59% of the 18:3n-3 dietary intake disappeared. This 30% relative decrease in 18:3n-3 disappearance could be explained by the 4.6-fold lower amount of dietary 18:3n-3 and twofold lower proportion of total fats in the diets in our study (19). Conversely, in the study the proportion of 18:3n-3 deposited in the whole body was higher (21% of the dietary intake vs. 10.9%) than that of total n-3 LC-PUFA (17% vs. 1.4%). However, expressed as total amount deposited daily, the whole-body deposition of n-3 LC-PUFA was very similar and represented around 3 mg. These observations are in acceptable agreement with the hypothesis that the bioavailability of 18:3n-3 to be converted to n-3 LC-PUFA (and mainly 22:6n-3) should be maintained, even if the energetic status or n-3 fatty acid dietary supply is insufficient (19). Altogether, our results are in accordance with the view that a large proportion of the ingested 18:3n-3 disappears, probably *via* β-oxidation, but our results do not support the notion that the biosynthesis pathway of n-3 LC-PUFA is quantitatively of minor importance.

We found that the disappearance of dietary 22:6n-3 was similar to that of 18:3n-3 (64.5% of the intake) and predominated over the n-3 fatty acid deposition. Although these data do not constitute conclusive proof, they argue for a high β-oxidation rate of 22:6n-3. This finding is unexpected because data from *in vivo* and *in vitro* radiotracer methodology show that the oxidation of LC-PUFA is generally low (9,11). But the new comprehensive view of the β-oxidation pathway of 22:6n-3 (first a peroxisomal retroconversion of 22:6n-3 to 20:5n-3 followed by a mitochondrial oxidation of 20:5n-3) and its regulation by dietary n-3 PUFA could partly explain it (reviewed in Ref. 20). Indeed, the presence of 22:6n-3 in dietary lipids induces an increase in the rate of peroxisomal β-oxidation of 22:6n-3 and activities of the key enzymes involved (21–24). Besides, the large disappearance of 22:6n-3 is consistent with the large deposition of its retroconverted products in organ compartments (14.1% of total n-3 fatty acids), either as 20:5n-3 or 22:5n-3, the 20:5n-3 elongation product (7.5 and 6.6% of total n-3 fatty acids, respectively). Studies conducted *in vitro* on rat liver and *in vivo* with or without labeled 22:6n-3 also showed an active retroconversion rate of 22:6n-3 in these two LC-PUFA (25,26).

Although dietary 18:3n-3 and 22:6n-3 disappeared in the same proportion, their partitioning between organ compartments was different, particularly their incorporation into adipose tissues. Adipose tissues were not of major importance in 22:6n-3 deposition (17% of the whole-body accumulation), whereas they represented the main storage compartment for 18:3n-3 (50%). This difference in incorporation may result from both differential uptake from blood lipids and release *via* lipolysis by adipocytes of 22:6n-3 and 18:3n-3 (27). n-3 Fatty acids stored in adipose tissues are considered a reservoir to cover body requirements, although the specific bioavailability of 22:6n-3 for other tissues, and particularly for nervous tissues, *via* its mobilization from adipose tissues is uncertain during suboptimal n-3 dietary supply (28).

*Whole-body balance of n-6 fatty acids.* In rats fed the ALA

diet, the proportion of dietary 18:2n-6 that disappeared was less than that of 18:3n-3. Moreover, and similarly as we noted above for 18:3n-3, it was 30% lower than that demonstrated by Cunnane and Anderson (10) (about 50% of the amount ingested in our study vs. 76%). However, in contrast to 18:3n-3, the 18:2n-6 dietary amount was the same, and only the proportion of lipids in our diet differed. So we may suggest that this lower partitioning toward disappearance of the two essential fatty acids resulted from the lower amount of total lipids in our diets. n-6 PUFA were mainly deposited as 18:2n-6 (84 to 88%) in adipose tissues (about 50% of total 18:2n-6 stored). In these tissues, the ratio of 18:2n-6 to 18:3n-3 was 50% higher than in dietary lipids (9:1 *vs.* 6:1) and thereby reflected the lower proportion of 18:2n-6 disappeared and converted to LC-PUFA than 18:3n-3. Thus, this ratio in adipose tissues reflects not only the dietary intake of 18:2n-6 and 18:3n-3 (29) but also the metabolic partitioning of these fatty acids. As regards the conversion activity of n-6 fatty acids, we found that 13.1 mg of 18:2n-6 was converted daily into n-6 LC-PUFA, i.e., 7.3% of the total dietary intake, a value higher than that found previously (9.8 mg/day, 3% of the dietary intake) (10). We assume that it likely resulted from the higher ratio of 18:2n-6 to 18:3n-3 used in our study (6:1 vs. 1.25:1), which could prevent the inhibition of 18:2n-6 desaturation–elongation induced by dietary excess of n-3 fatty acids.

The consumption of 22:6n-3 did not modify the whole-body metabolism of 18:2n-6, but it did alter the distribution of the n-6 fatty acids accumulated. Indeed, deposition of higher contents of 18:2n-6 and 20:3n-6 and lower contents of 20:4n-6 were noticed. Thus, the 20:3n-6/18:2n-6 and 20:4n-6/20:3n-6 ratios, which are indexes of Δ6- and Δ5-desaturation activities, respectively, were both decreased and thereby provided an indirect confirmation of the well-known inhibitory effect of dietary LC n-3 fatty acids on the activity of these enzymes (21).

The fatty acid balance analysis shows that disappearances of dietary 18:3n-3 and 22:6n-3 were comparable, which leads us to speculate that these two n-3 fatty acids were β-oxidized at similar rates. So further *in vivo* tracer studies measuring expired $CO_2$ in rats fed either 18:3n-3 or 22:6n-3 are needed, especially since an *in vitro* study did not confirm this hypothesis (30). The comparison of the data of Cunnane and Anderson (10) with that of the present study suggests that the amount of total fatty acids in the diet may influence the proportion of PUFA that disappeared and therefore implies that additional research on essential fatty acid partitioning in relation to energy balance is crucial to determine the n-6 and n-3 fatty acid requirements of humans, and notably of neonates.

## ACKNOWLEDGMENTS

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# Hyperthyroidism Affects Lipid Metabolism in Lactating and Suckling Rats

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**ABSTRACT:** Two per thousand pregnant women have hyperthyroidism (HT), and although the symptoms are attenuated during pregnancy, they rebound after delivery, affecting infant development. To examine the effects of hyperthyroidism on lactation, we studied lipid metabolism in maternal mammary glands and livers of hyperthyroid rats and their pups. Thyroxine (10 μg/100 g body weight/d) or vehicle-treated rats were made pregnant 2 wk after commencement of treatment and sacrificed on days 7, 14, and 21 of lactation with the litters. Circulating triiodothyronine and tetraiodothyronine concentrations in the HT mothers were increased on all days. Hepatic esterified cholesterol (EC) and free cholesterol (FC) and triglyceride (TG) concentrations were diminished on days 14 and 21. Lipid synthesis, measured by incorporation of $[^3H]H_2O$ into EC, FC, and TG, fatty acid synthase, and acetyl CoA carboxylase activities increased at day 14, while incorporation into FC and EC decreased at days 7 and 21, respectively. Mammary FC and TG concentrations were diminished at day 14; incorporation of $[^3H]H_2O$ into TG decreased at days 7 and 21, and incorporation of $[^3H]H_2O$ into FC increased at day 14. In the HT pups, growth rate was diminished, tetraiodothyronine concentration rose at days 7 and 14 of lactation, and triiodothyronine increased only at day 14. Liver TG concentrations increased at day 7 and fell at day 14, while FC increased at day 14 and only acetyl CoA carboxylase activity fell at day 14. Thus, hyperthyroidism changed maternal liver and mammary lipid metabolism, with decreased lipid concentration in spite of increased liver rate of synthesis and decreases in mammary synthesis. These changes, along with the mild hyperthyroidism of the litters, may have contributed to their reduced growth rate.

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Two per 1000 pregnant women have hyperthyroidism (HT) (1). Most of the symptoms of hyperthyroidism are attenuated during pregnancy, but the marked rebound after delivery has impact on infant development. Specific signs in the neonate include growth retardation, advanced bone age in relation to chronological age, and craniosynostosis (2). Hyperthyroxinemia and suppressed serum thyroid-stimulating hormone (TSH) in postnatal blood confirm the diagnosis (1). Similarly, in hyperthyroid rats, TSH secretion in the pituitary is suppressed, and serum and milk TSH concentrations are reduced as well (3).

Lactation is associated with widespread changes in whole-body lipid metabolism, the purpose of which is to direct lipids and lipid precursors to the mammary glands for milk-fat production (4,5). Fat is the major component of the milk, with 95% of this fat being triglycerides (TG) (4). Survival of all newborn mammals is dependent upon an adequate milk supply secreted from the mammary glands of the mother (6). Triiodothyronine ($T_3$) administration in physiological amounts is able to stimulate prolactin-induced synthesis of milk products and the enzymatic activities related to this process in differentiated rat mammary glands (7), but there is a paucity of studies concerning the effects of thyroid hormone excess on lactation and mammary gland function. Thyroid hormones affect a number of physiological processes including lipid, carbohydrate, and protein metabolism (8–10). Studies with litter-removed lactating rats have shown that hyperthyroidism depresses lipoprotein lipase (LPL) activity in mammary gland and white adipose tissue (7).

Rosato *et al.* (11,12) showed that the chronic administration of tetraiodothyronine ($T_4$) (100 μg/100 g body wt) produces marked changes in organ weight, lipid and protein content, and enzymatic activities (11) in virgin rats. These changes were markedly attenuated in pregnant rats near term (12) as well as in their fetuses (11), but the treatment produced advances in delivery and lactogenesis, with adverse effects on maternal behavior and milk release that resulted in death of the pups (12). The administration of 25 μg $T_4$/100 g body wt showed similar effects on delivery and pup mortality (13). Additionally, we previously showed that chronic administration of a lower dose of 10 μg/100 g body wt of $T_4$ in virgin females produced important changes in liver lipid metabolism (14).

In the present work, we investigated the effects of chronic administration of 10 μg $T_4$/100 g body wt on the lipid metabolism of the dams and their pups at days 7, 14, and 21 of lactation. This dose regimen was selected because it produced hyperthyroidism as measured by increases in circulating thyroid hormones and allowed the rats to nurse the litters until weaning.

## MATERIALS AND METHODS

*Chemicals and radioisotopes*. $[^3H]H_2O$ (3.70 GBq/g) and $[^{14}C]NaHCO_3$ (39.2 MBq/mmol) were purchased from Dupont, New England Company (Boston, MA). Lipid standards were acquired from Sigma Chemical Co. (St. Louis, MO). L-Tetraiodothyronine ($T_4$) was a generous gift from Glaxo (Buenos Aires, Argentina). All the other chemicals

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Abbreviations: ACC, acetyl CoA carboxylase; Co, control rat; CPT-1, carnitine palmitoyltransferase 1; DTT, dithiothreitol; EC, esterified cholesterol; FAS, fatty acid synthase; FC, free cholesterol; HT, hyperthyroid rat = thyroxine-treated rat; LPL, lipoprotein lipase; $T_3$, triiodothyronine; $T_4$, tetraiodothyronine; TG, triglycerides; TSH, thyroid-stimulating hormone.

were of reagent grade and were obtained from Merck Laboratory (Buenos Aires, Argentina).

*Animals and experimental design.* Adult female Wistar rats bred in our laboratory, 3- to 4-mon-old, and weighing 190–210 g at the onset of treatment were used. The rats were housed in an animal room and kept in a 22–25°C controlled environment with a light-dark cycle of 12 h each. Rat chow and tap water were available *ad libitum*. Hyperthyroidism was induced by daily subcutaneous injection of $T_4$ (at a dose of 10 µg/100 g body wt) dissolved in 0.9% NaCl and alkalinized with NaOH to pH 9. The presence of spermatozoa in the vaginal smear the morning after caging with a fertile male during the night of proestrus was indicative of pregnancy and this day was counted as day 0 of pregnancy. Thyroxine-treated (= hyperthyroid; HT) or vehicle-treated (= control; Co) rats were made pregnant approximately 14 d after commencement of treatment and sacrificed on day 7, 14, or 21 of lactation at 0900–1000 by decapitation. The rats were injected during six ($L_7$), seven ($L_{14}$), and eight ($L_{21}$) weeks.

On day 1 of lactation, the number of pups in each litter was standardized to eight. Trunk blood of the dams and the pups was collected, and serum was separated by centrifugation and stored at –20°C until used. The livers from dams and pups and inguinal mammary glands from the dams were removed, washed in a cold saline solution, and stored at –70°C until they were analyzed. The values are means ± standard error of the means (SEM) for groups of eight lactating rats.

Blood and tissues of the pups were pooled for each litter and thus the values represent the means of the values for the litters. The body weights of the pups are the means ± SEM for eight litter means.

Animal maintenance and handling were performed according to the *NIH Guide for the Care and Use of Laboratory Animals* (NIH publication No. 86–23, revised 1985 and 1991) and the United Kingdom requirements for ethics of animal experimentation [Animals (Scientific Procedures) Act 1986].

*Serum determinations*. Serum $T_3$ and $T_4$ total concentrations were determined by a commercial enzyme-linked immunosorbant assay kit purchased from Boehringer, Mannheim, Germany.

*Tissue preparation and enzymatic assays.* Liver portions (1 g for 4 mL of buffer) were homogenized in an Ultra Turrax T25 homogenizer (Jahnke & Kunkle, Stauffen, Germany) in 0.5 M potassium phosphate buffer (pH 7) containing 10 mM EDTA and 10 mM D,L-dithiothreitol (DTT). The homogenates were centrifuged at 100,000 × *g* for 1 h to yield the cytosolic fraction in a Beckman model L8-80M ultracentrifuge with a Ty-80 rotor.

Cytosolic fatty acid synthase (FAS) activity was determined spectrophotometrically by a modified version of the method of Alberts *et al.* (15). The reaction mixture contained a 0.5 M potassium phosphate buffer (pH 6.6), 1 µmol each of EDTA and DTT, respectively, 100 nmol NADPH, and 0.05 mL of the cytosolic fraction. The reaction was started by adding 100 nmol of malonyl-CoA, and the final assay volume was 1.05 mL. The oxidation of NADPH at 30°C was monitored at 340 nm.

Acetyl CoA carboxylase (ACC) activity in liver or mammary cytosols was measured as described previously by Allred and Rochringer (16). The enzyme activity was measured using a reaction mixture that, in a final volume of 700 µL, contained 60 mM buffer Tris-acetate (pH 7.8), 100 mM potassium acetate, 3 mM DTT, 8.5 mM potassium citrate, 1 mM ATP, 0.6 mg/mL bovine serum albumin, 0.35 mM acetyl CoA, 8 mM magnesium acetate, 25 mM sodium bicarbonate, and 2 µCi [$^{14}$C]$NaHCO_3$. For blanks, acetyl-CoA was omitted. The mixture was preincubated 1 min at 37°C. Then 50 µL of the cytosolic fraction was added. After 1 min of incubation at 37°C, 50 µL of concentrated HCl was added to stop the reaction. A 200 µL aliquot was transferred into the scintillation vial and dried under cold air flow. The dried extract was resuspended with 200 µL 50% ethanol, and the radioactivity was measured in 10 mL of scintillation fluid in a Wallac LKB 1409 liquid scintillation analyzer. ACC activity was expressed as units per mg of protein, where 1 unit equals 1 pmol of [$^{14}$C]bicarbonate incorporated into malonyl-CoA per minute at 37°C. The protein concentration was determined by the method of Lowry *et al.* (17), using fraction V bovine serum albumin as standard.

*Lipid determinations*. The lipids from the hepatic or mammary tissue were extracted with chloroform/methanol (2:1) according to the method of Folch *et al.* (18). An aliquot of the lipid extracts was taken to determine total cholesterol, and another one to separate the different lipid fractions by thin-layer chromatography with an *n*-hexane/diethyl ether/acetic acid (80:20:1, by vol) solvent system. After eluting the scraped bands, aliquots were used for the mass determination according to the methods of Sardesai and Manning (19) for TG and of Zack *et al.* (20) after saponification (21) for free cholesterol (FC) and esterified cholesterol (EC). A recovery from thin-layer chromatography averaging 90% of cholesterol mass was obtained.

*Incorporation of $^3H$ from $H_2O$ into lipids*. The groups of lactating rats were fed *ad libitum* and then injected intraperitoneally with $^3H_2O$ (3.7 mBq/rat in 9 g/L of NaCl). They were killed 1 h later to ensure that the newly synthesized lipids in the liver and mammary gland had been labeled. One gram of the liver and mammary gland was extracted with 20 mL of chloroform/methanol (2:1) according to the method of Folch *et al.* (18). The radioactivity incorporated into the lipids was counted in the different lipid fractions that had been separated by thin-layer chromatography (see section above on lipid determinations). The results are expressed as pg $^3H$ incorporated/h/g of tissue.

*Statistical analyses.* Significant differences among means were considered at a level of $P < 0.05$ and identified by one-way analysis of variance and the Tukey test. In all cases, the variances were homogeneous.

## RESULTS

*Serum $T_4$ and $T_3$ concentrations in Co and HT lactating rats and their pups.* Serum $T_4$ and $T_3$ concentrations in the HT group of lactating rats were increased significantly compared with the Co group (Fig. 1).

The serum $T_4$ concentrations of the pups of the HT lactating dams were also increased significantly compared with the offspring of Co lactating dams on days 7 and 14 of neonatal

life, but not on day 21. The serum $T_3$ concentrations increased significantly on day 14 compared with the pups of the Co lactating dams, but no differences were observed on days 7 and 21 (Fig. 1).

*Body, liver weight, and total protein of liver and mammary gland in HT and Co lactating rats and their pups.* Table 1 shows that the liver weight of the HT mothers was increased significantly compared with Co only on day 7 of lactation. No differences were observed in body weight or in liver or mammary gland protein concentrations in any of the other studied days. The body weights of the pups of the HT lactating dams were decreased significantly compared with the Co on all the days of lactation. The total protein concentrations and liver weights on day 7 of lactation were lower than in the Co group, but no differences were observed on days 14 and 21 of lactation.

*TG, cholesterol concentrations, and activities of FAS and ACC in livers of the pups of HT and Co lactating rats.* The liver TG concentrations in the HT group were significantly decreased on days 14 and 21 of lactation when compared with the Co group, but no significant differences were observed on day 7 (Table 2). FC and EC concentrations were also significantly lower in the HT group on days 14 and 21, but no changes in cholesterol concentrations were observed on day 7 (Table 2).

As shown in Table 2, the FAS and ACC liver activities in the HT groups were higher than those of the Co on day 14 of lactation, while no differences were observed on the other days of lactation.

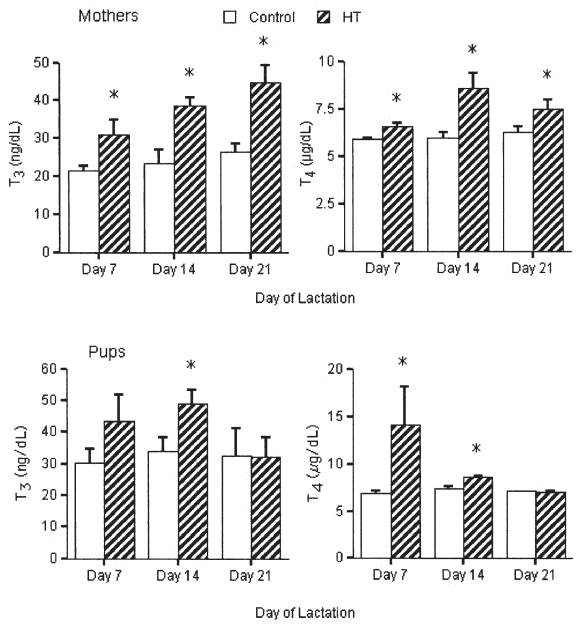


**FIG. 1.** Effects of hyperthyroidism on plasma thyroxine ($T_4$) and triiodothyronine ($T_3$) in lactating rats and their pups. Rats were made hyperthyroid (HT) by daily injections of $T_4$ at a dose of 10 µg/100 g body weight and mated 2 wk after beginning the treatments. Controls (Co) were injected with vehicle. The adult rats and their litters were sacrificed on days 7, 14, and 21 of lactation. $T_4$ and $T_3$ were measured by enzyme-linked immunosorbent assay. Values are means ± standard error of the means for groups of eight dams or eight litters. Asterisk (*) indicates $P < 0.05$ compared with the respective control groups using one-way analysis of variance and the Tukey test.

*Incorporation of $^3H_2O$ into liver of HT and Co lactating rats.* The incorporation of [$^3$H] from $^3H_2O$ into liver TG was significantly increased on day 14 of lactation compared to Co. On the other hand, incorporation into FC was significantly decreased on day 7, while incorporation into both FC and EC was increased on day 14. The incorporation in the EC was lower on day 21 compared with the Co group (Table 2).

*TG, cholesterol concentrations, and activities of FAS and ACC in livers of the pups of HT and Co lactating rats*. The TG concentrations in the HT group were increased significantly on day 7 and decreased on day 14 of lactation when compared with the Co group, but no differences were observed on day 21. FC concentrations in the pups of both HT lactating and Co dams were lower on day 14 compared with the other days of lactation. On this day the HT group had slightly higher values compared with the Co group, while there were no differences on days 7 and 21. On the other hand, there were no differences in the EC concentration between HT and Co groups on all the studied days, although an increase was observed in both groups on day 21 compared with the previous days (Table 2).

FAS liver activities decreased on day 7; however, there were no differences between HT and Co pups on days 14 and 21. The ACC liver activities decreased on day 14 but no differences were observed on days 7 and 21 of lactation (Table 2).

*Incorporation of $^3H_2O$ into lipids in liver of the pups of HT and Co lactating rats.* As shown in Table 2, no significant differences between HT and Co groups were observed in the incorporation of [$^3$H] from $^3H_2O$ into liver TG, FC or EC fractions. On the other hand, $^3H_2O$ incorporation into both fractions of cholesterol was increased on day 14 compared with day 7 and thereafter decreased to very low values on day 21 of lactation.

*Mammary gland TG and cholesterol concentrations and the incorporation of $^3H$ from $^3H_2O$ into the lipids of dams rats.* The mammary TG concentrations of the HT group showed lower values on day 14 compared with the Co group, while no changes were observed on days 7 and 21 of lactation. The concentration of TG was elevated on day 14 in both groups compared with the other days. FC concentrations were also decreased only on day 14 in the HT group when compared to Co, while no changes in EC concentrations were observed in the 3 d of lactation studied (Table 3).

In the mammary gland, there was a decrease in the incorporation of [$^3$H] into TG in the HT groups on days 7 and 21 of lactation compared with the Co, with no differences on day 14 of lactation. There was an increase in incorporation into FC in the HT group only on day 14 of lactation compared with the Co, while no differences were observed in the incorporation into EC fractions in the three studied days (Table 3).

## DISCUSSION

The present paper describes the changes in lipid metabolism caused by chronic $T_4$ treatment during the days previous to pregnancy, during pregnancy, and during lactation in the mammary gland and liver in the dams on days 7, 14, and 21 of lactation and the effects on the pups.

**TABLE 1**
**Effects of Hyperthyroidism on Body and Liver Weight and Liver Total Protein Concentration in Lactating Rats and Their Pups[a]**

| | Day 7 of lactation | | Day 14 of lactation | | Day 21 of lactation | |
|---|---|---|---|---|---|---|
| | Co | HT | Co | HT | Co | HT |
| Mothers | | | | | | |
| Liver weight (g) | 9.2 ± 0.5 | 11.2 ± 0.2[b] | 10.7 ± 1.1 | 12.0 ± 0.3 | 11.5 ± 0.4 | 10.8 ± 0.9 |
| Total liver protein (mg/g) | 104.2 ± 15.1 | 109.3 ± 16.4 | 187.4 ± 0.4 | 186.2 ± 2.9 | 181.9 ± 28.1 | 137.9 ± 16.9 |
| Total mammary protein (mg/g) | 121.6 ± 11.1 | 103.4 ± 6.8 | 128.0 ± 8.1 | 119.8 ± 8.8 | 122.5 ± 4.3 | 111.7 ± 3.5 |
| Body weight (g) | 250.7 ± 5.8 | 251.1 ± 11.1 | 253.0 ± 7.2 | 257.8 ± 5.2 | 260.2 ± 12.5 | 258.7 ± 2.3 |
| Pups | | | | | | |
| Liver weight (g) | 0.49 ± 0.03 | 0.40 ± 0.01[b] | 0.88 ± 0.04 | 0.84 ± 0.04 | 1.46 ± 0.17 | 1.65 ± 0.11 |
| Total liver protein (mg/g) | 146.2 ± 5.91 | 110.5 ± 7.41[b] | 168.00 ± 7.57 | 158.37 ± 7.34 | 167.4 ± 10.23 | 149.9 ± 8.64 |
| Body weight (g) | 14.71 ± 0.70 | 10.42 ± 1.01[b] | 25.74 ± 0.67 | 19.14 ± 1.81[b] | 39.60 ± 1.74 | 24.74 ± 2.95[b] |

[a]Values are means ± standard error of the means for groups of eight dams or eight litters. Co, control—vehicle-treated rats; HT, hyperthyroid—thyroxine-treated rats.
[b]$P < 0.05$ compared with the respective control groups using one-way analysis of variance and the Tukey test.

**TABLE 2**
**Effects of Hyperthyroidism on Liver Triglyceride and Cholesterol Concentrations and the Incorporation of $^3H$ from $^3H_2O$ into the Lipids and Lipogenic Enzyme Activities of Lactating Rats and Their Pups[a]**

| | Day 7 of lactation | | Day 14 of lactation | | Day 21 of lactation | |
|---|---|---|---|---|---|---|
| | Co | HT | Co | HT | Co | HT |
| Mothers | | | | | | |
| Triglyceride (TG) (μg/g of liver) | 1630 ± 115 | 2909 ± 686 | 3551 ± 377 | 1027 ± 128[b] | 2305 ± 244 | 1423 ± 275[b] |
| Free cholesterol (FC) (μg/g of liver) | 2700 ± 109 | 2561 ± 173 | 6473 ± 485 | 5158 ± 305[b] | 3527 ± 229 | 1960 ± 218[b] |
| Esterified cholesterol (EC) (μg/g of liver) | 515 ± 47 | 464 ± 27 | 6268 ± 1221 | 3370 ± 253[b] | 2775 ± 239 | 1362 ± 296[b] |
| FAS | 0.49 ± 0.03 | 0.62 ± 0.17 | 1.64 ± 0.11 | 3.30 ± 0.36[b] | 6.18 ± 0.75 | 6.19 ± 0.32 |
| ACC | 76.83 ± 1.91 | 87.65 ± 5.36 | 83.87 ± 4.65 | 115.73 ± 5.82[b] | 105.62 ± 15.1 | 128.8 ± 9.9 |
| $^3H$ Incorporation | | | | | | |
| To TG (ng $^3H$/h/g of liver) | 6.86 ± 0.73 | 5.44 ± 0.63 | 3.51 ± 0.43 | 5.38 ± 0.23[b] | 3.90 ± 0.44 | 3.90 ± 0.65 |
| To FC(ng $^3H$/h/g of liver) | 6.11 ± 0.76 | 3.00 ± 0.59[b] | 4.05 ± 0.32 | 5.17 ± 0.34[b] | 1.72 ± 0.24 | 1.57 ± 0.31 |
| To EC (ng $^3H$/h/g of liver) | 4.04 ± 1.26 | 3.63 ± 0.78 | 2.35 ± 0.61 | 4.16 ± 0.32[b] | 2.06 ± 0.09 | 0.82 ± 0.17[b] |
| Pups | | | | | | |
| Triglyceride (μg/g of liver) | 855 ± 132 | 1239 ± 65[b] | 2356 ± 227 | 1627 ± 132[b] | 1243 ± 177 | 1053 ± 147 |
| FC (μg/g of liver) | 1802 ± 336 | 2551 ± 621 | 333 ± 34 | 619 ± 57[b] | 2638 ± 334 | 3057 ± 184 |
| EC (μg/g of liver) | 471 ± 109 | 560 ± 90 | 401 ± 45 | 440 ± 67 | 1231 ± 106 | 1193 ± 146 |
| FAS | 0.93 ± 0.21 | 0.24 ± 0.05[b] | 1.68 ± 0.12 | 1.75 ± 0.4 | 1.12 ± 0.27 | 1.21 ± 0.26 |
| ACC | 16.1 ± 0.7 | 17.5 ± 1.9 | 19.9 ± 1.3 | 14.2 ± 0.6[b] | 29.3 ± 5.9 | 27.7 ± 3.3 |
| $^3H$ Incorporation | | | | | | |
| To TG (ng $^3H$/h/g of liver) | 1.48 ± 0.33 | 3.24 ± 0.82 | 2.57 ± 0.43 | 3.66 ± 0.32 | 3.81 ± 0.20 | 3.62 ± 0.52 |
| To FC (ng $^3H$/h/g of liver) | 1.40 ± 0.23 | 1.60 ± 0.40 | 2.88 ± 0.38 | 3.20 ± 0.37 | 0.30 ± 0.01 | 0.40 ± 0.11 |
| To EC (ng $^3H$/h/g of liver) | 0.67 ± 0.07 | 1.08 ± 0.18 | 2.53 ± 0.73 | 2.73 ± 0.35 | 0.23 ± 0.02 | 0.26 ± 0.01 |

[a]Values are means ± standard error of the means for groups of eight dams or eight litters. Fatty acid synthase (FAS) and acetyl coenzyme A carboxylase (ACC) activities were expressed as units/mg of cytosolic proteins; for other abbreviations see Table 1.
[b]$P < 0.05$ compared with the respective control groups using one-way analysis of variance and the Tukey test.

**TABLE 3**
**Effects of Hyperthyroidism on Mammary Gland Triglyceride and Cholesterol Concentrations and the Incorporation of $^3H$ from $^3H_2O$ into the Lipids of Lactating Rats[a]**

| | Day 7 of lactation | | Day 14 of lactation | | Day 21 of lactation | |
|---|---|---|---|---|---|---|
| | Co | HT | Co | HT | Co | HT |
| TG (μg/g of MG) | 3.18 ± 0.22 | 3.62 ± 0.43 | 40.37 ± 3.15 | 22.32 ± 3.90[b] | 2.10 ± 0.17 | 2.30 ± 0.20 |
| FC (μg/g of MG) | 2042 ± 330 | 1724 ± 290 | 1661 ± 73 | 1153 ± 119[b] | 3531 ± 353 | 3286 ± 204 |
| EC (μg/g of MG) | 849 ± 221 | 1346 ± 326 | 503 ± 82 | 515 ± 38 | 915 ± 86 | 964 ± 105 |
| Incorporation | | | | | | |
| To TG (ng $^3H$/h/g of MG) | 32.94 ± 1.37 | 28.05 ± 0.75[b] | 28.20 ± 2.89 | 29.36 ± 6.0 | 28.66 ± 8.74 | 13.34 ± 3.72[b] |
| To FC (ng $^3H$/h/g of MG) | 1.41 ± 0.24 | 1.42 ± 0.12 | 3.03 ± 0.11 | 3.98 ± 0.40[b] | 1.60 ± 0.26 | 1.31 ± 0.09 |
| To EC (ng $^3H$/h/g of MG) | 1.09 ± 0.13 | 1.12 ± 0.03 | 2.38 ± 0.13 | 2.60 ± 0.43 | 1.90 ± 0.94 | 0.98 ± 0.05 |

[a]Values are means ± standard error of the means for groups of eight dams. MG, mammary gland; for other abbreviations see Table 1.
[b]$P < 0.05$ compared with the respective control groups using one-way analysis of variance and the Tukey test. For abbreviations see Table 2.

*Effects of hyperthyroidism in lactating dams.* The dose of 10 μg of $T_4$ per 100 g body wt induced an increase in the circulating concentration of $T_3$ and $T_4$ in the dams at the three times of lactation studied, thus confirming the state of HT.

Long-chain fatty acids are structural components of all cells; they also serve as energy reserves and are involved in a variety of regulatory functions including their own synthesis and degradation (22). ACC is the enzyme that catalyzes the rate-limiting step in the biogenesis of long-chain fatty acids. It is well known that thyroid hormones increase FAS and ACC activities at a pretranslational step (23). In our experimental model the activities of FAS and ACC in the livers of HT rats increased on day 14 of lactation, corresponding to the increased synthesis of TG as observed by the $^3H_2O$ incorporation experiments, and accompanied by a paradoxical decrease in the mass of liver TG. Similar results were observed in HT virgin females (11), but no changes were found in enzyme activities on days 7 and 21 of lactation in spite of the decreased content of TG on day 21.

Increased malonyl-CoA, which is only generated by ACC, suppresses mitochondrial carnitine palmitoyltransferase I activity (CPT-I) (24); this may increase the level of cytosolic long-chain fatty acyl-CoA esters, which are known signals for insulin secretion (25). But, in the hyperthyroid state, fatty acid oxidation and ketogenesis are stimulated simultaneously along with a paradoxical stimulation of fatty acid synthesis (26). These processes may be linked, because thyroid hormones accelerate fatty acyl-CoA entry into the mitochondrial matrix by increasing mitochondrial CPT-I activity (27) and decreasing sensitivity of CPT-I to inhibition by malonyl-CoA (26). The liver plays a central role in the maintenance of whole-body cholesterol by integrating the regulation of a group of hepatic enzymes, receptors, and other proteins important for cholesterol homeostasis. During lactation the synthesis of cholesterol is increased, in part to provide *de novo* synthesized cholesterol for new membrane synthesis in the liver hypertrophy that occurs during lactation and also for lipoprotein secretion to provide cholesterol and triglycerides for milk production (28). Sixty percent of the total content of cholesterol in milk is of hepatic origin (29).

The lower content of FC in the livers of the HT mothers on day 14 of lactation compared to the control group, associated with the increased synthesis, may indicate that export from the liver to the mammary glands is also increased. On the other hand, in thyroxine-treated rats, an increase in the activity of hepatic acyl coenzyme A:cholesterol acyl transferase (ACAT) has been reported (30).

Consistent with this, we also observed an increase in the synthesis of EC on day 14 of lactation in the HT rat compared with the Co. The higher activities of hepatic lipogenic enzymes in the hyperthyroid rats suggest that there are sufficient fatty acids available for cholesterol esterification. However, the EC mass decreased in the HT rats, suggesting that export of EC from liver to mammary gland was also augmented.

In mammary tissue, acidic cholesterol ester hydrolase activities increase during lactation and fall 2 d after weaning, increasing the FC concentration destined to be secreted into milk (31). In our experimental model, we observed no differences in EC content and rate of synthesis in the HT rat compared with Co on day 7 or 21; however, on day 14 both groups showed the lowest EC content. On the other hand, in the HT group, FC concentration on day 14 of lactation was diminished, in spite of an increase in its synthesis, suggesting that there is an increased mobilization of cholesterol from the gland to milk on this day.

Milk TG and cholesterol are known to be derived from lipoproteins as well as originated from mammary *de novo* synthesis (28). On day 7 we observed a decrease in the synthesis of TG in the HT groups, without changes in TG tissue content. However, on day 14 there was a decrease in the mass of TG compared with the high values observed in the Co, while no changes were observed in the synthesis, which decreased on day 21. It is well known that milk stasis induces involution of the tissue (32). The decrease in TG synthesis and content observed on day 21, along with the reduced growth rate of the litter and histological changes (not shown), are suggestive of a premature mammary involution in the HT mothers.

Our results show that the most pronounced effects of HT in liver and mammary gland lipid metabolism were on day 14 of lactation, with effects that were similar to those observed previously in the virgin females. Day 14 may be considered the peak of lactation, when the growth rate of the litter is maximal, and, thus, the demand on maternal metabolism, especially mammary metabolism, may be at its greatest and the effects of HT most marked. In contrast, on day 7 no changes were observed, and on day 21 of lactation there was an amelioration of the effects observed on day 14. Moreover, the changes observed on day 21 could be associated with premature weaning and involution of the mammary glands.

*Effects of maternal HT on the metabolism of the pups.* We observed an increase in $T_4$ concentration in the HT pups at 7 and 14 d but not at 21 d old, suggesting that there is an important contribution of thyroid hormone from the milk. However, the $T_3$ concentration only increased in the pups at 14 d of life. Our results agree with previous data showing that the conversion rates of $T_4$ to $T_3$ in livers of pups before day 10 are very low and then rise to reach the maximal value at around day 23 of life (33). The $T_3$ and $T_4$ concentrations in pups at 21 d of life were not modified, probably because on this day the pups were already eating solid food and drinking water.

Chronic administration of $T_4$ to lactating rats negatively affected the growth rate of the litters in the 3 d studied, as is shown by the decrease in their body weights. These results agree with Rosato *et al.* (11), who showed that the administration of $T_4$ (100 μg/100 g body wt) produced an advance in lactogenesis in the mammary gland associated with a 100% mortality in the pups, suggesting deficient milk release, as the pups were unable to draw milk from the mothers in spite of vigorous suckling. Additionally we observed that the birth weights of the pups of hyperthyroid rats were smaller than the control group (HT: 6.11 ± 0.008 g and Co: 6.67 ± 0.11 g; $P < 0.001$). Moreover, we found that the liver weight in 7-d-old pups was also diminished. This decrease may be related to a loss in liver glycogen (HT: 47.15 ± 5.35 and Co: 110.86 ± 37.26 μM glucose/g of liver; $P < 0.05$) associated with a reduction in protein content.

Although thyroid hormones have been reported to stimulate the expression of ACC and FAS mRNA in the liver of the adult

rat, some evidence suggests that they do not play a primary role in the increase of lipogenic enzyme mRNA concentration after weaning to a high-carbohydrate, low-fat diet (34). Additionally, studies performed in suckling rats injected with $T_3$, during 2 d, reported no changes in the activity of the liver FAS (35). We observed in the liver of the pups a decrease in FAS activity at day 7 and of ACC activity at day 14 of lactation. On day 7 of lactation the liver TG of the pups are medium-chain TG and long-chain TG proceeding from the milk. The increased TG content at day 7 may be linked to the fact that thyroid hormone accelerates the absorption of lipids into the mucosal cells of the intestinal tract (36).

Maternal hyperthyroidism produces complex changes in liver and mammary lipid metabolism, which may be partially responsible for the diminished growth of the litter in combination with the hyperthyroid state of the pups themselves.

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# Evidence for Specific Ceramidase Present in the Intestinal Contents of Rats and Humans

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**ABSTRACT:** A neutral ceramidase activity stimulated by bile salt was previously identified in the intestinal content. Recently, bile salt stimulated lipase (BSSL) was found to have ceramidase activity. It is unknown whether the ceramidase activity previously found is attributable to BSSL. To address this question, we compared the behaviors of high quaternary aminoethyl (HQ) anion exchange chromatography, the distributions, the stability, and the responses to lipase inhibitor between ceramidase and pancreatic BSSL. The proteins from whole small intestinal contents of humans and rats were precipitated by acetone and dissolved in 20 mM Tris buffer pH 8.2. These proteins had neutral ceramidase activity but not BSSL activity against *p*-nitrophenyl acetate. When the proteins were subject to HQ chromatography, two peaks of ceramidase activity were identified, which had acid and neutral pH optima, respectively. Neither of them had BSSL activity against *p*-nitrophenyl acetate. Western blot using BSSL antiserum failed to identify BSSL protein in the fractions with high neutral ceramidase activity. In rat intestinal tract, pancreatic BSSL activity was high in the duodenum and declined rapidly in the small intestine, whereas neutral ceramidase activity was low in the duodenum and maintained a high level until the distal part of the small intestine. In addition, orlistat, the inhibitor of lipase, abolished human BSSL activity against *p*-nitrophenyl acetate and slightly reduced its activity against ceramide but had no inhibitory effect on ceramidase activity isolated by HQ chromatography. In conclusion, we provide the evidence for a specific ceramidase other than pancreatic BSSL present in the intestinal content. The enzyme may play important roles in digestion of dietary sphingolipids.

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In the gastrointestinal tract, there are several lipolytic enzymes, including gastric lipase, pancreatic lipase, pancreatic bile salt stimulated lipase (BSSL) and pancreatic phospholipase A2, that hydrolyze dietary lipids (1). While the enzymes responsible for digestion of fat and glycerophospholipids have been intensively studied, those for digestion of dietary sphingolipids have received less attention.

Sphingomyelin (SM) is the major form of sphingolipids. It is present not only in eukaryotic cell membranes but also in Western food such as milk, meat, fish, and egg in a considerable amount (2,3). SM is hydrolyzed by sphingomyelinase (SMase) that cleaves the phosphocholine head group and converts SM to ceramide. Ceramide is further hydrolyzed by ceramidase to sphingosine and fatty acids. In the intestinal tract, an SMase with an optimal alkaline pH was previously reported and characterized (4,5). The alkaline SMase may have important roles in triggering the digestion of dietary SM (4,6). A ceramidase activity was also previously identified in the intestinal mucosa of pig and duodenal content of human beings (7). The optimal pH of the activity is 7.4 to 7.6 in the presence of 2 mM taurodeoxycholate. Recently, Hui *et al.* (8) found that recombinant BSSL had lipoamidase activity. This result was confirmed and extended by Nyberg *et al.* (9), who showed that BSSL in human milk was able to hydrolyze ceramide at pH values ranging from 7 to 10 in the presence of 4 mM bile salt mixture. Pancreatic BSSL is the product of the gene of BSSL in milk and is present in pancreatic juice and released in the intestine (10). A question is thus naturally raised: Is the ceramidase activity found previously in the intestinal content actually contributed by pancreatic BSSL or is a specific ceramidase present in the intestinal content? In the present investigation, we address this question by comparing the chromatographic behaviors, the distributions, the stability, and the sensitivities to lipase inhibitor between the two enzymes.

## MATERIALS AND METHODS

*Materials.* Palmitoyl ceramide, taurocholate, and *p*-nitrophenyl acetate (*p*-NPA) were purchased from Sigma Co. (St. Louis, MO). 1-[$^{14}$C]Palmitoyl-labeled ceramide ([$^{14}$C]Cer) was provided by Astra Draco (Lund, Sweden), and was prepared by converting 1-[$^{14}$C]palmitic acid to the corresponding anhydride with palmitoyl chloride, followed by reacting the anhydride with sphingosine. The labeled ceramide was purified by flash-column chromatography, and the purified ceramide had a specific activity of 15.7 mCi/mmol with radiochemical purity more than 97% (9). Orlistat (capsule Xenical®) was obtained from University Hospital of Lund, Sweden. For *in vitro* use, the contents of one capsule were dissolved in ethanol (95%), followed by centrifugation for 10 min at 2,000 rpm to remove insoluble material (11). Antiserum against human BSSL was a gift from Dr. Berit Sternby, Lund University Hospital. The IgG purified from the antiserum has been previously shown to be able to inhibit BSSL activity against ceramide (9).

Human small intestinal content was collected from stomas of three individuals who had been previously treated with ileostomy due to ulcerative colitis and were fully recovered from the operation. The contents were freeze-dried first and

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Abbreviations: BSSL, bile salt stimulated lipase; [$^{14}$C]Cer, 1-[$^{14}$C]palmitoyl-labeled ceramide; *p*-NPA, *p*-nitrophenyl acetate; SM, sphingomyelin; SMase, sphingomyelinase; TBS, Tris-buffered saline.

then dissolved in ice-cold 0.15 M NaCl containing 1 mM benzamidine. The insoluble materials were removed by filtration through a nylon membrane. The proteins in the filtrate were precipitated by acetone to 50% at temperatures lower than −10°C. The precipitated proteins were dissolved in 20 mM Tris-HCl buffer, pH 8.2, containing 1 mM benzamidine. Insoluble materials were removed by centrifugation at 18,000 rpm at −10°C for 10 min. The supernatant obtained was used for the experiment.

Sprague-Dawley rats ranging from 200–250 g were obtained from Møllegård (Ry, Denmark). In one set of experiments, the intestinal content from whole small intestine was collected by rinsing the intestinal tract with 0.15 M NaCl containing 1 mM benzamidine. The proteins in the content were precipitated, and the precipitated proteins were dissolved as described above. In another set of experiments, the small intestine of rat was cut into five segments. The first one was 10 cm long and represented duodenum. The rest of the small intestine was cut into four segments of equal length. The contents of the five segments were collected, centrifuged at 3,000 rpm for 10 min, and the supernatants were used for studying enzyme activity distribution.

Human pancreatic juice was collected from a patient with pancreatic cyst *via* a drainage tube in the pancreatic duct. Human pancreatic BSSL was purified in the laboratory according to the method of Duan and Borgström (12).

*Ceramidase assay.* Ceramidase activity was determined by using [$^{14}$C]Cer as substrate (13). For each determination, 0.75 nmol [$^{14}$C]Cer (75,000 dpm) was dried under nitrogen. Tris-maleate buffer (90 μL of 50 mM) containing 10 mM taurocholate, pH 7.0, was added, and the mixture was sonicated for 2 min. The reaction was started by adding 10 μL of sample followed by incubation for 1 h at 37°C. The reaction was then terminated by addition of 0.6 mL methanol/chloroform/heptane (28:25:20, by vol) as described previously (14). The solution was alkalized by adding 0.2 mL of 0.05 M $K_2CO_3/K_2B_2O_4$, pH 10.0. The mixture was centrifuged at 10,000 rpm for 10 s and 200 μL of the supernatant was taken for liquid scintillation counting. The distribution of released fatty acids in the upper phase under this condition was about 80%, which was corrected for activity calculation (15). When the optimal pH for ceramidase was determined, 50 mM Tris-HCl buffer with pH 8–10 and 50 mM Tris-maleate buffer with pH 4–7 were used. All the buffers contained 10 mM taurocholate.

*BSSL assay.* The determination of BSSL activity was according to Shirai and Jackson (16), using *p*-NPA as substrate. The sample was added in 0.1 M Tris buffer, pH 7.2, containing 3 mM taurocholate and 0.5 mg *p*-NPA to a final volume of 200 μL. The incubation was performed at room temperature and the release of nitrophenyl was measured by the increase in absorbance at 405 nm, which was read at 0, 2, 5, and 10 min by a microplate reader (Bio-Rad). The activity was calculated as μmol/min/mg protein or per mL of the samples, with the adjustment of the blank values.

*Anion exchange chromatography.* The anion exchange high quaternary aminoethyl (HQ) cartridge (Bio-Rad, Hercules, CA) with total volume of 5 mL was equilibrated with 20 mM Tris-HCl buffer, pH 8.2, containing 1 mM benzamidine. About 30 mg of sample proteins in 5 mL equilibrate buffer were loaded on the cartridge at a rate of 2 mL/min. After washing the cartridge with 15 mL of the same buffer, the bound proteins were eluted with a gradient of NaCl from 0 to 0.25 M, and then the NaCl concentration rapidly increased to 0.5 M in a total volume of 50 mL of the equilibrate buffer. The fractions were collected every 1.5 min. The activities of ceramidase and BSSL in the fractions were determined as described above. The chromatography was performed at 4°C by a Bio-Logic HR chromatography system (Bio-Rad Co.) and the proteins in the fractions were monitored by an ultraviolet detector.

*Western blot.* Western blot against BSSL was performed using a kit purchased from Bio-Rad. Briefly, 10 μg of proteins from rat pancreatic homogenate and from the fractions with ceramidase activity were subject to 12% sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane electrophoretically. The membrane was blocked with Tris-buffered saline (TBS) containing 3% gelatin. After washing with TBS buffer containing 0.3% Tween 20, the membrane was probed with antiserum against human BSSL at 1:1000 dilution in TBS buffer containing 1% gelatin for 2 h. After washing, the membrane was incubated with IgG antibody conjugated with alkaline phosphatase. The binding bands were visualized according to the instructions of the kit manufacturer.

*Response to orlistat.* Orlistat was dissolved in ethanol and was added in human pancreatic juice, ceramidase fraction from HQ chromatography, or purified human BSSL at various concentrations. After incubation for 5–10 min, the ceramidase and BSSL activities were determined as described. The ethanol without orlistat was also added in the samples to check whether ethanol had any effect on the enzyme activity. The maximal concentration of ethanol added was 10%, and at this concentration, ethanol had no significant effect on both enzyme activities.

## RESULTS

*Ceramidase activity in the acetone-precipitated proteins of whole small intestinal contents.* The ceramidase activity in the acetone-precipitated proteins from small intestinal contents of rats and human beings were determined, and the results are shown in Figure 1. In both cases, there were dose-dependent increases of ceramidase activity. The results confirmed the earlier findings of Nilsson (7) that ceramidase activity exists in the intestinal contents of several species. The specific activity was about 15 nmol/h/mg proteins of rat intestinal contents and 28 nmol/h/mg of human intestinal contents.

*Ceramidase activity of human pancreatic BSSL.* The ceramidase activity in the purified BSSL from human pancreatic juice is shown in Figure 2. Human pancreatic BSSL hydrolyzed ceramide in a dose-dependent manner. The specific

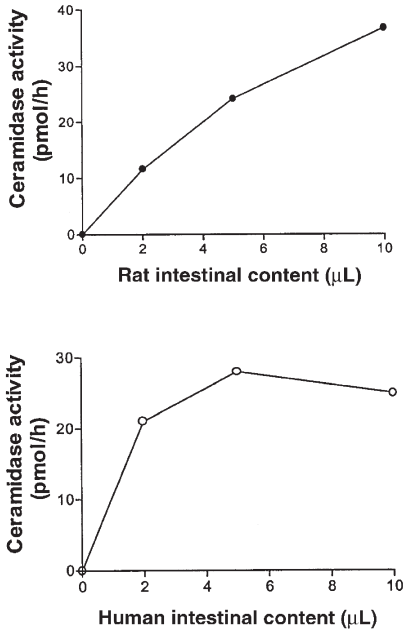


**FIG. 1.** Ceramidase activity in the proteins of intestinal contents of rats and human beings. The intestinal contents of the whole small intestine were collected from 10 rats. The proteins were precipitated by acetone at 50% and dissolved in 20 mM Tris-HCl buffer, pH 8.2. Human intestinal contents were collected from stomas of three patients after ileostomy and freeze-dried. The materials were resuspended in 0.15 M NaCl, and the proteins were precipitated and dissolved as above. Neutral ceramidase activity was determined using fatty acid-labeled [$^{14}$C]ceramide as substrate.

activity was about 155 nmol/h/mg. The results indicate that pancreatic BSSL, like BSSL in milk (9), has the ability to cleave the fatty acid from ceramide. Similar results were also obtained when the activity was determined in rat pancreatic homogenate (data not shown).

*Migration of intestinal ceramidase in HQ anion exchange chromatography.* To address whether the ceramidase activity shown in the intestinal content was contributed by pancreatic BSSL, the acetone-precipitated proteins from rat intestinal content were subject to HQ anion exchange chromatography. As shown in Figure 3, ceramidase activities were identified in two portions. The first one was in the proteins that were not retained in the HQ cartridge. The second one was in the proteins that were eluted by high concentrations of NaCl. The activity in the nonretained proteins was not caused by an overloading; we checked this by resubjecting these nonretained proteins to a new HQ cartridge. The result thus indicates the presence of at least two types of ceramidase activity in the intestinal contents.

*Optimal pH of the two ceramidases isolated after HQ chromatography.* The two portions with ceramidase activity were pooled and concentrated by ultrafiltration through a YM10 membrane (10 kDa cut-off; Millipore Co., Sundbyberg, Sweden). The ceramidase activities in both portions at various pH values were determined. As shown in the upper panel of Figure 4, the ceramidase that was not retained in the HQ cartridge preferred acid pH, whereas the retained one had high activity at neutral pH (lower panel).

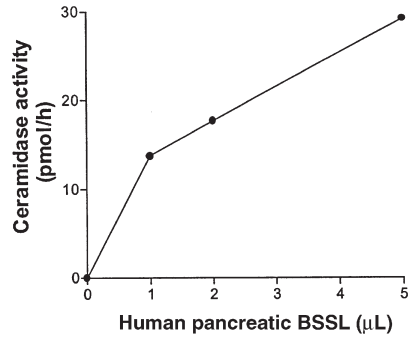


**FIG. 2.** Ceramidase activity of human pancreatic bile salt stimulated lipase (BSSL) at various doses. Human pancreatic BSSL was purified in the laboratory, and ceramidase activities were assayed using [$^{14}$C]ceramide as substrate.

*Determination of whether the fractions having ceramidase activity have BSSL activity.* We determined BSSL activities against *p*-NPA in both fractions having ceramidase activity after HQ chromatography with rat pancreatic homogenate and human pancreatic BSSL as positive controls. The results are shown in Table 1. Little activity in either nonretained or retained

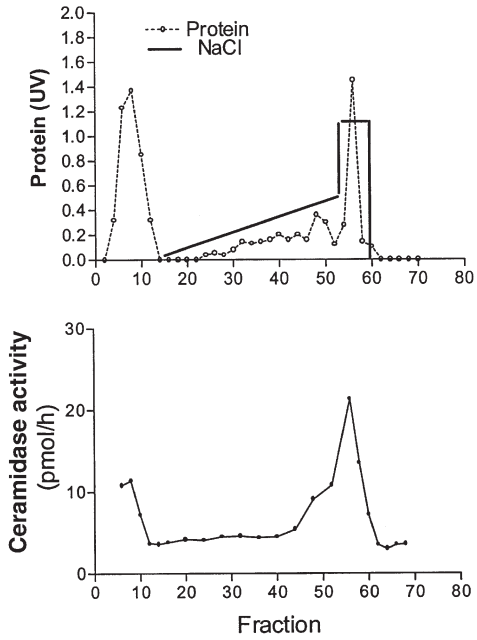


**FIG. 3.** Profile of high quaternary aminoethyl (HQ) anion chromatography of the proteins precipitated by acetone from rat intestinal tract. The precipitated proteins were dissolved in 10 mL of 20 mM Tris buffer pH 8.2 and loaded on an HQ cartridge that had been equilibrated with the same buffer. After washing the cartridge, the bound protein was eluted by a NaCl gradient from 0 to 0.25 M and the concentration of NaCl was then rapidly increased to 0.5 M. The flow rate was 2 mL/min, and the fractions were collected every 1.5 min. The proteins were monitored by an ultraviolet detector (upper panel). Chromatography was performed on a HR Bio-Logic system at 4°C. After chromatography, the ceramidase activity in the fractions was assayed (lower panel).

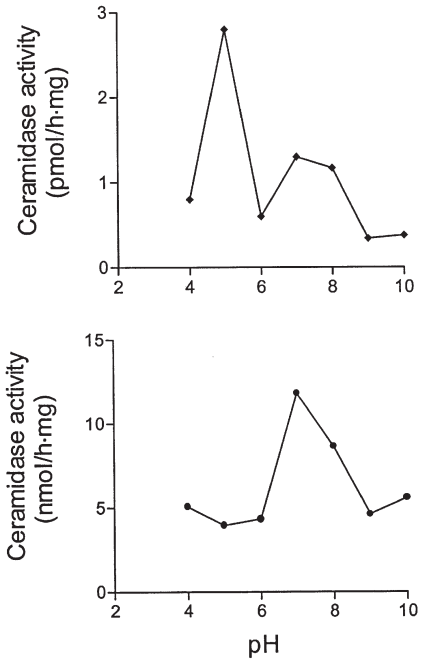


**FIG. 4.** The optimal pH of ceramidase activities isolated by HQ chromatography. The nonretained and retained portions with ceramidase activities were pooled, respectively, and the ceramidase activities of the two portions were determined in the buffers with various pH. Upper panel: ceramidase activity in the nonretained portions; lower panel: ceramidase activity in the retained portions. See Figure 3 for abbreviation.

fractions was detected. If the BSSL activities of nonretained and retained portions were expressed as μmol/min/mL, the activities were 2.31 and 2.24, respectively, which were lower than that of the blank value (Table 1). We further determined BSSL activities in all fractions of HQ chromatography as well as in the original sample loaded on the HQ cartridge. No BSSL activity could be identified (Table 1). The results show that in the proteins precipitated from whole small intestine by acetone, BSSL was denatured and ceramidase was preserved.

*Western blot of BSSL protein in fractions with high ceramidase activity.* To answer the question whether the negative esterolytic activity in the fractions with ceramidase is caused by the absence of BSSL or by the conformational changes under the sample preparation, western blot was performed. As shown in Figure 5, in rat pancreatic homogenate BSSL antiserum identified one protein band at 70 kDa, which corresponds to the molecular mass of rat BSSL (17). The antiserum also identified two smaller proteins in the pancreatic homogenate, which may represent degraded BSSL molecules. However, no band reacted with BSSL antiserum in the fractions with high ceramidase activity after HQ chromatography.

*Distribution of BSSL and ceramidase activities in the intestinal tract.* The distribution patterns of ceramidase and BSSL activity in rat intestinal tract were compared, and the results are shown in Figure 6. The pancreatic BSSL activity was high in the duodenum and sharply decreased in the small intestine, whereas ceramidase activity was low in the duodenum and high in the middle of the small intestine. The distribution patterns between BSSL and ceramidase are thus different.

**TABLE 1**
**Bile Salt Stimulated Lipase (BSSL) Activity in Various Samples**

| Sample | Activity (μmol/min/mg) |
|---|---|
| Blank control | 2.71[a] |
| Nonretained portion | 4.27 |
| Retained portion | 4.02 |
| Rat pancreatic homogenate | 23.82 |
| Human pancreatic BSSL | 208.13 |
| Acetone precipitated proteins of human intestinal content | 0.10 |
| Acetone precipitated proteins of rat intestinal content | 0.19 |

[a]0.15 M saline was used for blank. The unit of this value is μmol/min/ml.

*Response to lipase inhibitor, orlistat.* It is well known that orlistat is an inhibitor of several lipases, including pancreatic BSSL (18). Whether orlistat has similar inhibitory effects on pancreatic BSSL and ceramidase was studied. We found that orlistat dose-dependently inhibited the human BSSL activity against *p*-NPA and it also slightly reduced BSSL activity against ceramide by about 20%. However, orlistat had no inhibitory effect on the neutral ceramidase activity isolated from HQ chromatography (Fig. 7).

## DISCUSSION

Ceramidase activity in the intestinal content was first identified in 1969 (7). Recently an investigation showed that BSSL in milk had ceramide hydrolytic activity (9). Pancreatic BSSL is a product of the same gene of BSSL in milk (10) and is present in the intestinal tract. To investigate whether the ceramidase activity found previously in the intestinal content is contributed by pancreatic BSSL is important in understanding the events involved in the digestion of dietary sphingolipids. It also has clinical implications, because the ceramide levels in the gut are recently thought to have impact on mucosal cell proliferation and colon cancer development (19,20).

In the present study, we first confirmed the presence of enzymatic activities against ceramide in the intestinal content

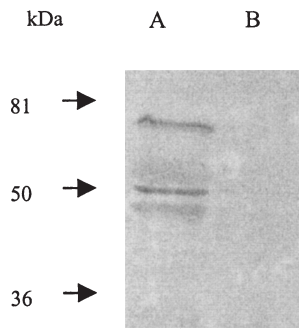


**FIG. 5.** Western blot of BSSL. Rat pancreatic homogenate (lane A) and the fraction with high ceramidase activity after HQ chromatography (lane B) were subjected to 12% sodium dodecylsulfate polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membrane electrophoretically. The membrane was probed with rabbit anti-BSSL antiserum first and then with IgG antibody conjugated with alkaline phosphatase. The bands were visualized by a kit from Bio-Rad Co. See Figures 2 and 3 for abbreviations.

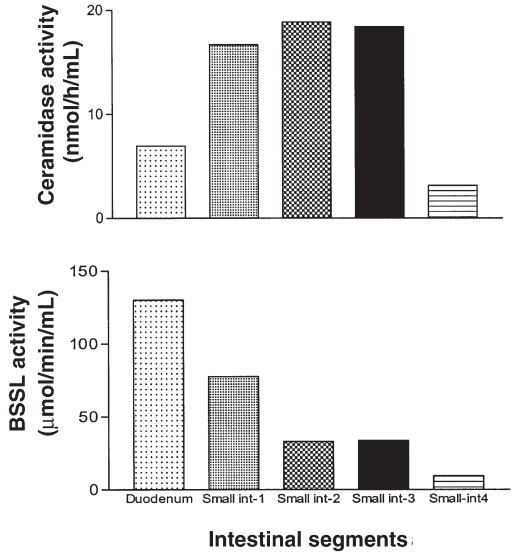


**FIG. 6.** Distribution of neutral ceramidase and BSSL activities in the rat small intestinal tract. The whole small intestine was cut into five parts. The first part was 10 cm long and represented duodenum. The rest of the small intestine was divided into four parts of equal length. The intestinal contents of each segment were suspended in 10 mL of saline containing 1 mM benzamidine. The activities of ceramidase and BSSL in each segment were assayed. For abbreviation see Figure 2.

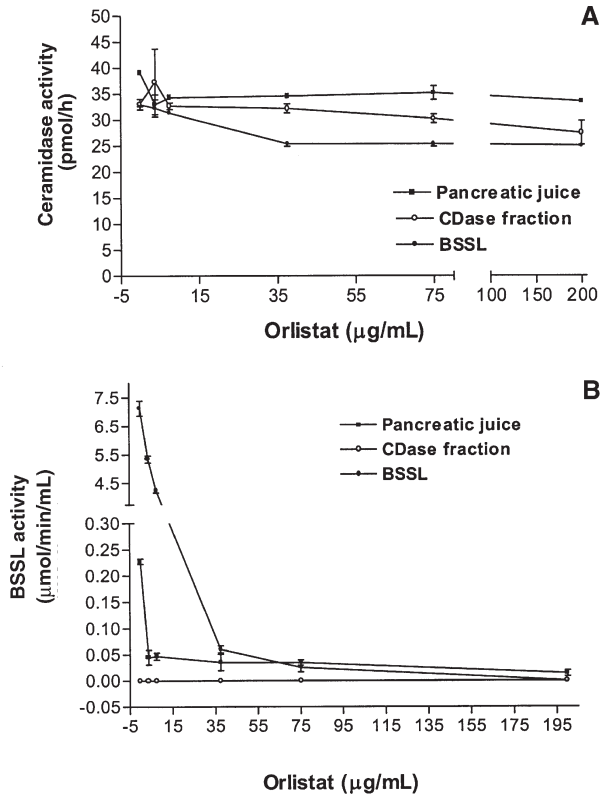


**FIG. 7.** Comparison of the effects of orlistat on ceramidase (CDase) and BSSL. Orlistat at various concentrations was incubated with pancreatic juice, neutral ceramidase fractions after HQ chromatography, and purified human BSSL for 10 min. The activities of ceramidase (upper panel) and BSSL (lower panel) after incubation were determined. For abbreviation see Figure 2.

and showed that pancreatic BSSL is able to hydrolyze ceramide in the same conditions. Then we provided several pieces of evidence supporting the presence of ceramidases in addition to BSSL in the intestinal content. First, when proteins from the intestinal contents of the whole small intestine were precipitated by acetone and subjected to HQ anion exchange chromatography, two portions of ceramidase activities were identified. Neither of them had esterolytic activity. Previous study suggested that the active sites of BSSL against ester and ceramide were different (8). However the negative esterolytic activity in HQ fractions is unlikely to be caused by a defect of a specific active site of BSSL, because Western blot failed to show BSSL band in the fractions with ceramidase activity. The two forms of ceramidase upon HQ chromatography preferred different pH values and were characterized as acid and neutral ceramidase, respectively. Based on this result, it seemed that BSSL has ceramidase activity, but not vice versa. Second, the distributions of neutral ceramidase and pancreatic BSSL in the intestinal tract differed significantly. The highest level of pancreatic BSSL was found in the duodenal content, and the activity sharply decreased in the jejunum and ileum. The activity of neutral ceramidase, in contrast, was low in the duodenal content but maintained a high level until the distal part of ileum. Since these results were obtained from the original intestinal content without acetone treatment, they also support the theory of the presence of two enzymes instead of one enzyme with two active sites for ester and ceramide in the intestine. The different distribution pattern is probably caused by the different stability of the enzyme in the presence of trypsin. It has been shown that intestinal ceramidase is resistant to trypsin digestion (15) whereas BSSL is not (21). This difference may also explain why BSSL activity is present in the duodenal content but is not detectable in the proteins precipitated by acetone from the contents of the whole small intestinal tract. Finally, we found that the responses of BSSL and ceramidase to orlistat, the inhibitor of pancreatic lipase and BSSL, are different. Orlistat strongly inhibited the BSSL activity against *p*-NPA but not ceramide hydrolytic activity in the fractions isolated from HQ chromatography. Orlistat also failed to strongly inhibit ceramide hydrolytic activity of BSSL at the doses that it completely inhibited esterolytic activity of BSSL. Because orlistat is an active-site directed inhibitor (22), the finding indicates that the structure of the active site of BSSL against *p*-NPA is different from that of ceramidase against ceramide. It also supports the previous findings that BSSL has different active sites for its esterolytic activity and ceramide hydrolytic activity (8).

In this study, we found at least three types of enzymes that can hydrolyze ceramide in the intestinal content: acid ceramidase, neutral ceramidase, and BSSL. These enzymes may contribute differently to the digestion of dietary ceramide. In the duodenum, acid ceramidase might be active due to the acid environment. Pancreatic BSSL might also be important in this part of the intestine due to the high levels of the enzyme. In the

rest of the small intestine, the hydrolysis of ceramide likely is dependent on the activity of neutral ceramidase, because the activity of neutral ceramidase, but not BSSL, is high in these parts of the small intestine. In addition, because dietary ceramide is mainly derived from the hydrolysis of SM, the digestion of ceramide is determined to some extent by the activity of SMase. The SMase in the intestinal tract responsible for digestion of SM is alkaline SMase (6). Its activity is high in the middle of the small intestine (5). The hydrolysis of SM and the formation of ceramide have been shown mainly in this part of the small intestine (6). It is reasonable to postulate that neutral ceramidase is more important than acid ceramidase and pancreatic BSSL in digestion of dietary ceramide.

Finally, it should be pointed out that orlistat had no effect on ceramide digestion by ceramidase and BSSL. We also determined previously that orlistat does not have a role in SM hydrolysis induced by purified alkaline SMase. The negative effects of orlistat on SM digestion might be interesting, because orlistat is used as a drug to reduce body weight by inhibition of hydrolysis of triglycerides (23). A high-fat diet is associated with an increasing risk of colorectal cancer, whereas SM hydrolysis in the gut may have protective effects on tumorigenesis in the colon (24–26).

Our work provides evidence for the presence of specific ceramidases other than pancreatic BSSL in the intestinal content. Different forms of ceramidase with different optimal pH in other organs such as skin (27) and liver (28) have been purified and characterized. Purifying and characterizing intestinal ceramidase will elucidate the physiological role of the enzyme in sphingolipid digestion.

## ACKNOWLEDGMENTS

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# Docosahexaenoic Acid Modulates Phorbol Ester-Induced Activation of Extracellular Signal-Regulated Kinases 1 and 2 in NIH/3T3 Cells

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**ABSTRACT:** Phosphorylation of extracellular signal-regulated kinases (ERK1/ERK2) has been implicated in cell proliferation of mammalian cells. In the present study, we investigated the role of docosahexaenoic acid (DHA) in the modulation of ERK1/ERK2 phosphorylation, stimulated either with phorbol 12-myristate 13-acetate (PMA) or transforming growth factor-alpha (TGFα) in NIH/3T3 cells. We observed that both PMA and TGFα induced ERK1/ERK2 phosphorylation within 5 min of stimulation. PMA acts upstream of MEK and *via* activation of protein kinase C (PKC), as GF109203X, a potent PKC inhibitor, and U0126, a MEK inhibitor, abolished its actions on ERK1/ERK2 phosphorylation. TGFα did not act *via* PKC because GF109203X failed to curtail the degree of ERK1/ERK2 phosphorylation in these cells. DHA alone failed to induce the phosphorylation of these mitogen-activated protein (MAP) kinases; however, this fatty acid significantly curtailed the PMA- but not TGFα-induced MAP kinase enzyme activity and phosphorylation in NIH/3T3 cells. Furthermore, we observed that DHA significantly inhibited PMA-induced translocation of two PKC isoforms, PKCα and PKCε, from cytosol to plasma membrane. Interestingly, DHA failed to inhibit the PMA-induced translocation PKCδ isoform in these cells. Furthermore, DHA decreased PMA-induced proliferation of NIH/3T3 cells. In this study, we show for the first time that DHA inhibits MAP kinase (ERK1/ERK2) activation and proliferation of NIH/3T3 cells *via* its inhibitory action on PKCα and ε isoforms.

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A number of receptors involved in growth and differentiation, such as those activated by epidermal growth factor (EGF) and platelet-derived growth factor, are themselves tyrosine-specific kinases (1). Transforming growth factor-alpha (TGFα), a mitogenic peptide, is an epidermal growth factor-ligand that is distinguished from EGF by its acid-labile structure and potent transforming structure. Lipeski *et al.* (2) showed that TGFα may activate EGF receptor (EGFr) coupled with Ras/MAP kinase pathway. EGF *via* EGFr is able to induce rapid activation of these kinases, which are thought to be key intermediate regulatory proteins functioning in signal transduction pathways (3). Binding of TGFα to its receptor induces intracellular receptor autophosphorylation on tyrosine residues and creates a recognition site for intracellular SH2-containing proteins, particularly the adaptator Grb2 implicated in $p21^{ras}$ dependent signaling pathway (4,5). Grb2 associated with SOS is translocated to the plasma membrane where $p21^{ras}$ is localized. Ras is active in the GTP-bound form, and the activation of Ras is mediated by the nucleotide exchange, as catalyzed by exchange factors such as SOS or Vav (6,7). The Ras-GTP binds directly to a serine/threonine kinase, Raf-1, which activates MAP kinase kinase (MAPKK or MEK1/2), which in turn activates MAPK isoforms (8). Raf-1 activates Raf/MEK/ERK cascade in a protein kinase C (PKC)-dependent manner. Hence, PKCα acts as a critical component of the MAPK signal transduction pathway as this enzyme phosphorylates Raf-1 (9). Moreover, EGF triggers MAPK activation *via* PLCγ-dependent pathway that converges with a Ras-dependent pathway on Raf-1 in NIH/3T3 cells (10). When EGFr is activated, a signal is transmitted to PLCγ, which leads to PKC activation, by the release of diacylglycerol cleaved from phosphatidylinositol and production of inositol 1,4,5-trisphosphate.

Numerous studies have been conducted on cell growth and differentiation to understand the consequences of MAPK cascade alteration and to determine the mechanisms implicated in downstream signaling (10,11). Reszka *et al.* (11) showed that MAPK activation is crucial for regulation of cell cycle progression. Hence, MAPK is associated with the cytoskeleton proteins such as microtubules in NIH/3T3 cells (11). In these cells, ERK2 is finally translocated into the nucleus where it regulates gene expression, involved in cell proliferation (12).

Several studies have demonstrated the involvement of polyunsaturated fatty acids, particularly of docosahexaenoic acid (DHA; 22:6n-3), in signal transduction and gene transcription (12,13). DHA has been shown to modify the segregation of membrane proteins, thus affecting fluidity of plasma membranes (14,15). Several authors have demonstrated that DHA modulates the production of diacylglycerol and

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Abbreviations: ALA, α-linolenic acid (18:3n-3); DHA, docosahexaenoic acid (22:6n-3); ECL, equivalent chain length; EGF, epidermal growth factor; EGFr, EGF receptor; ERK, extracellular signal-regulated kinase; LA, linoleic acid (18:2n-6); MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKK-ERKK (also known MEK1/2); PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; n-3 PUFA, polyunsaturated fatty acids of n-3 family; PVDF, polyvinylidine difluoride; SDS, sodium dodecyl sulfate; TGFα, transforming growth factor-alpha.

therefore inhibits DNA synthesis in different cell lines (15,16). In the present study, we investigated the role of this fatty acid in the regulation of MAP kinase signaling and in the proliferation of NIH/3T3 fibroblast cells.

## EXPERIMENTAL PROCEDURES

*Materials.* Antiphosphorylated MAP kinase (ERK1/ERK2) antibodies were obtained from New England Biolabs (Hitchen-Hertfordshire, United Kingdom). The MAP kinase assay kit, [$^3$H]thymidine (specific activity 20 Ci/mmol), and $\gamma^{32}$P-ATP were procured from Amersham (Orsay, France). GF109203X was obtained from Calbiochem (Meudon, France). PKC sampler kit for the detection of different isoforms was obtained from New England Biolabs. All other chemicals, including phorbol 12-myristate 13-acetate (PMA), TGFα and DHA, were purchased from Sigma (St. Quentin Fallavier, France). U0126 was a generous gift from Promega (Charbonniers, France).

*Cell culture and treatments.* NIH/3T3 fibroblasts, used in this study, were grown in 35-mm petri dishes in a humidified chamber with 5% $CO_2$ atmosphere at 37°C in RPMI 1640 medium supplemented with L-glutamine, penicillin (50 µg/mL), streptomycin (50 µg/mL), HEPES 20 mM, and 10% fetal calf serum.

Quiescent cells were obtained by serum-starvation for 18 h. Cells were incubated for 5 min with DHA (dissolved in ethanol, 0.01% wt/vol) and then stimulated with PMA (200 nM) or TGFα (50 ng/mL) for 5 min. Cells were lysed with 200 µL of Laemmli buffer (Tris, 62.5 mM; sodium dodecyl sulfate (SDS), 2%; glycerol, 25%; β-mercaptoethanol, 715 mM; bromophenol blue, 0.01%, pH 6.8) and used immediately or stored at −80°C for western blot detection of MAP kinase phosphorylation.

*Western blot analysis of MAP kinase phosphorylation.* Cell proteins were subjected to SDS-polyacrylamide gel electrophoresis (10%) and transferred to polyvinylidine difluoride (PVDF) membrane. The membrane was blocked by incubation with cream-free milk for 2 h and was then incubated with antiphosphorylated ERK1/ERK2 antibodies. MAP kinases were visualized using anti-rat peroxidase-conjugated antibody. These antibodies were used at 1:1000 dilution. Peroxidase activity was detected using the chemiluminescent equivalent chain (ECL) length system.

*MAP kinase enzyme activity.* MAP kinase enzyme activity was determined according to the instructions furnished with the kit. The enzyme activity was assessed by monitoring the incorporation of $^{32}P_i$ into a peptidic fraction of EGFr, containing a proline-leucine-serine/threonine-proline sequence that is a more specific substrate for MAP kinases than the myelin basic protein.

*Cell lysis and western blot detection of different isoforms of PKC.* After incubation of NIH/3T3 cells ($8 \times 10^6$ cells/dish) with DHA and/or PMA, cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, and the reaction was stopped by addition of a buffer that contained the following: Tris-HCl, 25 mM; EDTA, 0.5 mM; EGTA, 0.5 mM; β-mercaptoethanol, 10 mM; phenylmethyl sulfonyl fluoride, 50 µM; and leupeptin, 10 µg/mL, pH 7.5. Cells were scraped off, sonicated twice for 2 s at 4°C and then centrifuged ($500 \times g \times 10$ min at 4°C) to isolate the nuclear fractions. The supernatant was used to isolate plasma membrane and cytosolic fractions by centrifuging ($100{,}000 \times g \times 60$ min), essentially according to Szallasi *et al.* (17). The particulate and cytosolic fractions were used to detect PKC translocation after electrophoresis and transfer onto the PVDF membrane. The anti-PKC antibodies and secondary anti-rat antibody were employed at 1:1000 dilution. The different isoforms of PKC were visualized by detecting peroxidase activity using the chemiluminescent ECL system.

*Proliferation assay*. NIH/3T3 cells were seeded into 24-well plates at a density of $50 \times 10^3$ cells/well and cultured in complete RPMI 1640 medium. At subconfluence, cells were serum-starved for 24 h and incubated with DHA at increasing concentrations in the presence of PMA (1 µM) for another 24 h. Cell proliferation was determined by addition of [$^3$H]-thymidine (20 Ci/mmol; 1 µCi/well) for the last 6 h. The incubation was stopped by removing the medium and adding cold methanol. The cells were washed with PBS, fixed with 10% perchloric acid, and dissolved in NaOH (0.5 N) for scintillation counting.

*Statistical analysis.* Results are shown as mean ± standard deviation of quadruplicate assay samples. Statistical analysis of data was carried out using Statistica (version 4.1, Statsoft, Paris, France). The significance of the differences between mean values was determined by analysis of one-way variance, followed by a least significant difference (LSD) test.

## RESULTS

*Rapid activation of ERK1/ERK2 by PMA and TGFα.* In order to determine the time required to induce phosphorylation of ERK1/ERK2 by two mitogenic agents, cells were exposed to PMA or TGFα for different times (5, 10, or 30 min). Figure 1 shows that both PMA and TGFα phosphorylated MAP kinases within 5 min of incubation. Interestingly, PMA-induced ERK1 and ERK2 phosphorylation completely disappeared

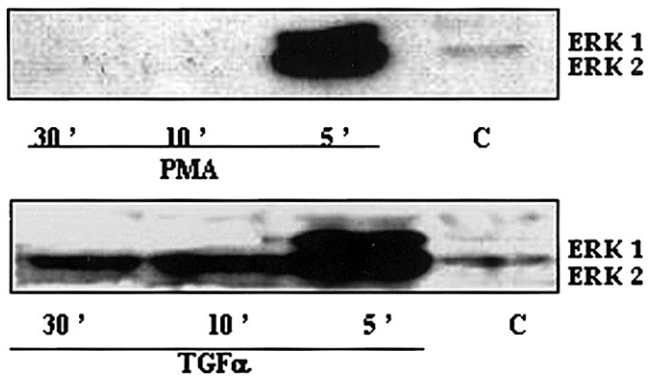


**FIG. 1.** Time course phosphorylation of extracellular signal-regulated kinases (ERK1/ERK2) by phorbol 12-myristate 13-acetate (PMA) and transforming growth factor-alpha (TGFα). NIH/3T3 cells ($3 \times 10^6$ cells/dish) plated in 60-mm petri dishes were growth-arrested at confluence by overnight serum starvation. Cells were treated or not (Control, C) with PMA (200 nM) and TGFα (50 ng/mL) at 37°C for different times. Phosphorylated mitogen-activated protein (MAP) kinases were detected by immunoblotting as described in the Experimental Procedures section.

after 10 min; although TGFα-induced ERK2, but not ERK1, phosphorylation was apparent until 30 min of incubation (Fig. 1).

*MAPK activation* via *MEK is partially PKC-dependent*. Pretreatment of cells with GF109203X, a specific inhibitor of PKC, curtailed PMA-induced ERK1/ERK2 phosphorylation; however, this inhibitor was inefficient when cells were activated by TGFα (Fig. 2). We also employed U0126, a specific inhibitor of MEK, and observed that this agent completely abolished the TGFα- and PMA-stimulated MAPK phosphorylation (Fig. 2).

*DHA inhibits PMA- but not TGFα-induced MAP kinase activation*. To determine the molecular mechanism of action of DHA on ERK1/ERK2 activation, we assessed two aspects; determination of the degree of phosphorylation, and the enzyme activity of MAP kinases. For the latter, we used a specific substrate that is a part of EGFr (see Experimental Procedures section). It is interesting to note that DHA alone did not induce any activation of MAPK under basal conditions (Figs. 3 and 4) whereas Figure 3D shows that linoleic acid and α-linolenic acid alone are capable to induce MAPK phosphorylation. DHA inhibited, from 5 to 40 μM, MAPK phosphorylation, stimulated by PMA (Fig. 3B). On the contrary, DHA failed to reduce MAPK phosphorylation significantly (Fig. 3C) and enzyme activity (Fig. 4) stimulated by TGFα. In parallel control assays, Figure 3A shows the presence of nonphosphorylated MAPK in NIH/3T3 cells.

*DHA seems to act* via *PKC-dependent pathway.* Because DHA curtailed PMA-induced MAPK activation, we assessed the effect of DHA in the presence or absence of PMA on translocation of different isoforms of PKC from cytosol to plasma membrane. We observed that DHA alone did not induce translocation of any isoform of PKC (Fig. 5). However, DHA completely inhibited the PMA-induced translocation of PKCα and PKCε isoforms in these cells. It is interesting to note that DHA failed to inhibit the PMA-induced translocation of PKCδ isoform (Fig. 5). On the other hand, PMA did not induce the translocation of PKCζ in these cells (Fig. 5). PKCβ and PKCγ were not significantly detectable in these cells (results not shown).

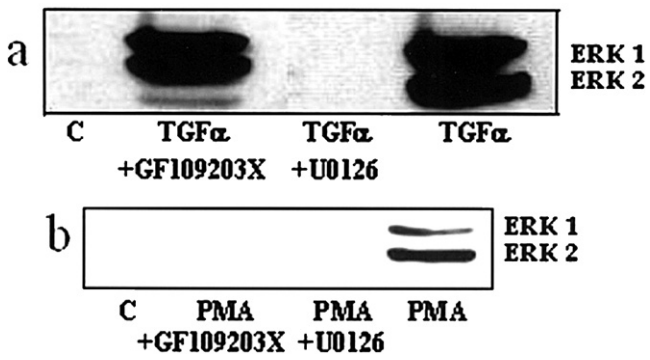


**FIG. 2.** Effect of U0126 and GF109203X on MAP kinase phosphorylation. NIH/3T3 cells (3 ×$10^6$ cells/dishes) plated in 60-mm petri dishes were growth-arrested at confluence by overnight serum starvation. Cells were pretreated or not (control, C) with GF109203X (500 nM) or U0126 (10 μM) and then incubated with TGFα (50 ng/mL) or PMA (200 nM) at 37°C for 5 min. MAP kinases were detected by immunoblotting as described in the Experimental Procedures section. See Figure 1 for abbreviations.

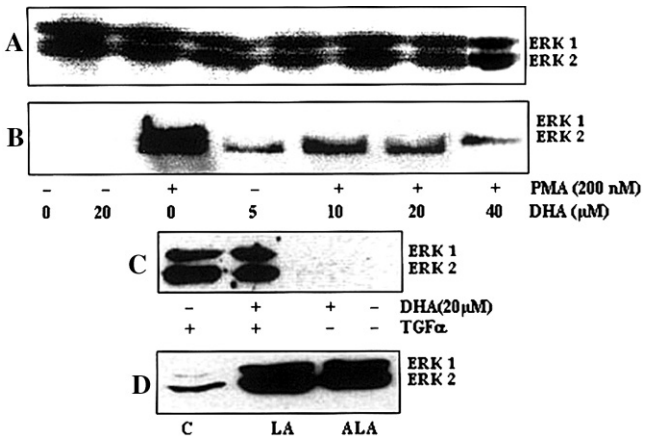


**FIG. 3.** Effects of docosahexaenoic acid (DHA) on PMA- and TGFα-induced MAPK phosphorylation. NIH/3T3 cells (3 × $10^6$ cells/dish) plated in 60-mm petri dishes were growth-arrested at confluence by overnight serum starvation. Panel A shows unphosphorylated MAPK present in unstimulated cells. In panel B, cells were pretreated or not (control) with DHA at increasing concentrations (5, 10, 20, 40 μM) in the presence of PMA (200 nM) for 5 min at 37°C. In panel C, cells were treated with DHA (20 μM) and stimulated with TGFα (50 ng/mL) for 5 min at 37°C. In panel D, cells were pretreated or not (control, C) with linoleic acid (LA; 20 μM) and α-linolenic acid (ALA; 20 μM). Cell lysates were prepared as described in the Experimental Procedures section. Similar amounts of proteins of each sample were loaded into sodium dodecyl sulfate polyacrylamide gel electrophoresis and MAPK were detected by immunoblotting as described in the Experimental Procedures section. See Figures 1 and 2 for other abbreviations.

*DHA diminishes cell proliferation stimulated by PMA*. To investigate the effects of DHA on proliferation of NIH/3T3 fibroblasts, we stimulated cells by PMA and treated or not by DHA at increasing concentrations. We observed that DHA inhibited PMA-induced cell proliferation dose-dependently (Fig. 6). It is noteworthy that DHA does not influence cell viability as assessed by trypan blue exclusion test.

## DISCUSSION

In NIH/3T3 cells, MAPK plays an important role in cell cycle progression (18). Several studies have demonstrated that inhibition of MAPK cascade inhibits cell growth (10,18). In these studies, authors have used ERK2 antisense constructs that were derived from epitope-tagged $p44^{mapk}$ cloned into pc DNA-neo vector (18). Hence, the transfection of rat fibroblasts with $p44^{mapk}$ cDNA constructs arrested these cells in G0 phase of the cell cycle. These experiments, which result in destruction of *de novo* synthesized MAP kinase RNA, clearly indicate that MAPK isoforms are necessary to cell growth and, particularly, to pass the restriction point of the cell cycle (18). In another recent study, Abbott and Holt (19) employed PD98059, a MEK inhibitor, in order to probe whether this kinase is specifically required during the progression of G2/M phase of the cell cycle in NIH/3T3 cells.

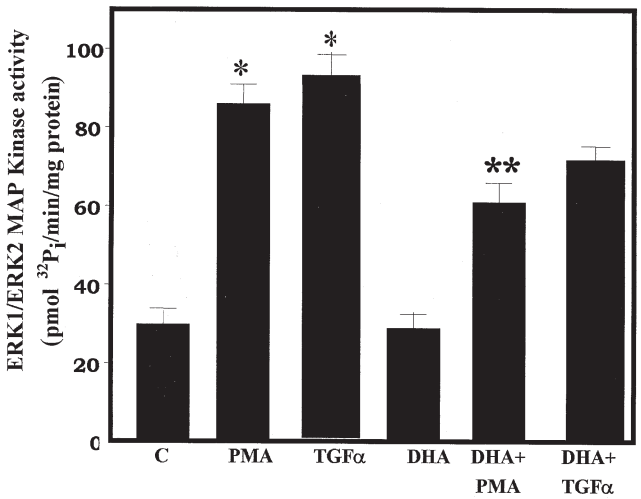


**FIG. 4.** Effects of DHA on PMA- and TGFα-induced MAPK enzyme activity. NIH/3T3 cells ($3 \times 10^6$ cells/dish) plated in 60-mm petri dishes were growth-arrested at confluence by overnight serum starvation. Cells were pretreated or not (control, C) with DHA (20 μM) and then exposed to PMA (200 nM) or TGFα (50 ng/mL). The enzymatic activity was determined as described in the Experimental Procedures section. Each value represents the mean ± standard deviation of quadruplicate assay samples, reproduced at least two times independently. Data are significant as compared to control (*$P < 0.001$) and PMA-activated cells (**$P < 0.001$), following the least significant difference test of significance. See Figures 1–3 for abbreviations.

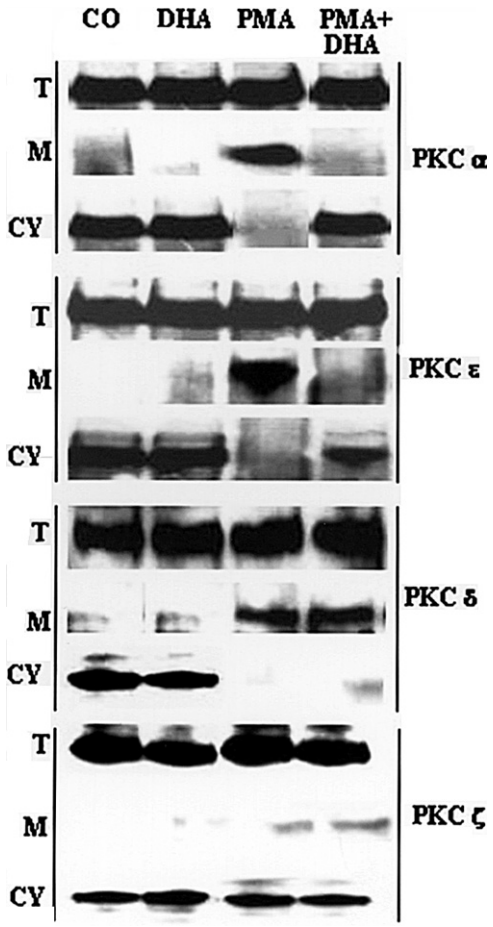


**FIG. 5.** Effects of DHA on PMA-induced translocation of protein kinase C (PKC) isoforms. NIH/3T3 cells ($3 \times 10^6$ cells/dishes) plated in 60-mm petri dishes were growth arrested at confluence by overnight serum starvation. Cells were pretreated or not (control) with DHA for 5 min. Cells were stimulated or not (control, CO) with PMA (200 nM). Upper panel (total, T) shows PKC in unphosphorylated/unstimulated cells. Different isoforms of PKC were detected in plasma membrane (M) and cytosolic (CY) fractions by immunoblotting as described in the Experimental Procedures section. See Figures 1 and 3 for abbreviations.

These investigators added this inhibitor to cells in late S phase of the cell cycle and observed that the phosphorylation of MEK was indispensable for progression through G2/M checkpoint arrest in NIH/3T3 cells exposed to ionizing radiation (19). These observations indicate that cell cycle progression is a MAP kinase-dependent phenomenon.

In the present study, we first investigated whether PKC was coupled to the activation of MAPK in NIH/3T3 cells. PKC represents a family of enzymes involved in signal transduction and cell proliferation (20,21). Down-regulation or overexpression of PKCα in NIH/3T3 has been shown to modulate growth rate and morphology of fibroblast cells (20). High levels of PKC in response to PMA reflected increased phosphorylation of critical cellular proteins involved in the control of cellular growth and morphology (20,21). As far as the activation of MAP kinases is concerned, our study shows that PMA and TGFα induced phosphorylation of ERK1/ERK2 after 5 min of incubation. To investigate whether PMA-induced MAPK phosphorylation is specifically PKC-mediated, we used GF109203X, a potent PKC inhibitor. GF109203X completely abolished the PMA-induced MAPK phosphorylation but exerted no effect on TGFα-induced MAPK phosphorylation. These results corroborate the observations of Ueda *et al.* (22), who reported that two activation pathways, a PKC-dependent and a PKC-independent, exist in NIH/3T3 fibroblasts. In fact, the Raf-independent pathway is stimulated by phorbol esters where PKC activates Raf-1 in a $p21^{ras}$-dependent manner in these cells (23). In NIH/3T3 cells, Raf-1 has been shown to be activated in response to EGF *via* PKC (24,25). It seems that PKC and Raf-1 are the key proteins in the MAP kinase cascade regulation. Furthermore, we have observed that U0126, a MEK inhibitor, completely abolished PMA and TGFα-induced MAPK activation in the NIH/3T3 fibroblasts (26), suggesting that MEK is an upstream regulator of MAPK whether the cells are stimulated by PMA or TGFα.

Most of the studies conducted hitherto have dealt with the role of arachidonic acid on MAP kinase activation (27–29). No study is available on the role of DHA in the modulation of MAPK activation and cell growth. In our study, eicosapentaenoic acid and arachidonic acid alone did not induce MAPK activation in these cells (results not shown). On the contrary, LA (18:2n-6) and ALA (18:3n-3) alone induced MAPK phosphorylation in NIH/3T3 cells. The fact that arachidonic acid stimulated MAPK activation in other studies (27–29) and failed to induce the same in our study may be due to the difference in cell type used. We used NIH/3T3 cells, whereas

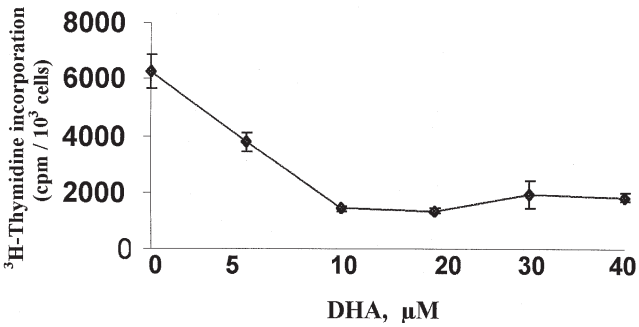


**FIG. 6.** Effects of DHA on PMA-stimulated proliferation of NIH/3T3 fibroblasts. NIH/3T3 cells were seeded in 24-well plates at a density of $50 \times 10^3$ cells/well and cultured in complete RPMI 1640 medium. At subconfluence, cells were serum-starved for 24 h and incubated with DHA at increasing concentrations in the presence of PMA (1 µM) for another 24 h. Cell proliferation was determined by addition of [$^3$H]-thymidine (20 Ci/mmol; 1 µCi/well) for the last 6 h. Data are mean ± standard deviation of quadruplicate assays, reproduced at least two times independently. See Figures 1 and 3 for abbreviations.

other investigators used rat epithelial cells (27), vascular smooth muscle cells (28), and rabbit renal proximal cells (29).

In our study, we observed that DHA alone did not induce MAPK activation under basal conditions in NIH/3T3 cells; rather, this fatty acid diminished PMA-induced, without affecting TGFα-induced, MAPK phosphorylation, suggesting that DHA may regulate MAPK pathway *via* PKC activation. Furthermore, we stimulated PKC activity by PMA in the presence or absence of DHA and observed that PKC enzyme activity was significantly curtailed when cells were pretreated with this fatty acid (results not shown). In order to trace out which isoform of PKC was implicated in the inhibition by DHA, we performed further experiments on translocation of cytosolic PKC toward plasma membrane. We observed that DHA alone did not induce the PKC translocation, but pretreatment by this fatty acid inhibited the PMA-induced translocation of PKCα and PKCε isoforms. It is noteworthy that DHA failed to inhibit the translocation of PKCδ isoform in these cells. Furthermore, we failed to detect PKCβ and PKCγ isoforms in these cells (results not shown). We also observed that PMA failed to induce translocation of PKCζ isoform. These observations are substantiated by the findings of Szallasi *et al.* (17), who reported that PKCβ and PKCγ are not expressed and PKCζ is not translocated by PMA in NIH/3T3 cells.

Several studies have shown that PKC activation is regulated by free fatty acids (30,31). In our study, whether DHA inhibits PKC activity directly or *via* the production of diacylglycerol containing DHA is not well understood. However, Vernhet *et al.* (31) showed that the replacement of arachidonic acid by an n-3 fatty acid into membrane phospholipid alters the DAG composition and, subsequently, inhibits PKC activity. In our study, DHA does not seem to act *via* lipoxygenase or cyclooxygenase pathways since this fatty acid is never metabolized into eicosanoids (32); rather, it is either degraded *via* its β-oxidation or reincorporated into plasma membrane phospholipids *via* acylation (32,33). However, our study shows that DHA modulates MAPK activation *via* its inhibitory action on two isoforms of PKC, PKCα and PKCε. These two isoforms of PKC were previously shown to regulate MAPK activation, upstream of MEK, in NIH/3T3 cells (9,17,24).

In order to assess the role of DHA in cell proliferation, we stimulated cell growth by addition of PMA, followed by treatment with DHA. Hence, we noticed that DHA inhibited PMA-stimulated cell proliferation. Our results show, for the first time, that DHA may modulate MAPK activation *via* MEK/PKC pathway and that DHA inhibits NIH/3T3 cell growth. Since activation of MAPK is known to be involved in expression of several genes essential for cell proliferation, DHA may modulate cell growth by inhibiting PKCα and PKCε upstream of MEK. Further studies are in progress in our laboratory to assess the implication of DHA in the expression of such genes.

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# Incubation of Lipid Emulsions with Plasma Lipoproteins Modifies the Fluidity of Each Particle

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**ABSTRACT:** Lipid emulsions (LE) contain triglyceride (TG)-rich particles (TGRP) and phospholipid-rich particles (PLRP). Various lipid and protein exchanges take place during *in vitro* incubations of LE with lipoproteins. These composition changes affect physical properties of particles. The aim of this study was to determine the role of different LE particles and the effect of TG composition on physical modifications. Low density lipoproteins (LDL: $1.025 < d < 1.040$ g/mL) or high density lipoproteins (HDL: $1.085 < d < 1.150$ g/mL) were incubated with the following four LE or their TGRP or PLRP, which were manufactured with the same phospholipid emulsifier: long-chain triglycerides (LCT): 100% soybean oil; medium-chain triglycerides (MCT)/LCT (MCT/LCT, 5:5, w/w); FO (100% fish oil); and MLF541 (MCT/LCT/FO, 5:4:1, by wt). After incubation, modified LE particles and lipoproteins were analyzed by fluorescence polarization. Observed physical modifications were significant in emulsion particles (ordering effect) but not in lipoproteins and also were significant for TG composition effect. Since intact emulsion contained a large excess of TGRP over PLRP, it is not surprising that intact emulsion had the same behavior as TGRP alone, and that PLRP had the same physical characteristics as lipoproteins. TG loss and cholesterol and protein acquisitions by emulsion particles rigidify their envelope. The two emulsions containing FO were less ordered after incubation. In conclusion, incubation of LE with lipoproteins changes physical properties of each kind of particle, and TG composition of the emulsion affects emulsion particle changes but has no effect on LDL and HDL. These order modifications induce more effective exchanges between LE particles and lipoproteins and modify their metabolism; HDL changes may increase the reverse cholesterol transport.

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Lipid emulsions (LE) used in parenteral nutrition mainly contain triglyceride (TG)-rich particles (TGRP), which resemble endogenous chylomicrons with respect to size and structural assembly. They only differ by the absence of structural apolipoproteins (apo) such as apo $B_{48}$ or apo A-I. These particles are suspended in a mesophase containing glycerol and some excess of emulsifier, which may be separated and recovered as liposomes that are also called phospholipid (PL)-rich particles (PLRP) (1).

Triglyceride-rich lipoproteins (TGRL), such as chylomicrons and very low density lipoproteins (VLDL), are known to undergo a series of interactions with high density lipoproteins (HDL) such as the transfer of several exchangeable apo and surface lipids, mainly during lipolysis (2). Similar to TGRL, emulsion particles interact with endogenous lipoproteins and acquire apo (namely, apo C and apo E) by transfer from plasma lipoproteins, mainly from HDL (3,4). In addition, exchanges of cholesteryl esters (CE) and TG, regulated by the cholesteryl ester transfer protein (CETP), take place between CE-rich lipoproteins [low density lipoprotein (LDL) and $HDL_2$] and TGRL. These exchanges affect lipid transport and lipoprotein remodeling in humans (5,6). TGRP are also involved in CETP-mediated lipid transfers (7). Furthermore, Kuksis *et al.* (8) suggested a rapid and extensive replacement of endogenous PL of plasma lipoproteins by exogenous PL during the infusion of LE. Lipoprotein composition changes induce modifications of the order of the particle (9,10).

Some lipid, apo, and other protein exchanges take place during *in vitro* incubations of LE in the presence of LDL or HDL (3,4). It appears that changes of LE and lipoprotein particle composition could affect the physical properties, notably lipid dynamics, of the respective components of the incubation mixture.

We recently demonstrated that modified LE fractions (M-LE), after incubation with LDL, were unexpectedly enriched in apo B as well as in CE (11). Apo B cannot freely exchange due to its very large structure, which is deeply anchored in the LDL particle. Each LDL particle possesses only one apo B molecule (12,13). The presence of apo B in the M-LE fraction strongly suggests an LDL binding to TGRP, which is corroborated by the CE enrichment of the M-LE fraction, independent of CETP activity. This LDL binding is a fast process and results in a strong interaction. In addition, LDL binding appears to involve both TGRP and PLRP and is markedly affected by the TG fatty acid (FA) composition of the emulsion (14), suggesting an important influence of the TG core on the physical properties of the PL surface layer.

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Abbreviations: apo, apolipoprotein; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; DPH, 1,6-diphenyl-1,3,5-hexatriene; FA, fatty acid; FO, fish oil; HDL, high density lipoprotein; LCT, long-chain triglycerides; LDL, low density lipoprotein; LE, lipid emulsion; MCT, medium-chain triglycerides; M-HDL, modified HDL; M-LDL, modified LDL; M-LE, modified lipid emulsion; MLF, MCT/LCT/FO emulsion; PBS, phosphate buffered saline; PL, phospholipid; PLRP, phospholipid-rich particle; *r*, fluorescence anisotropy; TG, triacylglycerol or triglyceride; TGRL, triglyceride-rich lipoprotein; TGRP, triglyceride-rich particle; VLDL, very low density lipoprotein.

Modifications of the chemical and physical properties of both LE particles and lipoproteins may induce more efficient exchanges between these particles, with eventual consequences on their metabolism.

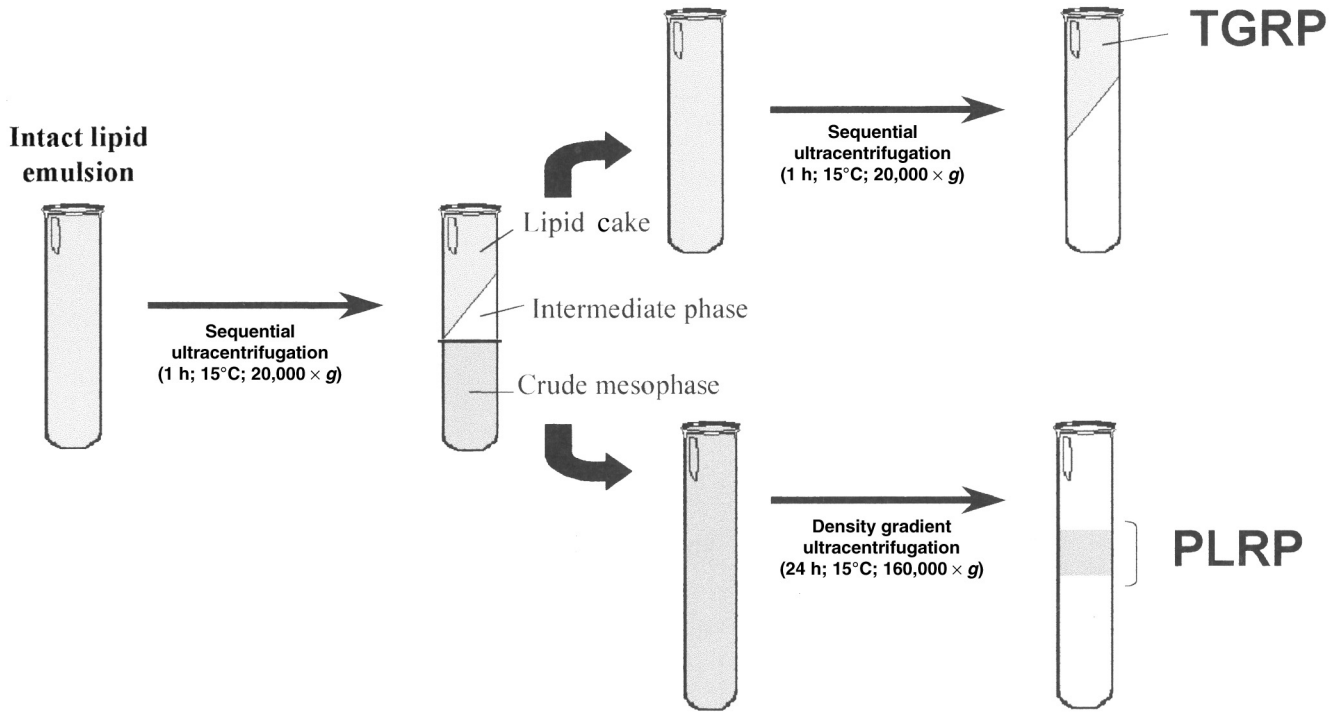
The aim of the present study was to elucidate the role *in vitro* of different components of LE (TGRP and PLRP) on physical property modifications of both LE and LDL or HDL and to explore if the TG core composition of LE plays a role in these modifications.

## EXPERIMENTAL PROCEDURES

*Plasma pool.* All 16 healthy overnight-fasted human subjects (2 males and 14 females, 25–50 yr old) were volunteers for the study, and informed consent was obtained from each volunteer. The blood was collected in tubes containing EDTA (1 mg/mL) and $NaN_3$ (0.2 mg/mL) and immediately placed in iced water. The plasma was separated at 4°C in a Beckman J2.21 (Beckman Instruments Inc., Fullerton, CA) at 3,000 rpm for 10 min. A pool of plasma was constituted, and the lipoprotein classes were immediately separated.

*Preparation of LDL and HDL fractions.* Individual lipoprotein classes were separated by sequential ultracentrifugation (227,000 × *g*; 20 h; 5°C) in a Beckman L8-55 ultracentrifuge using an angular Beckman 50.2Ti rotor (15). The LDL (density range: $1.025 < d < 1.040$ g/mL) and HDL (density range: $1.085 < d < 1.150$ g/mL) subfractions were selected to ensure a relatively homogeneous population of LDL or HDL particles (16,17). Isolated LDL and HDL were washed in KBr solutions of appropriate density. After isolation, the LDL and the HDL fractions were dialyzed in the dark against 40 vol of saline (0.19 M NaCl with 0.02% EDTA, $NaN_3$ 0.02%, pH 7.4). The dialysis solution was changed three times over a 24-h period (18).

*Emulsions.* The emulsions (supplied by B. Braun Melsungen AG, Melsungen, Germany) contained the same amounts of egg yolk PL emulsifier (12 g/L), TG (200 g/L), and glycerol (25 g/L). They differed only by their TG composition: 100% soybean oil [long-chain TG (LCT)]; 50% medium-chain TG (MCT)/50% soybean oil (MCT/LCT); 50% MCT/40% soybean oil/10% fish oil (FO) (MLF 541); 100% FO. Emulsion components (TGRP and PLRP) were fractionated as represented in Scheme 1 according to a method adapted from Férézou *et al.* (19). A 40-mL sample of each emulsion was ultracentrifuged (20,000 × *g*; 1 h; 15°C) in a heat-sealed polyallomer tube (Beckman) using an angular Beckman 50.2Ti rotor in a Beckman L8-55 ultracentrifuge. The tube was cut with a tube-slicer (Beckman) just under the limit of the lipid cake, which contained TGRP; and the crude mesophase (PLRP and glycerol) was discarded. Lipid cake was resuspended in a KBr solution of $d = 1.006$ g/mL. To isolate a pure fraction of TGRP, the lipid cake was washed by a second ultracentrifugation that was run under the same conditions (20,000 × *g*; 1 h; 15°C). TGRP were then resuspended to the initial volume with a solution of $d = 1.006$ g/mL at a TG concentration of 200 g/L. Moreover, PLRP of each emulsion were separated from a 2-mL sample of the crude mesophase by density gradient ultracentrifugation. The KBr gradient was carried out in Sorvall polyallomer tubes (ref. 03699) (Sorvall Instruments, E.I. du Pont de Nemours & Co. Inc., Wilmington, DE) by successively layering 2 mL of




**SCHEME 1**

crude mesophase, 1 mL of water, 1 mL of solution $d$ = 1.006 g/mL, 4 mL of $d$ = 1.019 g/mL, 2 mL of $d$ = 1.063 g/mL, and 1.9 mL of $d$ = 1.125 g/mL. After ultracentrifugation (24 h; 15°C; 160,000 × $g$) in a swinging-bucket rotor Beckman SW41Ti in a Beckman L8-55 ultracentrifuge, 24 fractions of 0.5 mL were successively needle-aspirated from the top of the tube and assayed for PL content. On the basis of the PL profile in the gradient, the PLRP fraction was isolated.

*Incubation protocol.* LDL (0.58 mg of apo B) or HDL (1 mg of apo A-I) fractions were incubated with various intact emulsions, with TGRP (7 mg of TG), or with PLRP (140 µg of PL) for 4 h in a shaking water bath at 37°C (Scheme 2) in sealed Beckman polyallomer Opti-Seal tubes (ref. 361621). After incubation, two fractions were separated by ultracentrifugation of the incubation mixture (163,000 × $g$; 20 h; 5°C) at the density $d$ = 1.006 g/mL in an angular Beckman 50.4Ti rotor using a Beckman L8-55 ultracentrifuge. The tubes were cut 1.5 cm from the bottom. The M-LE fraction containing TGRP or PLRP was in the supernatant, and modified LDL (M-LDL) or HDL (M-HDL) fraction was in the infranatant.

*Polarization fluorescence studies.* The fractions (intact emulsion, TGRP, PLRP, LDL, HDL, M-LDL, M-HDL, M-LE) were labeled with the classical lipophilic fluorescence probe: 1,6-diphenyl-1,3,5-hexatriene (DPH). In brief, 3 mL of a fresh dispersion of DPH in phosphate-buffered saline (PBS) (pH 7.4, 0.01 M) at a final concentration of $1 \cdot 10^{6}$ M were incubated with 100 µL of LDL, M-LDL, HDL, or M-HDL. Similarly, 10 µL of intact emulsion or TGRP or 40 µL of PLRP or M-LE were incubated, for 30 min at room temperature with constant and gentle agitation. The fluorescence anisotropy $r$ of each sample was obtained from fluorescence measurements with an Aminco SPF 500 spectrofluorometer equipped for fluorescence polarization as previously described (10). Each measurement was performed three times for each sample. Two independent samples were prepared for the intact emulsion, but anisotropy measurements were performed on a single sample for TGRP and PLRP. Our results are directly expressed as anisotropy $r$, which relates to the restriction of the probe movement when embedded in the sample (20). A high value of $r$ corresponds to a high structural order and vice versa (20,21). On the basis of our methodology, repeated $r$ values were within 0.002 of each other. Because analyses were performed on very small amounts of each fraction, we obtained values directly, which did not need correction for light scattering (10).

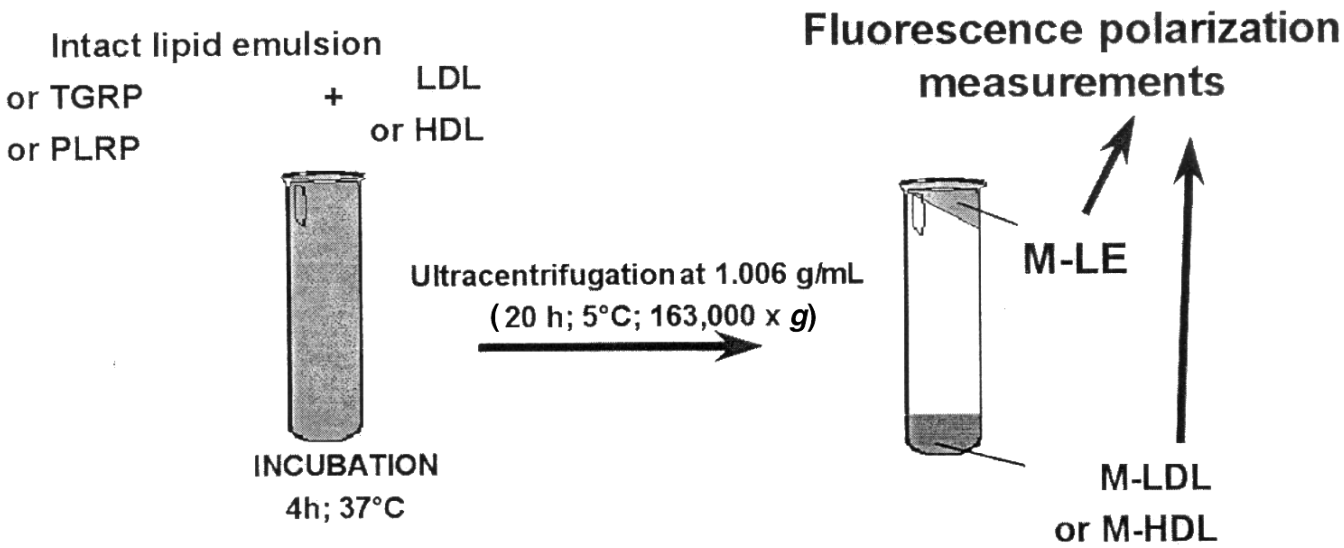
*Analytical procedures.* Lipid analyses were performed on each fraction. Total cholesterol concentration was determined by the cholesterol esterase–cholesterol oxidase method (CHOD-PAP, Roche Diagnostic, Mannheim, Germany). TG were measured enzymatically using a commercial test kit (triglycerides GPO-PAP, Roche Diagnostic). PL were determined by Bartlett method (22) and total protein was measured as described by Lowry *et al.* (23). Apo B and apo A-I were determined in each fraction using a specific enzyme-linked immunosorbent assay method as previously described (24). All reagents including KBr for density solutions were analytical grade products.

*Statistical analysis.* The results are expressed as mean values ± SEM. Student's unpaired test was used to assess the statistical significance between results obtained with different emulsion fractions and lipoproteins under different conditions. Differences were considered significant at $P \leq 0.05$ level.

## RESULTS

*Decrease of emulsion particle fluidity.* Table 1 shows anisotropy values at 24°C for emulsion particles after incubation with LDL or HDL.

Before incubation with lipoproteins, particles of intact emulsion and TGRP had anisotropy $r$ at 24°C that were less than 0.057; the comparable value for PLRP was 0.130. After




SCHEME 2

**TABLE 1**
**Decrease of Emulsion Particle Structural Order[a]**

| | Intact emulsion | | | TGRP | | | PLRP | | |
|---|---|---|---|---|---|---|---|---|---|
| | Initial | M-LE$_{LDL}$ | M-LE$_{HDL}$ | Initial | M-LE$_{LDL}$ | M-LE$_{HDL}$ | Initial | M-LE$_{LDL}$ | M-LE$_{HDL}$ |
| LCT | 0.026 | 0.073 | 0.078 | 0.027 | 0.076 | 0.080 | 0.130 | 0.230 | 0.238 |
| MCT/LCT | 0.028 | 0.066 | 0.066 | 0.017 | 0.065 | 0.065 | 0.115 | 0.215 | 0.231 |
| MLF 541 | 0.057 | 0.063 | 0.055 | 0.020 | 0.070 | 0.065 | 0.110 | 0.277 | 0.228 |
| FO | 0.017 | 0.043 | 0.060 | 0.025 | 0.065 | 0.070 | 0.125 | 0.273 | 0.240 |
| Mean ± SEM | 0.032 ± 0.009 | 0.061 ± 0.006 | 0.065 ± 0.005 | 0.022 ± 0.002 | 0.069 ± 0.003 | 0.070 ± 0.002 | 0.120 ± 0.005 | 0.249 ± 0.015 | 0.234 ± 0.003 |

[a]After incubation (4 h; 37°C) of intact emulsions, triglyceride-rich particles (TGRP), or phospholipid-rich particles (PLRP) with low density lipoproteins (LDL) or high density lipoproteins (HDL), M-LE$_{LDL}$ and M-LE$_{HDL}$ fractions were separated by ultracentrifugation (20 h; 5°C; 163,000 × *g*) at *d* = 1.006 g/mL. Fluorescence anisotropy *r* was measured at 24°C in the M-LE fractions. Abbreviations: M-LE, modified lipid emulsion; LCT, long-chain triglycerides; MCT, medium-chain triglycerides; FO, fish oil; MLF, MCT/LCT/FO present in the ratio of 5:4:1, respectively; SEM, standard error of the mean.

incubation with LDL, the mean anisotropy *r* for all LE were increased, from 0.032 to 0.061 for the intact emulsion, from 0.022 to 0.069 for TGRP, and from 0.120 to 0.249 for PLRP. After incubation with HDL, *r* values of all LE also were increased, from 0.032 to 0.065 for intact emulsion, from 0.022 to 0.070 for TGRP, and from 0.120 to 0.234 for PLRP. An increased of *r* indicates a more ordered particle. This result was confirmed by other measurements made in parallel. The emulsion particles acquired proteins and cholesterol and lost TG (Table 2). These composition changes of LE were in the same order of magnitude, whether incubation was carried out with LDL or with HDL. No significant difference was observed between incubation with LDL and with HDL.

*Increase of lipoprotein fluidity.* In plasma, anisotropy values at 24°C of LDL and HDL were, respectively, 0.257 and 0.220. The lipoprotein anisotropy measurements at 24°C (Table 3) showed smaller values for HDL than for LDL, before and after incubation.

After incubation with intact emulsion, *r* values decreased from 0.258 to 0.243 for LDL and from 0.220 to 0.198 for HDL; after incubation with TGRP, *r* values also decreased from a mean of 0.258 to 0.236 for LDL and from 0.222 to 0.204 for HDL; and after incubation with PLRP, from 0.258 to 0.246 for LDL and from 0.220 to 0.212 for HDL. Thus, lipoprotein structural order was decreased. In parallel, lipoproteins had lost proteins and cholesterol and had acquired some TG. No differences were observed in the TG core composition of incubated LE.

Moreover, whichever the emulsion fraction under incubation, no effect was observed on lipoprotein fluorescence anisotropy. HDL were always less ordered than LDL.

*Behavior of various emulsion components.* Anisotropy values at 24°C before and after incubation are shown for emulsion particles and for lipoproteins LDL and HDL in Tables 1 and 3, respectively.

Initial intact emulsion and initial TGRP displayed low anisotropy *r* values (less than 0.060), whereas PLRP had an anisotropy around 0.120. After incubation with plasma lipoproteins, the structural order of emulsion particles was increased. The *r* values of intact emulsion particles were around 0.063; *r* for TGRP were approximately the same as the intact emulsion, and *r* values for PLRP were much higher, between 0.215 and 0.277. Modifications observed for intact emulsions were in the same range as for TGRP. But PLRP anisotropy after incubation had a value similar to lipoprotein ones (0.249 for LDL and 0.234 for HDL). That observation demonstrated that the intact emulsion had the same behavior as TGRP. In contrast, the thermal susceptibility of PLRP was in the same range as that observed for lipoproteins. As far as modifications of physical properties of lipoproteins were concerned, no differences were observed between incubation with intact emulsion, TGRP or PLRP, and each lipoprotein, LDL or HDL.

*Effect of TG core composition of LE.* The role of LE TG core composition was also addressed, as it has been shown that the nature of the acyl chains of TG influences the physical properties of the particle surface. A series of emulsions varying in the nature of their acyl chains were incubated with LDL or HDL fractions. Table 1 shows differences for TG composition of LE after incubation with both LDL and HDL. The structural order of LE containing only very long chain TG (FO) was increased. After incubation, M-LE particles from LCT emulsion were more rigid, with *r* values from 0.026 to 0.073.

**TABLE 2**
**Composition Modifications of Emulsion Particles During Co-incubation[a]**

| | Triglycerides (μg) | | | Total cholesterol (μg) | | | Total proteins (μg) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Initial | M-LE$_{LDL}$ | M-LE$_{HDL}$ | Initial | M-LE$_{LDL}$ | M-LE$_{HDL}$ | Initial | M-LE$_{LDL}$ | M-LE$_{HDL}$ |
| LCT | 7000 | 4221 | 5615 | 68 | 99 | 85 | 8 | 121 | 125 |
| FO | 7000 | 5527 | 5728 | 31 | 185 | 71 | 16 | 179 | 133 |
| Mean ± SEM | 7000 ± 0 | 4874 ± 653 | 5671 ± 56 | 49 ± 19 | 142 ± 43 | 78 ± 7 | 12 ± 4 | 150 ± 29 | 129 ± 4 |

[a]After incubation (4 h; 37°C) of LCT or FO intact emulsions with LDL or HDL, M-LE$_{LDL}$ and M-LE$_{HDL}$ fractions were separated by ultracentrifugation (20 h; 5°C; 163,000 × *g*) at *d* = 1.006 g/mL. Triglycerides, total cholesterol, and total proteins were measured in the M-LE fractions. For abbreviations see Table 1.

**TABLE 3**
**Increase of Lipoprotein Structural Order[a]**

| | M-LDL (initial LDL = 0.258) | | | M-HDL (initial HDL = 0.220) | | |
|---|---|---|---|---|---|---|
| | Intact emulsion | TGRP | PLRP | Intact emulsion | TGRP | PLRP |
| LCT | 0.240 | 0.245 | 0.262 | 0.207 | 0.215 | 0.215 |
| MCT/LCT | 0.243 | 0.220 | 0.230 | 0.190 | 0.195 | 0.203 |
| MLF 541 | 0.243 | 0.230 | 0.247 | 0.200 | 0.195 | 0.217 |
| FO | 0.245 | 0.250 | 0.245 | 0.193 | 0.210 | 0.212 |
| Mean ± SEM | 0.243 ± 0.001 | 0.236 ± 0.007 | 0.246 ± 0.007 | 0.198 ± 0.004 | 0.204 ± 0.005 | 0.212 ± 0.003 |

[a]After incubation (4 h; 37°C) of intact emulsions, TGRP or PLRP with LDL or HDL, modified LDL (M-LDL) and modified HDL (M-HDL) fractions were separated by ultracentrifugation (20 h; 5°C; 163,000 × *g*) at *d* = 1.006 g/mL. Fluorescence anisotropy *r* was measured at 24°C in the M-LDL and M-HDL fractions. For abbreviations see Table 1.

However, the fluidity of lipoprotein fractions did not differ with the nature of the LE present in the incubation mixture (Table 3).

## DISCUSSION

Lipid emulsions used in parenteral nutrition interact with endogenous lipoproteins (4,6). Apart from their role as an energy source, some essential (ω-3 and ω-6) FA are largely incorporated in envelope PL and may markedly influence cell metabolism, with respect to lipid mediator production (25,26) and regulation of energy balance (27). *In vivo,* after their infusion into the blood stream, LE particles (devoid of apo) rapidly acquire several apo species (mainly C and E) by exchange from HDL (3,28). Such transfer, which also occurs spontaneously *in vitro*, is largely related to the physicochemical properties of emulsion particles and does not require any enzymatic mechanism. This transfer is, however, an absolute requirement for TGRP metabolism because it allows hydrolysis of an important part of TG by lipoprotein lipase and regulates the remnant particle uptake by cells in different tissues (4,29,30). To further characterize TGRP behavior in plasma from various kinds of lipid emulsions, an *in vitro* model was developed to address potential interactions of plasma LDL and HDL with artificial lipid particles.

LDL and HDL obtained from healthy human plasma were incubated with LE that differed only in TG composition and were prepared with an identical PL emulsifier. A fixed LE-TG/LDL-apo B or LE-TG/HDL-apo A-I ratio was used. The ratio was defined to ensure TG concentration similar to those currently in use in clinical practice; plasma apo B and apo A-I concentrations corresponded to the mean normal range.

These experiments allowed detection of an effect of the LE-TG core composition on structural order of LE particles after incubation with LDL and HDL. It was also possible to elucidate the role of different components of LE (TGRP and PLRP) in modifying physical properties of LE and LDL or HDL *in vitro*. During incubation of LE in the presence of LDL or HDL, modifications of physical properties and lipid dynamics took place both in emulsion particles and in lipoproteins.

With regard to LE particles, the structural order was increased after incubation in the presence of plasma lipoproteins. Yet, incubated emulsion particles remained less ordered. Moreover, the TG core composition of LE had an effect on the envelope modifications but these changes were of the same order of magnitude after incubation with LDL or with HDL. After incubation under almost physiological conditions, M-LE fractions were found unexpectedly enriched in apo B as well as in CE (11). Apo B cannot freely transfer due to its very large structure deeply anchored in the LDL particle. Each LDL particle possesses only one apo B molecule (12,13). The apo B presence in the M-LE fraction strongly suggests an LDL binding to TGRP. Thus, an association, aggregation, or fusion occurs between LE particles and LDL and accounts for apo B (and at least some CE) enrichment of the M-LE fraction. The LDL binding to LE was thus a specific property of lipid particles that was independent of the separation method and that was influenced by the particular chemical nature of TG in LE (14). An analogous event, i.e., a fusion of a TGRP (so-called nascent chylomicron) with a small apo B-containing particle, was recently described in the formation process of the endogenous chylomicron (31). A similar mechanism could account for apo B (and at least some CE) enrichment of the M-LE fraction. Besides the physical conditions, a number of factors related to the incubation mixture may modulate the formation of the complex. The most trivial ones are emulsion components other than TGRP, i.e., excess emulsifying PL present as liposomes (PLRP) and glycerol added as stabilizing agent. Preliminary observations have indicated that a number of proteins (mainly albumin present in large amounts in plasma) were able to bind to LE in our incubation conditions (32).

Major physical modifications were observed in intact LE and in two types of particles (TGRP and PLRP) that composed it. LE particles (intact, TGRP, and PLRP) were strongly ordered (Table 1) after incubation with lipoproteins. However, composition modifications owing to exchanges were different depending on the presence of LDL or HDL in the incubation mixture. LE envelope structural order changes were of the same order of magnitude in the two cases. Intact LE, which contains TGRP and PLRP, had almost the same behavior as TGRP. The presence of liposomal particles (PLRP) did not influence the intact emulsion behavior. After incubation, two LE containing FO (MLF 541 and FO) showed a higher envelope order, in spite of acquisition by FO of appreciable

amounts of ordering components (cholesterol and proteins). A more interesting issue in relation to the complex formation lies in the nature of FA contained in the TGRP. Striking differences in the modifications of physical properties of emulsions have been observed according to the TG core composition of LE particles. Isolated LDL, apo A-I, apo C, apo E, and other proteins bind far more to long-chain FA-containing emulsions (FO and LCT) than to MCT/LCT. Nevertheless, M-LE particles from FO emulsion remained the less ordered ones. M-LE from LCT emulsion, which also acquired ordering components (but less than FO), had a more decreased structural order. Along with liposoluble vitamins, TG are the main components of the LE particle core. Our results confirm that TG core composition largely affects surface layer properties (33), presumably by modulating the amount and the flexibility of TG dissolved in the PL surface (34). They also suggest that binding processes, whatever the resulting structure of the complex, regulate the physicochemical properties of the particle surface. On the other hand, PLRP, which are made of a PL bilayer, showed an envelope order similar to that of lipoproteins and did not play a significant role in modifications induced by incubations.

With regard to plasma lipoproteins, the magnitude of physical modifications observed was less important than that detected for LE particles. M-LDL and M-HDL were only moderately less ordered. The structural order of lipoproteins was decreased after incubation with emulsion. HDL, after incubation, remained less ordered than LDL, as in the plasma. The TG composition of the emulsion had no effect on lipoprotein modifications. The high order at 24°C obtained for HDL and LDL reflects a strong lipid–protein interaction (35,36). These molecular order modifications, notably in HDL, are susceptible to increased reverse cholesterol transport to the liver. Moreover, the TG nature of emulsions influenced changes in physical properties of emulsion particles but had no significant effect on LDL and HDL. The HDL fluorescence anisotropy *r* value is positively correlated with free cholesterol percentage of HDL and negatively correlated with TG content and TG/PL ratio of HDL (9). No significant correlation was noted with total and free cholesterol, PL, and proteins of HDL. Consequently, a decreased order might be related to the effect of TG in HDL, as previously shown for LDL (37,38). Lipoprotein fluidity may not reflect only the degree of polyunsaturation of the ingested fat (9). Moreover, relations exist between lipoprotein order, diet, and atherogenicity. Tall *et al.* (39) showed that lipoproteins after consumption of an atherogenic diet were more ordered than the lipoproteins after a normal diet. Soutar (40) suggested that the atherogenicity of lipoproteins increased as they became less ordered. This physical property of lipoprotein could be an important factor in the reduced incidence of atherosclerosis observed in populations consuming a "Mediterranean diet," which is rich in olive oil. In the study by Sola *et al.* (9), a diet rich in monounsaturated FA, i.e., the olive oil diet, induced a reduced order of HDL. The order is an important determinant of the capacity of $HDL_3$ to accept cholesterol from the cells. Thus, a decreased order in lipoprotein is suitable for the acquisition of more cholesterol, a well-know rigidifying substance (41). However, a low molecular order may lead to perturbations in lipoprotein functions, for example, alterations of lipoprotein affinity for the receptor or disturbance of interactions with other lipoproteins. According to the optimal fluidity, which inversely relates to the molecular order hypothesis (42), a specific degree of HDL fluidity may exist that favors an optimal HDL capacity to remove cellular cholesterol and therefore to avoid its accumulation. These modifications could alter the HDL and LDL spatial configuration, induce changes in the apo configuration at the surface of these lipoproteins, and alter the interactions between different lipoproteins or between lipoproteins and cells.

Besides strictly physicochemical interactions, other plasma factors may play a role in the interplay between lipoproteins and emulsion components. Just like chylomicrons, TGRP can transfer TG and accept CE during exchange with LDL (7,43,44). These neutral lipid exchanges are influenced by CETP concentration (5,45). These physical modifications could induce more efficient exchanges between emulsion particles and lipoproteins and so influence metabolism of these different exogenous and endogenous particles.

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# Comparative Studies on Individual Isomeric 18:1 Acids in Cow, Goat, and Ewe Milk Fats by Low-Temperature High-Resolution Capillary Gas–Liquid Chromatography

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**ABSTRACT:** The *trans*- as well as the *cis*-18:1 isomer profiles were established in cow, goat, and ewe cheese fats, with the assumption that these are representative of the corresponding milks. Argentation thin-layer chromatography was combined with low-temperature high-resolution gas–liquid chromatography on 100-m highly polar capillary columns, thus adding precision to earlier data for these species. Despite differences in the absolute content of *trans*-18:1 isomers between species, the relative profiles were essentially similar. Except for the minor *trans* Δ6–Δ8 group, all *trans*-18:1 isomers with their ethylenic bonds between positions Δ4 and Δ16 (including the resolved critical pair Δ13/Δ14) were separated and quantitated individually. As expected, vaccenic (*trans* Δ9-18:1) acid was the main isomer, accounting for as much as 37 to 50% of the total fraction. It was observed that the goat *trans*-18:1 isomer profile was usually rather close to that of cows in winter (barn feeding), whereas that of the ewe shows a seasonal dependence. The *trans*-18:1 profile of ewe milk fats from this study resembles that of cows in the transition period between winter and summer (pasture) feeding. Regarding the *cis*-18:1 acid fraction, two isomers (oleic and *cis*-vaccenic acids) accounted for *ca.* 97% of that fraction for the three species, with the *cis*-Δ12 isomer ranked third. The analytical procedure employed here appears a convenient alternative to oxidative-based procedures (generally ozonolysis), taking less time and alleviating some drawbacks of the latter procedure.

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As a result of earlier studies on the daily *trans*-18:1 acid consumption by European populations (1,2), it is well known that ruminant fats [mainly from dairy products (80–90%) and secondarily from meat fat and tallow (10–20%)] can constitute the main dietary source of *trans*-18:1 acids in many countries. A wealth of data has been published on the content and distribution of these isomers in cow milk fat, mostly by authors Precht and Molkentin (3–7) and Wolff (1,2,8), and these were recently reviewed in a book chapter (9) on natural edible sources of dietary *trans* fatty acids. On the other hand, ewe and goat milk fats are less well-documented, but useful data are available in the same chapter (9) and elsewhere (2). Also, important analytical data are to be found in an early paper by Bickerstaffe *et al*. (10) for goat milk fat.

To complete our knowledge on the distribution profile of *trans*-18:1 isomers in cow, goat, and ewe milk fats, we adopted for the present study a procedure that combines an argentation thin-layer chromatography (Ag-TLC) step with gas–liquid chromatography (GLC) on 100-m cyanopropyl-polysiloxane coated capillary columns operated under optimal temperature and carrier-gas pressure conditions. In France, cheeses are always manufactured with a single kind of milk. Cheeses were chosen because goat and ewe milks are almost exclusively employed for cheese processing. In all instances, total fatty acids starting with 4:0 acid were quantitated using a polyethylene-glycol coated 25-m capillary column, as well as polyunsaturated $C_{20}$ and $C_{22}$ acids (not reported here). *Trans*-18:1 acids were freed from overlapping *cis*-18:1 isomers, and from early-eluting isomeric 18:2 acids, thus ensuring reliable and accurate quantitative data. When the temperature of the columns is sufficiently low (e.g., 120°C is "low-temperature" GLC), base-line resolution is obtained for most individual *trans*-18:1 isomers, i.e., for the Δ4, Δ5, Δ9, Δ10, Δ11, Δ12, Δ15, and Δ16 isomers. The Δ6 to Δ8 isomers (minor), however, remain unresolved, whereas the Δ13 and Δ14 isomers are resolved with $R \approx 1$.

Data obtained under such conditions for the fat from French cow, ewe, and goat cheeses (12 samples each) were compared with earlier less well-detailed values for similar food categories (4,9). Differences among the three species are shown to be apparently species-dependent and are in fact most probably linked to the feed of the animals (abundance and nature of polyunsaturated acids in the feed). Data for ruminant fats may be compared with those for partially hydrogenated vegetable oils recently published for French processed foods (11). Also included in the present study are data for individual *cis*-18:1 positional isomers, not reported previously for goat and ewe milk fat.

## EXPERIMENTAL PROCEDURES

*Samples*. Three lots each of 12 different cheeses made with cow, goat, or ewe milks (produced in several regions of France) were purchased locally in supermarkets near Bordeaux (France) in May–June 1999. Genuineness of the samples was

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Abbreviations: Ag-TLC, argentation thin-layer chromatography; FAME, fatty acid methyl ester; FFA, free fatty acid; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; TMSH, trimethyl sulfonium hydroxide.

ascertained by establishing the ratios of 12:0/10:0 acids (2). In this study, it was assumed that cheese fat fatty acids are representative of milk fat fatty acids, as milk fat globules are retained in the cheese matrix during cheese processing.

*Fat extraction*. Cheese fat was extracted according to Wolff *et al*. (8) using isopropanol and hexane. Representative portions of the cheeses (*ca*. 40 g) were homogenized in a mortar with a pestle, and 5 g of the homogenates were dispersed in 10 mL of isopropanol in a Teflon beaker with an Ultraturrax T25 (Janke & Kunkel GmbH & Co. KG, Staufen, Germany) before adding a sufficient amount of anhydrous $Na_2SO_4$ and 15 mL of hexane and performing a second dispersion with the Ultraturrax. The suspension was then filtered on a glass column containing a lower layer of anhydrous $Na_2SO_4$ and an upper layer of Celite 545 (*ca*. 2 cm in height each). The lipid solution was eluted three times successively with 25-mL portions of hexane/isopropanol (3:2, vol/vol) and collected in a 250-mL flask. After removal of the solvents in a rotary evaporator at 45°C, the fat was weighed and transferred into 5-mL vials and stored at 4°C until used.

*Fatty acid methyl ester (FAME) preparation*. To choose a suitable transesterification method, FAME were prepared from cheese fat samples with particularly high content of free fatty acids (FFA) using trimethyl sulfonium hydroxide (TMSH) as well as sodium methoxide. However, the results showed that the fatty acid composition was not significantly influenced by the present FFA contents. As compared to the TMSH method, the sodium methoxide method was found to be more accurate and reproducible. FAME were prepared by vigorously shaking for 3 min a mixture of 1.2 mL *n*-heptane, 0.3 mL of a 10% fat solution in *n*-heptane, and 30 µL of a 2 N sodium methoxide solution in methanol. After centrifugation, the supernatant was directly used for GLC analysis. For Ag-TLC, a 20% fat solution was used. The fat solutions used for FAME fractionation by Ag-TLC were obtained by blending equal amounts of fats from each of the 12 cheeses from a given species. Data reported here for individual isomeric 18:1 acids are thus for composite samples.

*Fractionation of FAME by Ag-TLC*. FAME were fractionated according to the number and geometry of double bonds by TLC on silica-gel plates impregnated with $AgNO_3$. The plates were prepared by immersion in a 20% aqueous solution of $AgNO_3$ as described by Precht and Molkentin (3). The developing solvent was the mixture *n*-heptane/diethyl ether (90:10, vol/vol). At the end of the chromatographic runs, the plates were briefly air-dried, lightly sprayed with a solution of 2′,7′-dichlorofluorescein, and viewed under ultraviolet light (234 nm). The *trans*- and *cis*-monoenoic acid bands were scraped off separately and eluted several times with diethyl ether. Complete evaporation of the combined eluates of each fraction was achieved with a light stream of $N_2$. The residues were dissolved in an appropriate volume of *n*-heptane for further GLC analysis.

*Analysis of FAME by GLC*. The total fatty acid composition covering *ca.* 70 fatty acids in the range 4:0 to 24:0, including all polyunsaturated fatty acids, was determined by GLC analysis of the methyl esters prepared from each individual cheese sample on a CP 9001 chromatograph (Chrompack, Middelburg, The Netherlands) equipped with a split injector (split ratio, 1:50) and a flame-ionization detector, using a 25-m capillary CP-Wax 58 CB column (0.25 mm i.d., 0.20 µm film thickness; Chrompack). The operating conditions were as follows: $H_2$ as the carrier gas at an inlet pressure of 40 kPa; injector and detector temperature, 265°C; and oven program of 50°C for 1 min, then 5°C/min to 225°C, 15 min isothermal, and finally 5°C/min to 260°C. Calibration of the individual fatty acids was achieved using the reference butterfat CRM 164.

Analyses of the *cis*- and *trans*-18:1 isomers in total FAME as well as in 18:1 isomer fractions isolated by Ag-TLC were performed using a gas chromatograph CP 9000 (Chrompack) with a split injector, a flame-ionization detector, and a fused-silica capillary column (100 m × 0.25 mm i.d.) coated with 0.20 µm CP-Sil 88 (Chrompack) under the following conditions: $H_2$ as the carrier gas; injector temperature 255°C; and detector temperature 280°C. The unfractionated FAME were analyzed isothermally at 172°C with a column head pressure of 160 kPa (split ratio, 1:50). Monoenoic TLC fractions were analyzed isothermally at a lower temperature, 120°C, with a column head pressure of 220 kPa (split ratio, 1:25) (5,12). Under these conditions, elution of *cis*- as well as *trans*-18:1 isomers was achieved in approximately 3.5 h.

The calibration of positional isomers of *trans*-18:1 acids present in the *trans*-monoenoate fraction was achieved with the isomer *trans* Δ11-18:1 that was quantitated by stearic acid in the isothermal chromatography of the unfractionated FAME on the CP-Sil 88 column. Before, stearic acid was determined by total FAME analysis on the CP-Wax 58 CB column. Further, *cis*-18:1 isomers found in the *cis*-monoenoate fraction were calibrated by *cis* Δ11-18:1 acid that could be determined in the isothermal chromatogram of unfractionated FAME. The high content of *cis* Δ9 was calculated from the unfractionated FAME subtracting the overlapping *trans* Δ13/Δ14 and *cis* Δ6-Δ8 isomers.

Identification of individual isomeric *cis* and *trans* octadecenoates was achieved by comparison of retention times with FAME standards of the 18:1 isomers Δ6, Δ7, Δ9, Δ11, Δ12, Δ13, and Δ15 (Sigma, St. Louis, MO,). *Trans*-18:1 acids isolated from butterfat were used as a secondary standard (2,3). Integration and quantitation were accomplished with an HP 3365 II ChemStation system (Hewlett-Packard, Palo Alto, CA). Calibration of GLC data included the conversion from FAME to FFA. Thus, results expressing absolute concentrations are given as g/100 g of total fatty acids.

## RESULTS AND DISCUSSION

In earlier studies on the distribution profile of *trans*- and *cis*-18:1 isomers in ruminant milk fats, except for data obtained by oxidative cleavage and further GLC analysis of the resulting fragments (10,13,14), GLC analyses of these isomers on 50-m columns allowed only partial insight into the pattern of individual isomers. The Δ6–Δ9 isomer group was largely

unresolved, as were the Δ10/Δ11 and the Δ13/Δ14 pairs. Shoulders could be observed in some instances, but their accurate quantitation was questionable (1–3,5,9,15). Since 1995 (16,17), the use of 100-m columns has permitted some improvements in their separation (i.e., the Δ6–Δ8 isomer group from the Δ9 isomer, and the Δ10 from the Δ11 isomer). A further improvement was obtained by greatly decreasing the oven temperature to 125 or 120°C, allowing resolution of the critical Δ13/Δ14 isomers pair (4,5). Improvements in the resolution *R* were due to an increase of both the column length *L* and the capacity factor *k′*, as well as the selectivity factor α that is known to vary with the temperature. Separation of the Δ13/Δ14 isomers pair is remarkable, as these two acids have equivalent chain lengths determined on both 50- and 100-m CPSil 88 capillary columns measured with authentic standards at 160°C that differ by no more than 0.01 carbon unit (Wolff, R.L., and Destaillats, F., unpublished results). An apparent drawback is a considerable increase in the time of analysis (*vide infra*). Despite this enhanced resolution, fractionation of *cis*- and *trans*-monoenoic acids is still a prerequisite to their accurate measurement because of unavoidable overlaps between some components of these two fractions, i.e., when direct GLC of FAME is used.

A typical chromatogram showing the resolution obtained with *trans*-18:1 isomers prepared from ewe cheese fat is shown in Figure 1, and Table 1 gives the relative proportions of all individual *trans*-18:1 isomers (except for the Δ6–Δ8 group) in cow, goat, and ewe cheese fats. As expected, genuineness of cheese samples was confirmed by the 12:0/10:0 acids ratios: cow, 1.11; goat, 0.45; and ewe, 0.57, which compare well with earlier data (2). The total contents of *trans*-18:1 acids were 4.5, 3.1, and 4.9% of total fatty acids, respectively. For the latter two species, data obtained in the present study are in fairly good agreement with previous data for the same species, 2.7 and 4.5% (2), respectively. Our data for goat milk fat, which are the lowest among the three species studied, are close to those reported for goat milk from Spain by Alonso *et al.* (18), 2.1%. As expected, the value for cow cheeses is close to the value for French spring butterfats (4.3%) (8). Considerably lower values are reached in winter (barn-feeding), *ca.* 2.4% (8) or 2.7% (4). This supports the well-known observation that grass, likely because of its high α-linolenic acid content, is the main factor that affects accumulation of *trans*-18:1 acids in milk fat. In France, goats are mostly barn-fed, whereas sheep are pasture-fed, as are cows in spring.

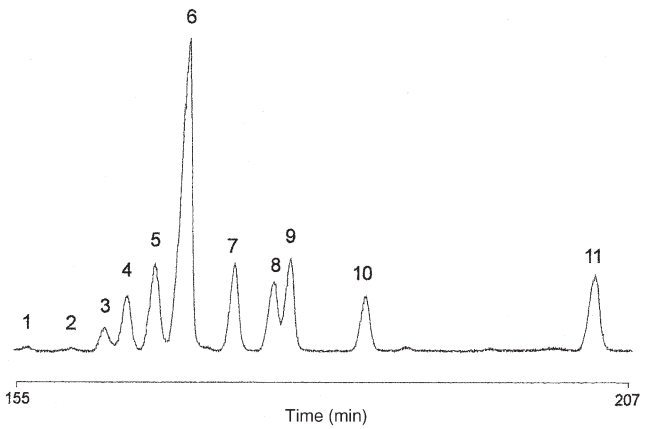


**FIG. 1.** Representative chromatogram of *trans*-isomeric octadecenoic acid methyl esters prepared from ewe cheese fat and fractionated by argentation thin-layer chromatography prior to analysis by low-temperature capillary gas–liquid chromatography on a 100-m CP-Sil 88 capillary column (Chrompack, Middelburg, The Netherlands; isothermal at 120°C, with $H_2$ as the carrier gas at an inlet pressure of 220 kPa). Peak numbering as in Table 1.

**TABLE 1**
**Relative Distribution of *trans*-Positional Isomers of Octadecenoic Acid in Cow, Goat, and Ewe Cheese Fat**

| Peak number[a] | Double bond position[b] | Total *trans*-18:1 fatty acids[c] (wt%) | | |
|---|---|---|---|---|
| | | Cow | Goat | Ewe |
| 1 | Δ4 | 0.5 | 0.7 | 0.6 |
| 2 | Δ5 | 0.4 | 0.5 | 0.5 |
| 3 | Δ6–Δ8 | 2.0 | 2.8 | 2.4 |
| 4 | Δ9 | 4.3 | 6.1 | 4.7 |
| 5 | Δ10 | 6.4 | 10.2 | 8.9 |
| 6 | Δ11 | 50.9 | 37.2 | 47.0 |
| 7 | Δ12 | 6.3 | 9.0 | 6.6 |
| 8 | Δ13 | 7.1 | 8.0 | 7.1 |
| 9 | Δ14 | 7.9 | 9.3 | 8.3 |
| 10 | Δ15 | 6.0 | 6.4 | 5.6 |
| 11 | Δ16 | 8.0 | 9.8 | 8.3 |

[a]Peak numbers refer to Figure 1.
[b]The position of the double bond is counted from the carboxylic end.
[c]Values established with composite samples (see Experimental Procedures section).

As a matter of comparison with most recent and extensive studies of cow milk fat in European countries, our data are between the values for the transition period in spring (3.8%; 236 samples) and the pasture-feeding period (5.08%; 593 samples) determined by Precht and Molkentin (4) for a large area of milk collection in Germany. The mean annual value for German butterfat (1756 samples) is 3.6% (3). However, our average *trans*-18:1 content for French cow cheese fats is similar to the annual mean content determined by the same authors (3) for milks collected in all other European countries (4.2%; 111 samples). These geographical variations are likely indicative of different climatic conditions and, thus, of different rearing habits in the countries considered (duration of pasture feeding vs. barn feeding). On the other hand, the present data and those quoted in the preceding references are significantly higher than those reported in the industry-sponsored TRANSFAIR study (19) covering 14 western European countries, for which a mean geographical content of 2.7% can be calculated. This discrepancy arises from differences in analytical procedures. In the TRANSFAIR study, *trans*-18:1 isomers were assessed by direct GLC runs, without Ag-TLC prefractionation. This procedure is known to give access only to the Δ4 to Δ11 isomers, with the Δ12 to Δ16 isomers masked by oleic and *cis*-vaccenic acid or even eluting after them (3). If we take as a reference the data from Precht and Molkentin (3) for European countries, established with the combined Ag-TLC/GLC procedure, a correction factor of 1.56 should be applied to data from the TRANSFAIR study, which consequently appear to

be underreporting by *ca*. 35%. This mean correction factor is exactly the same as that established for approximately 2,000 samples of milk or butterfat based on chromatographic considerations and taking into account seasonal as well as geographical variations (15). However, this correction factor can by no means be used to correct data for individual samples of ruminant fats, as it varies with the feed of the cattle (15).

It is common knowledge that vaccenic acid, the *trans*-Δ11 18:1 isomer, is the main constituent of *trans*-18:1 acids in ruminant fats. This acid was named from the Latin *vacca*, cow, by Bertram (20), who characterized it structurally for the first time in beef and sheep tallow as well as in butterfat as early as 1928. Here, it varies between 37 and 50% of the total fraction, depending on the species. The second main *trans*-18:1 isomer is either the Δ10 or the Δ16 isomer. The latter acid is frequently and unjustifiably ignored, though it is not negligible (between 8 and 9% of the *trans*-18:1 isomer group). In principle, this acid can be detected by single GLC analysis, as it is the only *trans*-18:1 isomer that elutes after *cis*-vaccenic acid, with the structure *cis* Δ11-18:1. This is due to a considerable retardation during GLC as compared to other isomers. However, quantitation of the Δ16 *trans* isomer by single GLC is impaired by an overlap with the *cis* Δ14-18:1 isomer. Some older papers already reported on this isomer (21) identified on chromatograms of total FAME. In partially hydrogenated vegetable oil, the *trans*-Δ16 18:1 isomer represents on average only 1% of total *trans*-18:1 acids (11,22). In fact, more than 90% of the *trans*-18:1 acid fraction in ruminant fats have their ethylenic bond between positions Δ9 and Δ16.

Within the *trans* Δ13/Δ14 pair, which can be resolved only under low-temperature conditions, the Δ14 isomer appears to be present in slightly higher amounts than the Δ13 isomer. This holds for the three species considered (Table 1). Unfortunately, even under such conditions, the group *trans* Δ6–Δ8 remains unresolved, but its total is rather low (5–7%). Parodi (14) found five times as much *trans*-Δ8 18:1 acid compared to *trans*-Δ6 or *trans*-Δ7 18:1 acids in butterfat using ozonolysis, and this may be the real reason for the lack in resolution. It is worth noting that elaidic (*trans*-Δ9 18:1) acid, the main *trans*-18:1 isomer in PHVO (11,22), is present in considerably lower amounts in ruminant milk fats, as it represents on average only 4 to 7% of total *trans*-18:1 acids.

In Table 2, data from the present study are compared with earlier data also obtained by the combination Ag-TLC/GLC, but employing a shorter (50-m) CP-Sil 88 capillary column. On this kind of column, the Δ6–Δ9 group and the Δ10/Δ11 and Δ13/Δ14 pairs of *trans*-18:1 isomers are not resolved. So, data from the present study are summed as a matter of comparison with earlier data from France (9). Two remarks can be made. The first one relates to the good reproducibility of data for goat and ewe milk fats, despite an interval of 5 yr between the two studies and some differences in the experimental procedures (e.g., 100- vs. 50-m columns, FAME instead of fatty acid isopropyl esters, different integration apparatuses). There is an obvious resemblance between milk fat from cows reared in winter and goat milk fats. This is attributable to the fact that goats in France are mostly kept in barn throughout the whole year. Second, the *trans*-18:1 acid isomeric profile of the ewe cheese fats from this study resembles that of cow milk fat from the transition period in Germany (1997) or from March in France (1995) as well as that of the present cow cheese samples. However, the ewe milk fats from 1995 are similar to cow milk fats from the pasture feeding period in France (1995) as well as in Germany (1997). In particular, the Δ10/Δ11 pair is within the narrow range 45–48% in cow milk in March and in goat milk, whereas it is about 10% more in cow milk in spring as well as in ewe milk (range 56–58%; Table 2). From this it can be inferred that the similar *trans*-18:1 isomer profile for cows and ewes likely results from similar feeding regimens and thus may vary throughout the year. The simultaneous course of *trans*-18:1 isomer profiles for cows and ewes results from a similar keeping of the species, different from goats. Similarities between ewes and cows can also be recognized from the absolute contents of *trans*-18:1 isomers shown in Figure 2. Among these fats originating all from the same season, ewe and cow milk fats exhibit almost the same content of *trans*-vaccenic acid (2.0%), while goat milk fat contains only one-half that quantity, 1.0%.

The *cis*-18:1 acid fraction is less complex than its *trans* counterpart, with only seven peaks (Fig. 3), of which those

**TABLE 2**
**Comparison of *trans*-Octadecenoic Acid Profiles (% of total *trans*-18:1 isomers) in Goat and Ewe Milk Fat with That of Cow Milk Fat at Different Periods of the Year**

| | Goat | Goat | Cow | Ewe | Ewe | Cow | Cow |
|---|---|---|---|---|---|---|---|
| Double bond position | 1995[a] | 1999[b] | 1995 (March) | 1995 | 1999 | 1995 (May–June) | 1999 (April–May) |
| Δ4 | —[c] | 0.7 | — | — | 0.6 | — | 0.5 |
| Δ5 | — | 0.5 | — | — | 0.5 | — | 0.4 |
| Δ6–Δ9 | 12.6 | 8.9 | 6.7 | 8.3 | 7.1 | 7.2 | 6.3 |
| Δ10/Δ11 | 44.9 | 47.4 | 47.8 | 56.7 | 55.9 | 58.2 | 57.3 |
| Δ12 | 8.7 | 9.0 | 10.2 | 6.7 | 6.6 | 6.2 | 6.3 |
| Δ13/Δ14 | 18.2 | 17.3 | 19.4 | 15.6 | 15.4 | 15.4 | 15.0 |
| Δ15 | 6.8 | 6.4 | 7.4 | 5.2 | 5.6 | 5.7 | 6.0 |
| Δ16 | 8.8 | 9.8 | 8.4 | 7.5 | 8.3 | 7.3 | 8.0 |

[a]Data for 1995 from Reference 9.
[b]Data for 1999 are from the present study.
[c]Not reported.

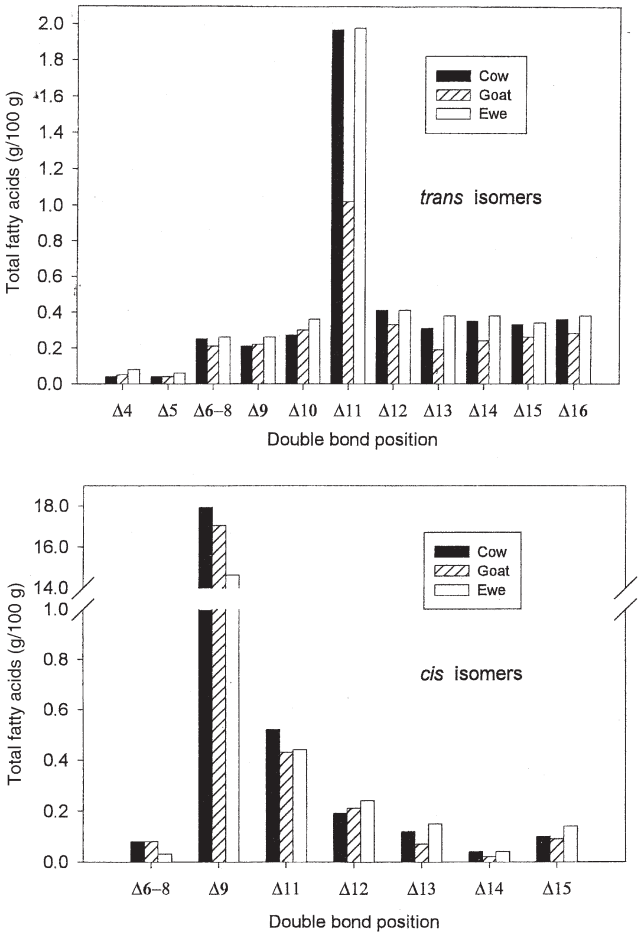


**FIG. 2.** Comparison of the absolute distributions of *trans*- and *cis*-octadecenoic acids (wt% relative to total fatty acids from 4:0 to 22:6n-3) in cow (black bars), goat (hatched bars), and ewe (open bars) cheese fats.

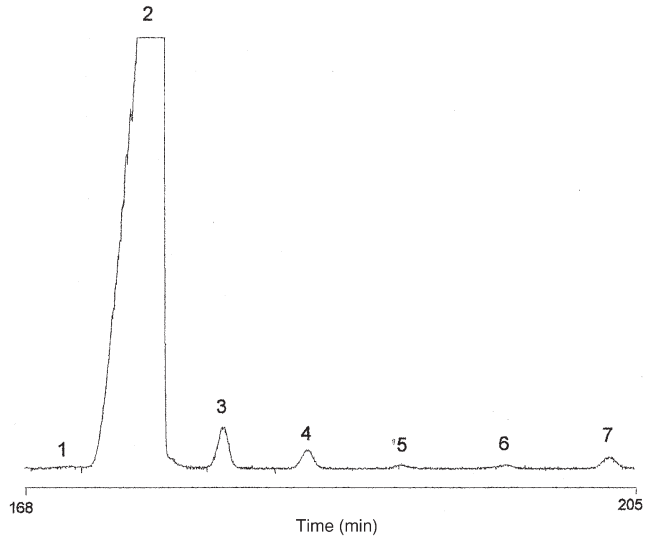


**FIG. 3.** Representative chromatogram of *cis*-isomeric octadecenoic acid methyl esters prepared from ewe cheese fat and fractionated by argentation thin-layer chromatography prior to analysis by low-temperature high-resolution gas–liquid chromatography on a 100-m CP-Sil 88 capillary column (same commercial source and operating conditions as in Fig. 1). Peak numbering as in Table 3.

corresponding to oleic and *cis*-vaccenic acids account for about 97% of the total fraction (Table 3). Other components are the Δ6–Δ8, and Δ12 to Δ15 isomers, most often each less than 1% of total *cis*-18:1 acids. The *cis* Δ6–Δ8 peak that elutes before oleic acid is of minor importance but slightly interferes with *trans* Δ13/Δ14-18:1 isomers during direct GLC analyses of FAME without prefractionation by Ag-TLC. Similarly, the *cis* Δ14-18:1 isomer interferes with the *trans* Δ16-18:1 isomer (results not shown). The *cis* Δ15-18:1 isomer is possibly derived from α-linolenic acid by biohydrogenation (23). Much more than for *trans*-18:1 acids, the relative *cis* isomer profiles do not seem to be species-dependent (Table 3).

In an earlier paper (2), it was noted that ewe and goat milk fats may not be negligible as a source of dietary *trans*-18:1 acids in those countries where cow rearing is not easily feasible, in particular in some Mediterranean countries (e.g., Greece) or regions. Whether data established in France can be extrapolated to such countries, however, remains to be established. We are aware of only one study on goat milk fat comparable to ours, that from Alonso *et al.* (18), who also employed the Ag-TLC/GLC procedure with a 100-m capillary CP-Sil 88 column. However, in that study, the operating conditions led to chromatograms similar to those obtained with 50-m columns, and integration might not have been perfect. Nevertheless, the total *trans*-18:1 isomer content was similar to, though slightly lower than, that reported in the present study.

Regarding the procedure for isomer profiling described here, it compares advantageously with ozonolysis-based procedures and requires less equipment. In the latter procedure, an argentation chromatography is also a prerequisite for further analysis. However, if the sample contains both *trans*-16:1 and -18:1 acids, as in ruminant fats (or other *trans*-monoenes, as in partially hydrogenated fish oils), a supplementary step is necessary to fractionate monoenoates as a function of their chain length: either by preparative GLC, or by reverse-phase high-performance liquid chromatography (HPLC). The purified fractions then have to be ozonolyzed, prior to GLC of the resulting fragments. Drawbacks of that procedure are: (i) dubious quantitative extraction of all fragments from the ozonolyzis medium, (ii) loss of the more volatile molecules

**TABLE 3**
**Relative Distribution of *cis*-Positional Isomers of Octadecenoic Acid in Cow, Goat, and Ewe Cheese Fat**

| Peak number[a] | Double bond position[b] | Total *cis*-18:1 fatty acids[c] (wt%) | | |
|---|---|---|---|---|
| | | Cow | Goat | Ewe |
| 1 | Δ6–Δ8 | 0.2 | 0.3 | 0.2 |
| 2 | Δ9 | 96.0 | 95.6 | 94.9 |
| 3 | Δ11 | 2.0 | 2.0 | 2.2 |
| 4 | Δ12 | 0.7 | 1.1 | 1.2 |
| 5 | Δ13 | 0.4 | 0.3 | 0.3 |
| 6 | Δ14 | 0.3 | 0.4 | 0.4 |
| 7 | Δ15 | 0.6 | 0.5 | 0.9 |

[a]Peak numbers refer to Figure 3.
[b]The position of the double bond is counted from the carboxylic end.
[c]Values established with composite samples (see Experimental Procedures section).

during any solvent evaporation step, and (iii) application of specific response factors for each fragment. With the Ag-TLC/GLC procedure, including low-temperature GLC, these drawbacks are avoided. The *trans*-16:1 as well as the *trans*-18:1 isomers can be determined during the same GLC run, evaporation will not lead to any loss, and no response factors are needed. Even if the time of analysis is about 3.5 h, there are no extra manipulations except for the Ag-TLC fractionation step. If the absolute contents are to be calculated, an additional analysis of the total FAME composition, common to both procedures, has to be performed. The equipment and supplies needed for the Ag-TLC/GLC are standard in most laboratories and there is no requirement for an HPLC and an ozonizer. The Ag-TLC/GLC procedure thus is a relatively fast and economical means of analysis of individual *trans*-monoenoates. Except for the minor Δ6–Δ8 group, data for individual isomers are thought to be more accurate and reliable than those obtained using ozonolysis.

Finally, it should be added that columns different from the CP-Sil 88 can be used, as well as derivatives of fatty acids other than FAME [isopropyl esters (8,16), or 4,4-dimethyloxazoline derivatives (24,25)].

## ACKNOWLEDGMENTS

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# Enrichment of Eggs with n-3 Polyunsaturated Fatty Acids: Effects of Vitamin E Supplementation

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**ABSTRACT**: Eggs enriched with n-3 polyunsaturated fatty acids (PUFA) could contribute to dietary intake of these healthful fatty acids (FA). Because n-3 PUFA are highly susceptible to peroxidation, a first part of the study with Leghorn laying hens was carried out to investigate the influence of different levels of fish oil (0, 0.7, 1.4, 2.8, or 5.6%, respectively) in the diet on n-3 PUFA, cholesterol, vitamin E, and lipid peroxidation product contents in eggs. Addition of fish oil to a complete diet based on wheat, rye, tapioca, and soybean constituents containing 11 IU vitamin E/kg resulted in increased n-3 PUFA content in egg yolk, mainly due to accumulation of docosahexaenoic acid. Cholesterol was not altered up to 2.8% fish oil in the diet. The vitamin E content of the yolk was insufficient for the protection of PUFA from peroxidation. Addition of up to 2.8% fish oil to laying hen diets increased the n-3 PUFA content of yolks with a concomitant imbalance between vitamin E and PUFA, leading to increased levels of cytotoxic aldehydic lipid peroxidation products such as malondialdehyde (MDA). In a second part of the studies, the balance between vitamin E, PUFA, and lipid peroxidation was analyzed during the period of storage of n-3 PUFA-enriched eggs produced after feeding the laying hens with 1.5% fish oil diets with different concentrations of vitamin E (0, 5, 10, 20, 40, 80, 160 IU/kg). Storage of eggs resulted in a marked loss of vitamin E in yolk. In stored eggs, the cytotoxic lipid peroxidation products MDA, 4-hydroxynonenal, and 4-hydroxyhexenal were reduced in response to vitamin E supplementation. To prevent the increase of cytotoxic aldehydic lipid peroxidation during production and storage of n-3 PUFA-enriched eggs, a high vitamin E supplementation with at least 80 IU vitamin E/kg is needed.

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As components of membrane phospholipids n-3 polyunsaturated fatty acids (PUFA) play a key role in the structure and function of biological membranes. They influence membrane fluidity, receptor and enzyme activities, ion channel properties, and signal transduction pathways. Additionally, the n-3 PUFA eicosapentaenoic acid (EPA, 20:5n-3) serves as a source for eicosanoid synthesis (1).

However, the typical Western diet provides much more n-6 PUFA than n-3 PUFA. In this diet the content of linoleic acid (LA, 18:2n-6) is especially high. That fatty acid is abundant in vegetable oils, e.g., corn oil, sunflower oil and safflowerseed oil (2). LA undergoes desaturation and elongation to form arachidonic acid (AA, 20:4n-6), which is the principal precursor for prostanoids of the 2 series and leukotrienes of the 4 series. Metabolically active PUFA from the n-3 PUFA family—EPA and docosahexaenoic acid (DHA, 22:6n-3) found in high concentrations in fish oil—are synthesized from α-linolenic acid (α-LNA, 18:3n-3), the major n-3 PUFA in vegetable oils like linseed oil and soybean oil. PUFA of both n-6 and n-3 families compete for the same enzymes for their metabolic pathways. Remarkably, prostanoids of the 3 series and leukotrienes of the 5 series derived from EPA are in general less prothrombotic and proinflammatory than eicosanoids originating from AA. The dietary ratio between n-6 and n-3 PUFA is important for a more or less inflammatory status (3,4). Hence, the changes in dietary habits leading to an imbalance between n-6 and n-3 PUFA in the diet may make a person more vulnerable to inflammatory diseases, e.g., rheumatoid arthritis, asthma, atopic dermatitis, diabetes, cancer, and AIDS (3,5,6). n-3 PUFA also have beneficial effects on a number of diseases associated with civilization, such as coronary heart disease (7). These are likely due to the hypolipidemic, antithrombotic, and antiarrhythmic effects of n-3 PUFA (1,8,9). Therefore, dietary recommendations encourage increased consumption of fish. However, dietary intake has been decreasing; therefore, it is important to raise the dietary intake of n-3 PUFA in populations of Western countries. Because it is difficult and time-consuming to change the nutrition habits of a population and because seasonal availability and consumer preference may limit fish consumption, enriching the n-3 PUFA content of food products is a reasonable approach. The enrichment of hens' complete diet based on wheat, rye, tapioca and soybean meal with n-3 PUFA has been tested (10). Alternatively, eggs enriched with n-3 PUFA also can contribute to the dietary intake of these healthful PUFA, n-3 providing them in a highly bioavailable phospholipid form (11,12).

Since n-3 PUFA are highly susceptible to peroxidation, a study with laying Leghorn hens was carried out to investigate the effects of different supplements of fish oil to the hens' diet on n-3 PUFA, vitamin E, and lipid peroxidation products of freshly laid eggs. The balance between n-3 PUFA, vitamin E, and lipid peroxidation products was also analyzed during the

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Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HHE, 4-hydroxyhexenal; HNE, 4-hydroxynonenal; HPLC, high-performance liquid chromatography; LA, linoleic acid; α-LNA, α-linolenic acid; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; TE, α-tocopherol equivalent; TLC, thin-layer chromatography.

egg storage for 4 wk. The latter part of the investigations was carried out with eggs produced from hens fed a constant content of fish oil (1.5%) but different contents of vitamin E. Therefore, we addressed the questions whether it is necessary to stabilize a high n-3 PUFA content in eggs to prevent a significant accumulation of cytotoxic aldehydic lipid peroxidation products and whether vitamin E supplementation is sufficient for the stabilization.

## MATERIAL AND METHODS

*Animals.* Experiments were performed in conformance to accepted standards. For part one of the study, on the n-3 PUFA enrichment of eggs produced by hens consuming diets with different levels of fish oil, 120 white Leghorn laying hens with an age of 38 wk were used. For the second part of the study, the influence of different vitamin E contents at one defined fish oil concentration on changes in eggs during 4 wk of storage was investigated with 120 additional Leghorn hens, age 35 wk.

*Diets.* During a 2-wk pre-test period an additional supplementation of 1% arachis oil as a source for LA was given (no linolenic acid). During the actual test period, combined additions of fish oil (12.5% EPA, 9% DHA) and arachis oil (25% LA) (3.5:1) were used to give dietary levels of 0, 0.7 ± 0.2, 1.4 ± 0.4, 2.8 ± 0.8, or 5.6 ± 1.6%, respectively. The test period was 4 wk.

For evaluating the antioxidant effects of added vitamin E on the n-3 PUFA enrichment of eggs with different fish oil contents [a blend of fish-oil (12.5% EPA, 9% DHA) and arachis oil (25% LA) (3.5:1) was used to give dietary levels of 0, 0.7, 1.4, 2.8, or 5.6%, respectively], a supplementation with 5 IU vitamin E/kg was performed. As vitamin E, all-*rac*-α-tocopherylacetate was used. The resulting average vitamin E content was 11 IU/kg diet. After the indicated times eggs were collected and pooled (3 eggs per pool) for analytical procedures. The mean egg weight was 60.2 g. The average amount of an egg yolk is about 16.8 g per fresh egg.

The total length of time for delivery of eggs to the laboratory, sampling, and homogenization of yolk until it had been frozen was less than 24 h. The influence of different vitamin E contents (at one defined fish oil concentration) on the changes in eggs during 4 wk of storage (room temperature, ~25°C; 70% humidity) was investigated by using a complete diet based on wheat, rye, tapioca, soybean meal, 1.5% fish oil, and vitamin E contents of 0, 5, 10, 20, 40, 80, and 160 IU/kg.

*Analytical procedures. (i) Fatty acids*. The extraction of egg yolk for fatty acid determination was carried out according to Brandt *et al.* (13). The fatty acid methyl esters were analyzed by means of gas chromatography according to the scheme of the Association of Official Analytical Chemists (14).

*(ii) Cholesterol*. The cholesterol content of egg yolk was determined spectrophotometrically (15).

*(iii) Vitamin E.* The vitamin E content of egg yolk was measured according to Bassler and Buchholz (16) by means of high-performance liquid chromatography (HPLC) separation and detection.

*(iv) Vitamin E requirement*. The tocopherol equivalent (TE) requirement—a value which is important for the protection of PUFA from peroxidation—was estimated on the basis of mathematical models according to Gassmann and Kübler (17).

*(v) Malondialdehyde (MDA)*. The concentration of MDA was determined according to Wong *et al.* (18). To 25 mg egg yolk, 0.75 mL phosphoric acid (0.44 mol/L) was added. The sample was thoroughly mixed in the presence of 10 mM butylated hydroxytoluene (BHT). The mixed sample (250 µL) was boiled in the presence of 0.25 mL thiobarbituric acid (42 mmol/L), 0.5 mL phosphoric acid, and 0.3 mL water for 60 min. Then the reaction was stopped by cooling the samples in an ice bath. Immediately before analysis on HPLC an equal volume of 1 M NaOH was added. The extract was then centrifuged. The HPLC equipment consisted of a Waters 510 pump and a Shimadzu fluorescence detector RF-530 (525/550 nm) and integrator C-R6A. The column was a Supelcosil 150 × 4 mm LC-18-S (5 µm) (Supelco). As eluent, a 50-mM potassium phosphate buffer solution at pH 6.8 with 40% methanol was used.

*(vi) 4-Hydroxynonenal (HNE) and 4-hydroxyhexenal (HHE)*. The aldehydes were analyzed according to Sommerburg *et al.* (19): 1 g of egg yolk was reacted with 1 mL of dinitrophenylhydrazine (DNPH) solution (1.8 mM in 1 M HCl) in presence of BHT in final concentration of 10 mM for 2 h in the dark. The sample was then kept for 1 h in an ice bath in the dark, extracted three times with 4 mL of dichloromethane, centrifuged at 900 × *g,* and evaporated on a rotary evaporator to dryness at a temperature of 35°C. The residue was transferred with 1.0 mL of dichloromethane into small vials for spotting on thin-layer chromatography (TLC) plates. The TLC plates were developed along with known standard compounds with dichloromethane. In this way the dinitrophenylhydrazones of carbonyl compounds were separated into three zones. Zone I contained the 4-hydroxyalkenals. This zone was scraped off, extracted three times with 5 mL each of methanol and evaporated to dryness. The residue was dissolved in 1 mL of methanol. An HPLC Gold system from Beckman (Unterschleissheim, Germany) was used. The eluent for the isocratic separation was methanol/water (4:1, vol/vol). The flow rate was 1 mL/min. A Supelcosil column LC-18 150 × 4 mm i.d. (5 µm) was used. Peak identification was performed by comparison of the retention times of peaks of samples and of standards, by the coelution of yolk extracts with the reference compound, and by comparison of the spectra. Quantification of HHE and HNE was achieved by separating HHE-DNPH and HNE-DNPH standard solutions of different concentrations.

*Statistical evaluation.* Data were compared and evaluated by means of variance and regression analysis. The analysis of variance and Duncan tests were carried out for six-yolk groups in the experiments with different fish oil contents and with different vitamin E concentrations at defined fish oil content. Mean differences with $P < 0.05$ were considered significant.

## RESULTS

Feeding fish oil to laying hens increased the n-3 PUFA content of egg yolk which is mainly owing to increases in EPA

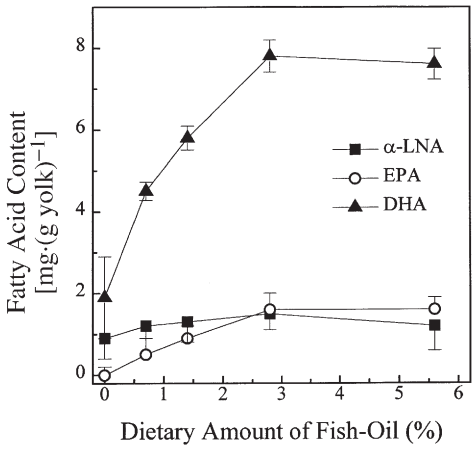


**FIG. 1.** Amount of n-3 polyunsaturated fatty acid (PUFA) as a function in egg yolk of dietary fish oil supplementation to hens. α-LNA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Error bars represent standard deviation.

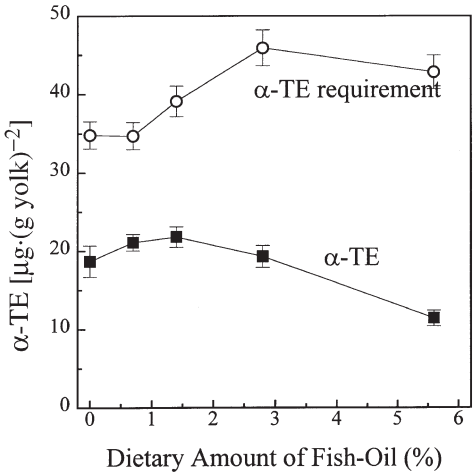


**FIG. 2.** Content of α-tocopherol equivalents (TE) in egg yolk in comparison to the TE requirement, which was calculated according to References 20 and 21. Error bars represent standard deviation.

and DHA. n-3 PUFA content (EPA + DHA) increased owing to fish oil supplementation in the up to 2.8% fish oil in the diet, $P < 0.001$ (Fig. 1). At 5.6% fish oil in the diet there was no further increase in n-3 PUFA content in egg yolk. Even though the EPA content was higher than the DHA content in the original fish oil, the DHA content of egg yolk was five to nine times higher than the EPA content. Because the content of several n-3 fatty acids was influenced by the diet, we decided to check for other lipid components. Table 1 shows that at fish oil contents up to 2.8% the cholesterol concentration of egg yolk and of total egg tissue remained unchanged. Only higher concentrations of fish oil were able to increase the cholesterol by 30%.

Figure 2 shows the content of α-TE in egg yolk compared to the TE requirement, which was calculated according to References 17, 20, and 21. At the highest fish oil concentration in the diet (5.6%), vitamin E content was markedly reduced. About 28% of vitamin E was transferred into the egg yolk at 0.7 to 2.8% fish oil addition. It was reduced to 18% at 5.6% fish oil addition. The vitamin E content was insufficient to protect PUFA from peroxidation as indicated by an imbalance between n-3 PUFA and the calculated physiological TE requirement. The gap between the TE determined and the TE required to balance PUFA became wider with increasing fish oil supplementation (Fig. 2). This imbalance between pro-oxidant and antioxidative defense should result in enhanced oxidation of lipids in the egg. Therefore, we measured MDA, one of the most abduant lipid peroxidation products. At concentrations beyond 1.4% fish oil in the diet, there was an increase in MDA concentration in the egg (Fig. 3).

Owing to the inability of the egg to protect this increased amount of the highly peroxidizable fatty acids, we investigated whether vitamin E supplementation can provide better protection. (Of course, the storage time from egg production to the usage by the consumer can influence the prooxidative-antioxidative balance dramatically.) We tested whether vitamin E supplementation of hens' diet would increase the vitamin E content in the egg and how stable this vitamin E was during storage. There was a 90% increase in vitamin E in yolks from fresh eggs at dietary levels up to 80 IU/kg vitamin E (Fig. 4). A level of 160 IU vitamin E/kg drastically

**TABLE 1**
**Cholesterol Concentration of Egg Yolk and of Total Egg After Feeding the Hens with Diets Containing Different Fish Oil Concentrations ($n$ = 3, 3 eggs per pool)**

| | Fish oil content of diet (%) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.7 | 1.4 | 2.8 | 5.6 |
| Cholesterol (mg/g yolk) | 13.2 ± 0.9[a] | 12.8 ± 0.3 | 12.9 ± 0.4 | 12.3 ± 0.7 | 17.0 ± 1.1 |
| Cholesterol (mg/egg) | 220 ± 15 | 214 ± 6 | 218 ± 7 | 223 ± 13 | 259 ± 17 |

[a]Mean ± standard deviation.

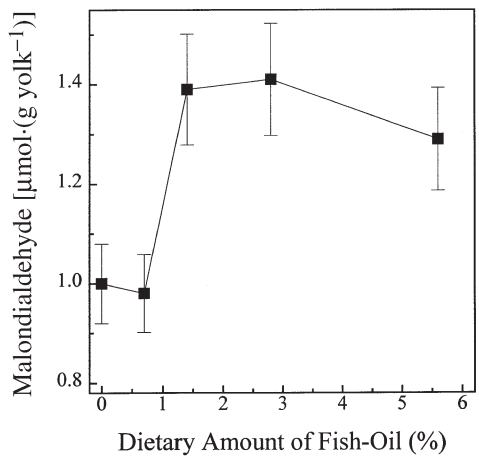


**FIG. 3.** Malondialdehyde (MDA) content in yolk of freshly laid eggs after treatment of the Leghorn hens with diets containing different fish-oil concentrations. Error bars represent standard deviation.

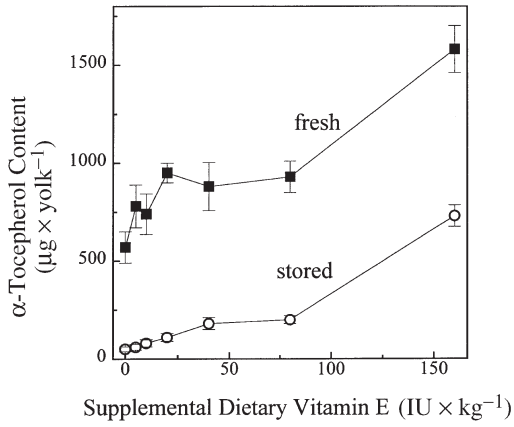


**FIG. 4.** Vitamin E concentration in yolk of fresh and stored eggs as a function of hens' diet supplementation with vitamin E. Error bars represent standard deviation.

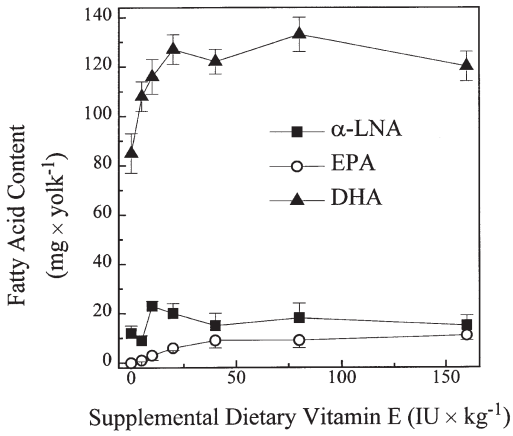


**FIG. 5.** n-3 PUFA in fresh egg yolk after feeding laying hens with 1.5% fish oil-supplemented diets containing different vitamin E concentrations. For abbreviations see Figure 1. Error bars represent standard deviation.

increased the vitamin E concentration in both fresh and stored eggs. Although at high amounts of vitamin E in the diet a remarkable amount of the vitamin was left after the storage at room temperature, there was also a notable loss of vitamin E in egg yolk (Fig. 4). Since we were able to enrich the eggs with vitamin E with dietary supplementation, we measured the influence of vitamin E on the n-3 PUFA concentrations in egg yolk (Fig. 5). Dietary vitamin E supplementation enhanced PUFA transfer to the egg to a certain degree. The increased PUFA content was mainly due to the accumulation of DHA. Increasing vitamin E supplementation up to 40 IU/kg led to PUFA/DHA increases (Fig. 5). Vitamin E concentrations higher than 40 IU/kg did not lead to further increases of PUFA/DHA contents.

During egg storage, high amounts of aldehydic lipid peroxidation products accumulated. Figure 6 demonstrates the concentrations of MDA, HNE, and HHE in egg yolk of fresh eggs and of eggs which were stored for 4 wk. The largest differences between fresh and stored eggs were for MDA and HNE. In stored eggs, MDA, HNE, and HHE were reduced in response to vitamin E supplementation (Fig. 6). Interestingly, the difference in HHE concentrations between stored and fresh eggs from hens without any vitamin E in the diet was notable, whereas this difference decreased with increasing vitamin E concentrations. Therefore, Figure 6 illustrates that vitamin E concentrations above 80 IU/kg diet are sufficient to protect eggs enriched with n-3 PUFA.

## DISCUSSION

During evolution the human being adapted biologically to foods with a relatively low content of total fat and saturated fatty acids. The n-6/n-3 ratio of unsaturated fatty acids was about one (2). As a result of social, agricultural, and industrial development, the n-6/n-3 ratio in a modern Western diet

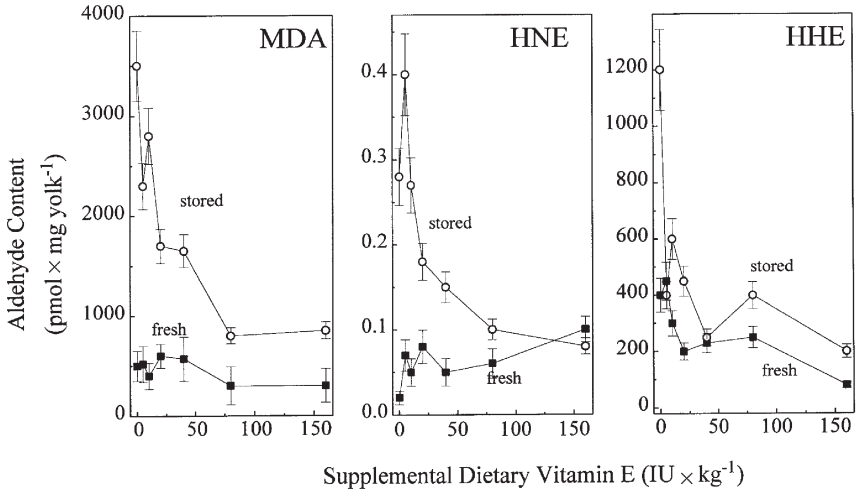


**FIG. 6.** Concentrations of MDA, 4-hydroxynonenal (HNE), and 4-hydroxyhexenal (HHE) in egg yolk of fresh eggs and of eggs stored at room temperature for 4 wk. Eggs were laid by hens fed diets with 1.5% fish oil and different vitamin E contents. Error bars represent standard deviation.

is now 20–25:1 (2). The main sources of EPA and DHA are fish and poultry (22).

It was shown earlier that the n-3 PUFA content of egg yolk may be an important source of n-3 PUFA for human nutrition (11,23). In our study the supplementation of hens' diet with fish oil resulted in increased n-3 PUFA content in egg yolk, mainly from accumulation of DHA. The present study considered DHA content of egg yolk as a function of different fish oil concentrations at one defined vitamin E concentration of the diet. The vitamin E content of the yolk was insufficient to protect PUFA from peroxidation, as calculated from mathematical models and as indicated by an increase of MDA. Therefore, it is reasonable to increase the vitamin E content in the hens' diet to ensure an adequate vitamin E provision for the animals.

That the addition of vitamin E to hens' diet is accompanied by an increased accumulation of vitamin E in the egg yolk was demonstrated Chen *et al.* (24) and Cherian *et al.* (25). The balance between vitamin E, PUFA, and lipid peroxidation in n-3 PUFA-enriched egg yolk was also analyzed. A notable increase in vitamin E content in the egg yolk was found, especially at high levels of dietary vitamin E supplementation. Storage of eggs resulted in a remarkable loss of vitamin E in the yolks. During the period of storage of n-3 PUFA-enriched eggs produced after feeding the laying hens a diet with a defined fish-oil concentration (1.5%) but different concentrations of vitamin E (0, 5, 10, 20, 40, 80, 160 IU/kg), the loss of vitamin E in all vitamin E supplementation groups was comparable. The high vitamin E content in eggs produced by hens supplemented with 160 IU/kg allowed eggs to be stored for 4 wk without a complete loss of the important lipid soluble antioxidant vitamin E. After storage, the amount of vitamin E in the yolk was still higher than the vitamin E content of fresh eggs from hens without any vitamin E supplementation. On the other hand, the supplementation of hens' diet with vitamin E enhanced the accumulation of n-3 PUFA in the egg. Therefore, the supplementation of hens' diet with vitamin E stabilized the antioxidant defense systems of the egg and enriched of n-3 PUFA in the egg.

Since the vitamin E functions in the egg to increase the antioxidant capacity of the yolk compartment, we tested for the protective role against spontaneous lipid peroxidation in the egg. For our measurements we chose three of the major cytotoxic lipid peroxidation products: MDA, HNE, and HHE. These three aldehydes are formed during lipid peroxidation of various fatty acids: MDA is formed from fatty acids with three or more double bonds, HNE is formed from n-6 PUFA with two or more double bonds, and HHE is formed from n-3 unsaturated fatty acids (26). None of the aldehydes detected in fresh eggs was found to change its content significantly due to the supplementation of hens' diet with vitamin E. Cherian *et al.* (25) reported a significant decrease of egg MDA content due to vitamin E supplementation of the hens' diet. However, nothing was said about the storage of eggs in that study. We found a notable accumulation in stored eggs of cytotoxic aldehydic lipid peroxidation products such as MDA, HNE, and HHE. That MDA is formed by peroxidation processes in large amounts during storage of eggs is not remarkable, since this aldehyde may be formed from a number of different PUFA (26). The increases in HHE and HNE during storage of eggs from hens fed with a non-supplemented diet are also obvious, since high amounts of n-3 fatty acids are present in the egg yolk and these are susceptible to oxidation. In the chain propagation of the lipid peroxidation process the n-3 and n-6 fatty acids—the precursors of HNE—are subject to enhanced oxidation (26). MDA, HNE, and HHE formation during storage at room temperature was reduced by vitamin E supplementation. At vitamin E doses of ≥ 80 IU/kg diet, no difference in the content of aldehydic lipid peroxidation products was found after 4 wk of storage. Therefore, these high doses of vitamin E are sufficient to protect high contents of n-3 and n-6 PUFA in eggs.

To produce n-3 PUFA-enriched eggs—so-called DHA eggs—without increased levels of cytotoxic aldehydic lipid peroxidation products, a 1.5% fish oil-containing diet needs a very high vitamin E addition. To produce DHA eggs without an accumulation of lipid peroxidation products, the hens' diet must be supplemented with at least 40 IU vitamin E/kg diet.

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# Diferuloylputrescine and *p*-Coumaroyl-feruloylputrescine, Abundant Polyamine Conjugates in Lipid Extracts of Maize Kernels

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**ABSTRACT:** Extraction of corn bran or corn fiber with polar solvents such as methylene chloride, ethanol or chloroform/methanol yielded common lipids and two unknown high-performance liquid chromatography (HPLC) peaks, each with an ultraviolet absorbance maximum at 320 nm. HPLC–mass spectrometry revealed that the unknowns were diferuloylputrescine (DFP) and *p*-coumaroyl-feruloylputrescine (CFP). When compared to extracts of corn fiber (a pericarp-enriched fraction from the wet milling of corn), comparable extracts of corn bran (a pericarp- enriched fraction from the dry milling of corn) yielded three- to eightfold higher levels of DFP and CFP. Extraction of corn bran or fiber with an accelerated solvent extractor revealed that elevated temperatures greatly enhanced the extraction of DFP and CFP by methylene chloride and ethanol. "Corn bran oil," prepared by extraction of corn bran with hot methylene chloride, contained 14 wt% DFP and 3 wt% CFP. However, when hexane was used as a solvent, accelerated solvent extraction of the corn bran or fiber did not extract any DFP or CFP. Extraction of wheat bran or psyllium hulls with hot methylene chloride did not yield any detectable DFP or CFP. Because it has been suggested that polyamine conjugates such as DFP and CFP may function as natural pesticides, a rapid method was developed to purify them so that their biological activity could be evaluated.

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Previously, we reported that high levels of three phytosterol classes (free phytosterols, phytosterol fatty acyl esters, and phytosterol ferulate esters) could be obtained by extraction of corn fiber with hexane, ethanol, or supercritical $CO_2$ (1). The present study was undertaken to investigate other lipids and lipid-like components that are present in lipid extracts of ground corn, corn fiber, and other corn processing streams. In addition to the polar and nonpolar lipids that have previously been reported (2), we now report that when corn kernels are extracted *via* routine organic extractions and analyzed *via* chromatographic methods, high levels of polyamine conjugates such as diferuloylputrescine (DFP) and *p*-coumaroyl-feruloylputrescine (CFP) will also be extracted and will appear as peaks interspersed among common lipids. In the only previous quantitative reports, the levels of DFP in kernels of 62 corn hybrids ranged from 8 to 560 μg/g ground kernel (3); and in a second study of isolated corn pericarp, embryo, and endosperm, the levels of DFP were reported to be 65, 15, and 5 μg/g, respectively (4). DFP was also reported to occur in extracts of gibberella ear rot-resistant corn (5). CFP has not previously been reported in any plant materials.

## EXPERIMENTAL PROCEDURES

*Materials.* Corn fiber [consisting of a combination of coarse fiber (mainly from the pericarp) and fine fiber (mainly from the endosperm)] was obtained from a conventional corn wet-milling plant and kindly provided by T. Carlson, Cargill, Inc. (Dayton, OH). Corn bran (NC02080, Coarse, DIETFIBER, from a corn dry-milling plant) was kindly provided by W. Duensing, Lauhoff/Bunge Foods (Danville, IL). Yellow dent #2 corn kernels, H3361, were kindly provided by Pioneer Hi-Bred International (Johnston, IA). Wheat bran and psyllium (*Plantago ovata*) husk powder (Yerba Prima Botanicals, Ashland, OR) were purchased locally. Corn fiber, corn kernels, and wheat bran were milled to 20 mesh with a Wiley mill (Thomas Scientific, Philadelphia, PA), whereas corn bran and psyllium had already been milled to a comparable particle size.

*Extraction.* Most extractions were performed with a Dionex Accelerated Solvent Extractor, Model ASE 200 (Dionex Inc., Sunnyvale, CA), using hexane, methylene chloride, or ethanol. The sample (2–4 g) was placed in an 11-cc stainless steel extraction vessel, and the remaining volume was filled with sand. The extractor was programmed to extract at a pressure of 1000 psi (69 bar), and a temperature of either 40 or 100°C; each sample was extracted with a total of 22 mL of solvent, delivered in three 10-min extractions. For purification studies, a sample was extracted with methylene chloride, first at 40 and then at 100°C. Alternatively, samples were extracted using the Bligh and Dyer chloroform/methanol extraction method (6), either with or without sample homogenization (Polytron; Brinkman, Westbury, NY), 60 s,

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Abbreviations: ASE, accelerated solvent extraction; CFP, *p*-coumaroyl-feruloylputrescine; DFP, diferuloylputrescine; EI, electron impact; ELSD, evaporative light-scattering detector; HPLC, high-performance liquid chromatography; LC/MS-EI, HPLC-mass spectrometry-electron impact ionization; MS, mass spectrometry; PDA, photodiode array detector; SPE, solid-phase extraction.

high speed. Purified pericarp and aleurone layers were prepared by steeping corn kernels (0.2% $SO_2$ and 0.55% lactic acid, at 50°C for 24 h), removing the hull with a scalpel, and separating it into the pericarp and aleurone layers *via* hand dissection with forceps, followed by Bligh and Dyer extraction and high-performance liquid chromatography with evaporative light-scattering detector (HPLC-ELSD) analysis of each. Two separate extractions were performed for each sample, and two HPLC injections were made from each extract.

*HPLC analyses.* HPLC analyses were performed on a Hewlett-Packard Model 1100 HPLC (Agilent Technologies, Palo Alto, CA) with an autosampler, with detection *via* two detectors in series; the effluent first entered a Hewlett-Packard Model 1100 Diode Array ultraviolet (UV)-visible detector, and then a Sedex Model 55 Evaporative Light Scattering Detector (Richard Scientific, Novato, CA) operated at 40°C, with nitrogen as a nebulizing gas, at a pressure of 2.0 bar. The HPLC column was a LiChrosorb Diol 5 micron (3 × 100 mm, packed by Varian/Chrompack, Walnut Creek, CA), and the isocratic mobile phase was hexane/isopropanol/acetic acid, 66.6:33.3:0.1, by vol, at a flow rate of 0.5 mL/min. A sample of purified DFP/CFP mixture (determined to be at least 99% pure by HPLC-ELSD, and estimated to be 85% DFP and 15% CFP by HPLC with UV absorbance measurement 320 nm) was used to construct a calibration curve which exhibited a linear relationship between area of UV 320 nm and mass in the range of 1–30 μg DFP and 1–10 μg CFP.

*HPLC/mass spectrometry-electron impact ionization (LC/MS-EI).* The LC/MS-EI instrument consisted of a Waters HPLC 2690 Separation Module (Waters Co., Milford, MA) connected in series to a Waters 996 Photodiode Array Detector (PDA) and a Waters Thermabeam Mass Detector (Integrity System). The HPLC portion used a Valco LiChrosorb Diol 5 micron column (2 × 250 mm) (Varian/Chrompack) with an isocratic solvent system of hexane (0.1% acetic acid)/isopropanol, 60:40 vol/vol, at a flow rate of 0.3 mL/min. The PDA was set to scan from 200 to 400 nm, and the MS-EI detector was set to scan in the mass range of *m/z* 60–600 at 1 scan/s with an ionization energy of 70 eV, a source temperature of 200°C, and a nebulizer temperature of 44°C.

*High-resolution electrospray MS.* Electrospray-MS was performed using a VG BioQ (Quattro II Upgrade) triple quadrupole mass spectrometer (source) scanning the range of 360–475 *m/z*; dwell 1.0 ms; pause 5.0 ms, calibrated using polyethylene glycol.

*Purification of a DFP- and CFP-enriched fraction* via *solid phase extraction (SPE).* Corn bran (25 g) was extracted by weighing five 5-g samples into accelerated solvent extraction (ASE) vessels as described above. Each vessel was extracted first with 22 mL of methylene chloride at 40°C, and then with an additional 22 mL of methylene chloride at 100°C. The 40°C methylene chloride extract was evaporated, weighed, analyzed for DFP and CFP, and then discarded. The 100°C extract, which was enriched in DFP and CFP, was then applied to an SPE cartridge (Mega Bond Elute, Silica, 10 g/60 mL, Varian), as follows: the cartridge was rinsed with 50 mL methanol and 50 mL chloroform; the sample (112 mL of extract, methylene chloride at 100°C, from 25 g corn fiber) was then applied to the SPE cartridge, and it was then sequentially eluted with 50 mL each of 99:1, 98:2, 95:5, 90:10, and 85:15 chloroform/methanol, vol/vol; and the solvent was evaporated in each fraction and the mass and levels of DFP and CFP were quantitatively analyzed *via* HPLC-UV at 320 nm.

## RESULTS AND DISCUSSION

Extraction of corn bran with hexane yielded an oil that contained only triacylglycerols, phytosterols, and other minor components reported previously (1). Extraction of corn bran with more polar solvents, such as methylene chloride or ethanol, yielded two unknown peaks (at retention times of about 6.5 and 8.5 min), and both peaks exhibited an absorption maximum of about 320 nm (Fig. 1). The UV absorption spectra of both unknowns were very similar to the UV spectrum of ferulic acid (data not shown), suggesting that the unknowns may contain esterified ferulic acid or a similar compound. With LC/MS-EI and the same column and chromatographic conditions as above (Fig.1), the two peaks each possessed unique mass spectra (Fig. 2). The molecular weights of the 6.5- and 8.5-min unknown peaks appeared to be *m/z* 410 and 440, respectively.

High-resolution electrospray-MS analysis of the larger peak fraction (8.5 min) provided an accurate mass for the protonated molecular ion $(M + H)^+$ signal of 441.1996. The mass

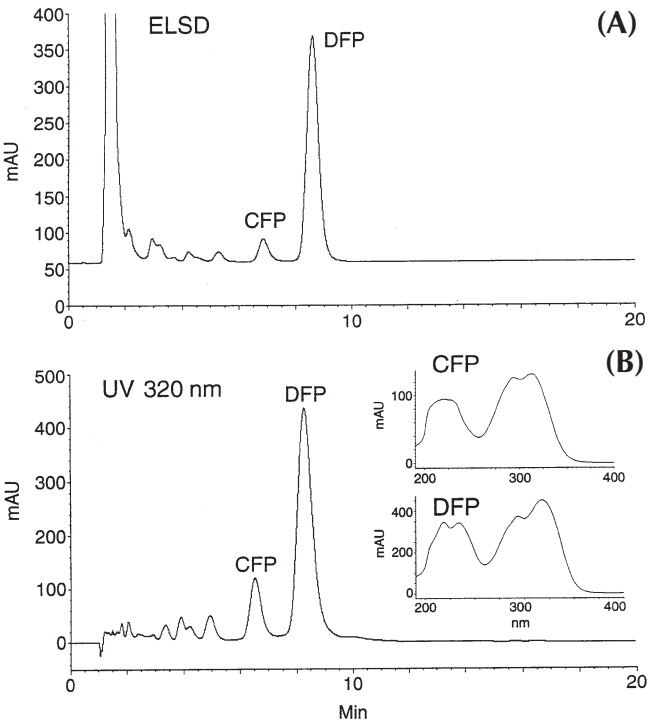


**FIG. 1.** Chromatograms of the 100°C methylene chloride accelerated solvent extraction lipid extract from corn bran (A) with detection *via* evaporative light-scattering detection (ELSD), and (B) with detection in the ultraviolet (UV) at 320 nm. CFP, *p*-coumaroyl-feruloyputrescine; DFP, diferuloylputrescine.

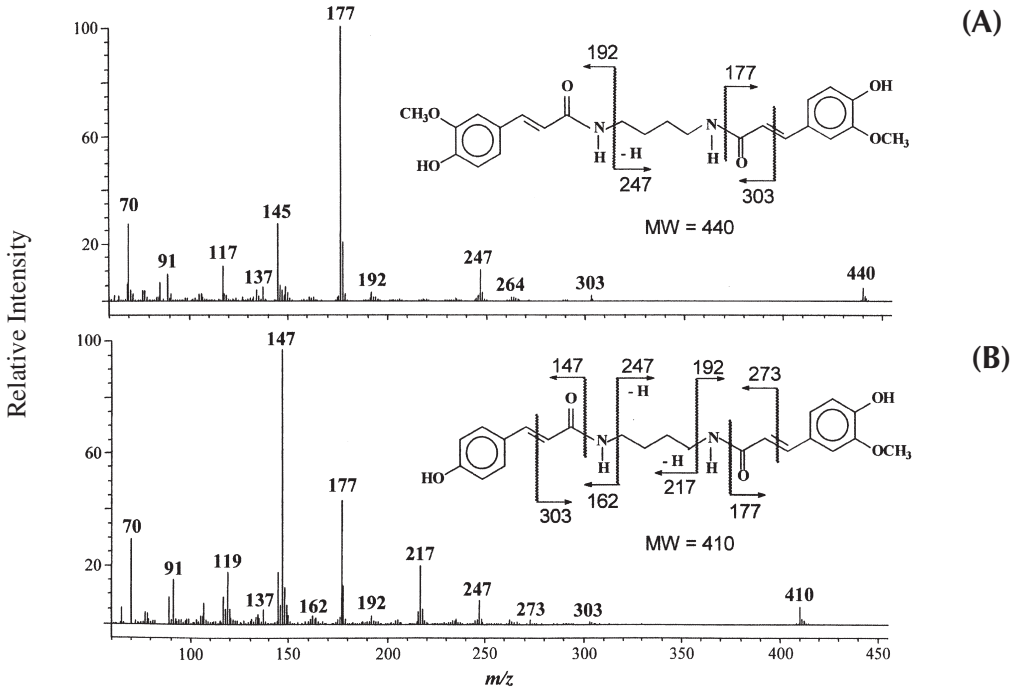


**FIG. 2.** Mass spectra (electron impact) and proposed structures with fragmentation patterns of the two unknown UV (320 nm)-absorbing compounds found in corn bran extracts. (A) DFP and (B) CFP. For abbreviations see Figure 1.

of the 8.5-min unknown compound matches the structure $C_{24}H_{29}N_2O_6$, calculated to be 440.2025620, which corresponds to the structure and MS-EI of diferuloylputrescine (see structure and fragmentation pattern in Fig. 2) as reported in the in the NIST database (*trans N,N′*-diferuloylputrescine, CAS #042369-86-8, molecular formula $C_{24}H_{28}N_2O_6$, National Institute of Standards and Technology, Gaithersberg, MD). Miller *et al.* (5) previously reported similar major MS-EI fragments from DFP at *m/z* 70, 117, 145, 177 (base peak), 191, and 247. The other unknown peak (the 6.5-min peak), with a molecular mass 30 mass units lower, was then proposed to be *p*-coumaroyl-feruloylputrescine, and the MS-EI fragmentation pattern was consistent with this identification (Fig. 2), although no previous reports of the occurrence of a mass spectrum of this compound are known.

DFP was previously reported to occur in tobacco (7), in corn kernels (3–5), and in the male reproductive organs of corn (8,9). This current report is the first evidence of CFP in plants. Sen *et al.* (4) reported that DFP was the most abundant polyamine conjugate in corn embryo, endosperm, and pericarp, but they also reported significant levels of monoconjugates (feruloylputrescine and *p*-coumaroylputrescine) and di-*p*-coumaroylputrescine. In the current chromatograms (Fig. 1) DFP and CFP were the major UV-absorbing peaks (320 nm), and if these other types of polyamine conjugates were present, they occurred in much lower levels than DFP and CFP (perhaps the small peaks at 320 nm and 3–5 min in Figure 1 are polyamine monoconjugates).

Upon identification of DFP and CFP in extracts of corn bran, we next investigated the effect of various extraction conditions on the levels of these compounds in corn bran, corn fiber, and whole ground corn (Table 1). A Dionex Accelerated Solvent Extractor was used to evaluate the effect of elevated temperatures on the levels of DFP and CFP extractable from corn bran. For these studies a constant solvent pressure of 1000 psi (69 bar) enabled the use of temperatures of up to 100°C and was used for all extractions. Extraction of corn bran with hexane, at either 40 or 100°C, yielded 1.27 and 1.44 g oil/100 g bran, respectively, but this oil contained no detectable levels of DFP or CFP. Extraction of corn bran with methylene chloride at 40°C yielded an oil that contained DFP and CFP (6.72 and 1.58 wt%, respectively), and much higher levels of both (approximately 2.5 times higher) were extracted when the temperature was increased to 100°C. Extraction of corn bran with ethanol yielded an oil with even higher levels of CFP and DFP, at both 40 and 100°C. Extraction of corn bran using the Bligh and Dyer (6) chloroform/methanol extraction (commonly used for extraction of polar membrane lipids) at ambient temperature (25°C) yielded DFP and CFP levels that were similar to those obtained with ASE ethanol extraction at 40°C. Polytron homogenization of corn bran during the Bligh and Dyer (6) extraction had no significant effect on the levels of DFP and CFP in the oil. The highest yields of extractable DFP were observed in two slightly different sets of conditions when yields were expressed in two different units: when expressed in units of wt% DFP in the oil, the highest concentration of DFP (13.6%) was recorded with the methylene chloride extraction at 100°C, and when expressed in units of mg DFP per 100 g dry wt of bran, the highest level of DFP (327.85 mg) was obtained with the ethanol extraction at 100°C.

Previously we reported (2) that corn fiber (a pericarp-enriched by-product obtained during the wet milling of corn)

**TABLE 1**
**Extraction and Quantitative Analysis of CFP and DFP in Corn Bran, Corn Fiber, Wheat Bran, and Psyllium Hulls[a]**

| Sample | Extraction | Extractable oil, w% of sample | wt% of oil | | mg/100 g dry wt of sample | |
|---|---|---|---|---|---|---|
| | | | CFP | DFP | CFP | DFP |
| Bran | ASE Hex40°C | 1.27 ± 0.03 | 0 | 0 | 0 | 0 |
| Bran | ASE Hex100°C | 1.44 ± 0.01 | 0 | 0 | 0 | 0 |
| Bran | ASE MC40°C | 1.57 ± 0.01 | 1.58 ± 0.04 | 6.72 ± 0.21 | 24.78 ± 0.39 | 105.30 ± 2.01 |
| Bran | ASE MC100°C | 1.97 ± 0.01 | 3.06 ± 0.03 | 13.64 ± 0.11 | 60.19 ± 0.54 | 268.07 ± 1.97 |
| Bran | ASE Et40°C | 1.88 ± 0.04 | 3.02 ± 0.01 | 9.61 ± 0.08 | 56.72 ± 1.05 | 180.42 ± 3.89 |
| Bran | ASE Et100°C | 2.88 ± 0.12 | 3.60 ± 0.09 | 11.41 ± 0.37 | 103.31 ± 1.73 | 327.85 ± 3.5 |
| Bran | CM | 1.72 ± 0.01 | 2.80 ± 0.04 | 10.52 ± 0.10 | 48.15 ± 1.17 | 180.86 ± 3.80 |
| Bran | CMP | 1.83 ± 0.06 | 2.63 ± 0.05 | 9.87 ± 0.18 | 47.98 ± 2.69 | 180.42 ± 9.59 |
| Fiber | ASE MC40°C | 0.89 ± 0.15 | 0.073 ± 0.003 | 0.321 ± 0.068 | 0.65 ± 0.13 | 2.79 ± 0.14 |
| Fiber | ASE MC100°C | 1.33 ± 0.00 | 0.562 ± 0.069 | 2.54 ± 0.49 | 7.47 ± 0.79 | 33.80 ± 6.56 |
| Pericarp | CMP | 0.59 ± 0.12 | 2.93 ± 0.81 | 11.95 ± 2.48 | 17.29 ± 3.52 | 70.5 ± 19.35 |
| Aleurone | CMP | 7.03 ± 0.75 | 0.01 ± 0.00 | 0.04 ± 0.01 | 0.70 ± 0.07 | 2.81 ± 0.26 |
| GC | ASE MC100°C | 3.55 ± 0.03 | 0.48 ± 0.02 | 1.69 ± 0.08 | 17.01 ± 1.10 | 60.8 ± 2.3 |
| WB | ASE MC100°C | 4.41± 0.02 | 0 | 0 | 0 | 0 |
| Psyl | ASE MC100°C | 1.16 ± 0.02 | 0 | 0 | 0 | 0 |

[a]Abbreviations: GC, ground corn; CM, Bligh and Dyer (6) chloroform/methanol extraction without homogenization; CMP, Bligh and Dyer chloroform/methanol extraction with Polytron homogenization; ASE, accelerated solvent extraction; Hex, hexane; MC, methylene chloride; Et, ethanol; WB, wheat bran; Psyl, psyllium hulls; CFP, *p*-coumaroyl-feruloylputrescine; DFP, diferuloylputrescine.

contained higher levels of phytosterols than corn bran (a pericarp-enriched by-product obtained during the dry milling of corn). Extraction of corn fiber with the ASE/methylene chloride, or Bligh and Dyer/chloroform/methanol resulted in oils with similar yields (expressed in g oil/100 g fiber), but the levels of DFP and CFP were much lower (three- to eightfold lower) in these corn fiber oils than in comparably extracted corn bran oils (Table 1). We recently reported ultrastructural evidence (10) that corn bran is primarily composed of only pericarp layers whereas corn fiber is a mixture of pericarp, aleurone layers, and fine fiber (cellular fiber from the endosperm). This report of higher levels of CFP and DFP in corn bran vs. corn fiber suggests that both compounds are primarily localized in the pericarp layers and not in the aleurone layer (cells). To further investigate the possible pericarp localization of the polyamine conjugates, corn kernels were steeped (0.2% $SO_2$ and 0.55% lactic acid at 50°C for 24 h), and the hulls were carefully removed with a scalpel and forceps and hand-dissected to yield separate pericarp and aleurone layers. Extraction and analysis of the lipids in both fractions confirmed that the pericarp layers contains much higher levels of DFP and CFP than the aleurone layers (Table 1).

Extraction of whole ground corn with hot methylene chloride yielded an oil with levels of DFP and CFP that were about twice the levels found in extracts of corn fiber. Additional studies are needed to determine whether all of the DFP and CFP is localized in the outer layers of the corn kernel or if some may occur in the internal portions (i.e., germ and endosperm). It is also possible that some of the differences in the levels of DFP and CFP in Table 2 may be due to hybrid variability, since commercial samples of bran and fiber were obtained from unknown and/or blended hybrids.

Because of the high levels of DFP and CFP in corn bran and corn fiber, we next extracted two other commercial pericarp-enriched grain products from related species—wheat bran and psyllium hulls. Surprisingly, we found that neither of these pericarp-enriched fractions contained any detectable levels of DFP or CFP (Table 1).

**TABLE 2**
**Purification of a CFP and DFP-Enriched Fraction from 25 g of Corn Bran *via* Temperature-Sequenced Accelerated Solvent Extraction (ASE) and Solid-Phase Extraction (SPE)**

| | Extractable oil (mg) | CFP (mg) | DFP (mg) |
|---|---|---|---|
| ASE, $CH_2Cl_2$, 40°C | 366.3 ± 2.0 | 4.8 ± 0.1 | 26.7 ± 0.3 |
| ASE, $CH_2Cl_2$, 100°C[a] | 100.8 ± 0.8 | 8.2 ± 0.9 (100)[b] | 40.7 ± 1.4 (100) |
| SPE 1, eluent of ASE, 100°C extract | 15.5 ± 3.4 | 0 | 0 |
| SPE 2, 99 vol $CHCl_3$/1 vol $CH_3OH$ | 4.5 ± 0.3 | 0 | 0 |
| SPE 3, 98 vol $CHCl_3$/2 vol $CH_3OH$ | 6.5 ± 0.9 | 0 | 0 |
| SPE 4, 95 vol $CHCl_3$/5 vol $CH_3OH$ | 14.2 ± 1.6 | 0.8 ± 0.1 (10) | 0 |
| SPE 5, 90 vol $CHCl_3$/10 vol $CH_3OH$ | 34.5 ± 1.3 | 5.7 ± 0.2 (70) | 28.7 ± 1.4 (71) |
| SPE 6, 85 vol $CHCl_3$/15 vol $CH_3OH$ | 9.5 ± 0.6 | 0.9 ± 0.1 (11) | 3.8 ± 0.2 (9) |

[a]Sample applied to SPE cartridge.
[b]Numbers in parentheses are the percent recoveries of CFP or DFP in the SPE fractions, relative to the amount that was applied to the SPE cartridge. For abbreviations see Table 1.

In the only previous quantitative reports, the highest levels of DFP were 560 μg DFP/g in ground kernels (3) and 65 μg/g of corn pericarp (4). Much higher levels of DFP were observed in the current study, with the ASE 100°C ethanol extraction yielding 3279 μg DFP/g of corn bran (equal to the value of 327.85 mg/100 g bran reported in Table 1). These findings suggest that DFP and CFP are difficult to extract quantitatively and that previous reports of these compounds need to be reexamined. It is also possible that additional studies may reveal solvent extraction methods that yield oils with even higher levels of DFP and CFP than are reported herein.

Because corn bran was found to be an abundant source of DFP and CFP, a process was developed to purify these compounds in levels sufficient for evaluation of their biological activity (Table 2). After examining the data in Table 1, it appeared that if corn bran was first extracted with methylene chloride at 40°C (extracting most of the phytosterols and other nonpolar lipids), followed by the same solvent at 100°C, then the latter extract is enriched in CFP and DFP and would be a good starting material for chromatographic purification. Accordingly, when the 100°C methylene chloride extract was applied to a SPE column, the 90:10 chloroform/methanol fraction was a highly enriched mixture of DFP and CFP (at a ratio of about 85% DFP and 15% CFP). HPLC analyses of the preparation with detection by both UV 320 nm and ELSD indicated that it was highly enriched in DFP and CFP. Several very small unidentified peaks were observed in chromatograms of the preparation, but their total area was <1%, even when the polarity of the mobile phase was increased to 44.9:50:5:0.1, hexane/isopropanol/water/acetic acid, by vol (which was sufficiently polar to separate very polar lipids such as phospholipids, if any had been present). We are in the process of developing a preparative HPLC method to further purify DFP and CFP, but we feel that this SPE process to yield a purified mixture of these two structurally related compounds is a rapid method that can be used to purify large quantities of material for evaluation of biological activity of this simple mixture.

The physiological role of DFP and CFP in corn is not known, but this report of their high levels in corn pericarp suggests that their potential physiological role(s) should be investigated. It has been suggested that polyamine monoconjugates may function as fungicidal (5), insecticidal (11), and viricidal (12) compounds. Polyamine monoconjugates have also been reported to occur in wheat (13) and in the barley root-arbuscular mycorrhizal fungi symbiotic system (14). Others have reported that DFP is a radical scavenger and may function as a protectant against ozone damage (15). Treatment of barley leaves with methyljasmonate was reported to cause an elevation of the levels of polyamine monoconjugates (16). Free putrescine and other polyamines have been reported in corn kernels (17), and their levels remained constant during seed storage and showed no correlation with loss of seed viability. Although free polyamines are likely to be metabolic precursors to polyamine conjugates, we are not aware of studies of possible interrelationships between free polyamines and polyamine conjugates in corn kernels. Clearly, more work is needed to elucidate the function and relationship between the polyamine diconjugates that we have herein quantitatively analyzed in corn and the polyamine monoconjugates that others have reported in corn and other plants.

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# The Genus *Thapsia* as a Source of Petroselinic Acid

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**ABSTRACT:** We describe the results from the isolation and structural identification of the acylglycerol constituents of fruits from wild plants belonging to different species of *Thapsia* (Apiaceae). The isolated lipid fractions were analyzed and characterized by chemical, chromatographic, and spectroscopic means. In particular, $^{13}C$ nuclear magnetic resonance data allowed the identification of petroselinic acid as the major fatty acid esterified to glycerol in the fruit oils from all the plant samples. This was also confirmed by gas chromatography (GC) and GC–mass spectrometry analyses of fatty acid methyl and butyl esters derivatives from *Thapsia* oil. The genus *Thapsia* should be regarded as a useful source for the extraction of petroselinic acid, which represents an important oleochemical raw material.

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The Apiaceae (Umbelliferae) represent one of the best-known plant families, widely distributed in temperate climate regions where they are often used as spices or drugs owing to the presence of useful secondary metabolites such as coumarins, essential oils and sesquiterpenes (1–3).

In addition, more recently the plant family has attracted attention for the high level of petroselinic acid (*cis*-6-octadecenoic acid) produced by some of the genera belonging to it. This unsaturated fatty acid, isomer of oleic acid, represents in fact an interesting oleochemical for the food, cosmetics, and pharmaceutical industries (4–7). In particular, petroselinic acid can be oxidatively split at the double bond into lauric (12:0) and adipic (6:0) acids, both with important applications in the manufacture of soaps and plastics (4–7).

Attempts to engineer industrial oilseed crops, e.g., by introducing genes from coriander, to increase the yield of petroselinic acid have not yet been very successful (4–7). Therefore, we believe that the identification of new plants which can provide high amounts of petroselinic acid is still appropriate.

In recent years, within the Apiaceae family, the genus *Thapsia* has been the subject of several scientific investigations (8–10), especially for the medical properties of *T. garganica*. Phytochemical studies of plants belonging to different species of *Thapsia* have shown that they are an important source of bioactive compounds, and may be useful in providing new insights into the present taxonomy of the genus (11–17). In the present paper we report the results from the isolation and structural identification of the acylglycerol components of fruits from wild plants of different *Thapsia* species.

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Abbreviations: COSY, correlation spectroscopy; 2D, two-dimensional; FTIR, Fourier transform infrared spectroscopy; FAME, fatty acid methyl esters; GC, gas chromatograpy; GC–MS, GC–mass spectrometry; ME, methyl ester; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

## EXPERIMENTAL PROCEDURES

*Plant material.* Fruits of *T. garganica* were collected, during 1999, from wild-growing plants at Sammichele (Bari, Italy). Fruits of *T. villosa* (two samples, 88-16, 2n = 44, and 88-9, 2n = 66), *T. laciniata*, *T. minor*, *T. maxima* I and *T. maxima* II were obtained from wild-growing plants as described before (12,16,17). Fruit vouchers are deposited at Dipartimento Farmaco-Chimico, Universitá di Bari (Italy).

*Oil extraction and purification*. Finely pulverized fruits (10 g) of *Thapsia* species were extracted in a Soxhlet apparatus with refluxing petroleum ether (bp 35–60°C) for 3 h. Evaporation of the solvent under vacuum gave an oily product, which was further investigated.

Each oil sample was first characterized by precoated silica gel 60 F254 TLC aluminum sheets ($10 \times 20$ and $5 \times 7.5$ cm; Merck, Darmstadt, Germany) developed in $CHCl_3$. Lipid visualization was obtained with phosphomolybdic acid reagent (10% in EtOH; Sigma, Milan, Italy) and by charring the plates at 110°C.

Oils extracted from *T. garganica* consisted of a nearly pure fraction of acylglycerols; fruit extracts from the other species of *Thapsia* resulted instead in a mixture of acylglycerols plus other compounds (identification not reported here) mainly originating from the terpene pool of metabolites. All extracts were, however, purified by column chromatography (silica gel 60H, Merck) using $CHCl_3$ as the eluent to recover pure triacylglycerols.

*Saponification and methylation*. Triacylglycerols (15 mg) from *Thapsia* species were treated with 5% NaOH in MeOH (3 mL) for 20 min at room temperature. Methanolic 6 N HCl was then added to stop the reaction. After evaporation of the solvent, the residue was further reacted with $CH_2N_2$ according to Reference 18. The methylated fatty acid fractions were then analyzed by gas chromatography (GC) and GC–mass spectrometry (MS). For comparison, commercial tripetroselinin (Sigma) was also subjected to the above reactions. Free fatty acids (stearic, palmitic, oleic, linoleic, petroselinic, and linolenic; Sigma) were also methylated with $CH_2N_2$ and used as reference compounds for GC and GC–MS analyses.

*Preparation of fatty acid butyl esters.* Triacylglycerols (5 mg) from *Thapsia* species and commercial tripetroselinin were reacted with 1 mL 0.1 M Na *tert*-butylate in *n*-butanol

(Fluka, Milan, Italy). The reaction was performed for 1 h at room temperature, and then 5% $NaHSO_4$ in $H_2O$ was added as described (19). The organic layer was used for GC and GC–MS analyses. Standard butyl esters of stearic, palmitic, petroselinic, oleic, and linoleic acids were also prepared and submitted to GC and GC–MS analysis.

*GC analysis.* A Carlo Erba HRGC 5160 gas chromatograph equipped with a flame-ionization detector and an on-column injector was used. Hydrogen was the carrier gas; air and $H_2$ were adjusted to yield optimal separation. Data were processed by a Spectra Physics SP 4290 computing integrator. All the samples were dissolved in $CHCl_3$.

Triacylglycerols from *Thapsia* oil samples, commercial tripetroselinin, triolein, trilinolein, tristearin, and tripalmitin were analyzed with an Easy 1 (Analitica, Milan, Italy) bonded phase fused-silica capillary column (25 m × 0.32 mm i.d.; 0.1–0.15 μm film thickness) either in isothermal conditions at 280°C or under programmed conditions, from 260°C, 7°C/min, up to 360°C. The detector port was kept at 380°C.

Determination of fatty acid methyl esters (FAME) and fatty acid butyl esters was carried out on a DB-5 (Superchrom, Milan, Italy) fused-silica capillary column, 30 m × 0.32 mm i.d.; 0.25 μm film thickness. The chromatographic conditions were as follows: detector temperature 300°C; column temperature was programmed from 70°C, 20°C/min to 170°C and then up to 280°C (5 min isothermal) at 5°C/min. Alternatively, FAME and fatty acid butyl esters from *Thapsia* acylglycerols were analyzed on a DB-23 (Superchrom) fused-silica capillary column (30 m × 0.32 mm i.d.; 0.25 μm film thickness) at the following conditions: 70°C, 15°C/min to 110°C and then up to 230°C (20 min isothermal) at 3°C/min. Detector port was maintained at 300°C.

*GC–MS analysis*. GC–MS analyses of FAME and fatty acid butyl ester derivatives were performed with a Hewlett-Packard 6890-5973 mass spectrometer interfaced with an HP 59970 Chemstation. The chromatographic conditions were as follows: column oven program from 70 (4 min isothermal) to 280°C (20 min isothermal) at 20°C/min; injector, 250°C. Helium was the carrier gas (flow rate, 1 mL/min). An HP-5-MS capillary column (30 m × 0.25 mm; 0.25 μm film thickness) was utilized. MS operating parameters were: ion source, 70 eV; ion source temperature, 230°C; electron current, 34.6 μA; vacuum $10^{-5}$ torr. Mass spectra were acquired over 40–800 amu range at 1 scan/s. The ion source was operated in the electron impact mode. The samples (1 μL) were injected using the splitless sampling technique.

*Spectroscopic methods*. Fourier transform infrared (FTIR) spectra were recorded using NaCl cells on a PerkinElmer Spectrum One.

Proton ($^1$H NMR) and carbon nuclear magnetic resonances ($^{13}$C NMR) were recorded on a DRX500 Avance Bruker instrument equipped with probes for inverse detection and with *z* gradient for gradient-accelerated spectroscopy. Standard Bruker automation programs were used for two-dimensional (2D) NMR experiments. 2D correlation spectroscopy (COSY) experiments were performed using COSYDFTP (double-quantum-filtered phase-sensitive COSY) and COSYG (gradient-accelerated COSY) sequences. Inverse detected normal and long-range $^1$H-$^{13}$C heterocorrelated 2D NMR spectra were obtained by using the gradient-sensitivity enhanced pulse sequences INVIEAGSSI and INV4GPLRND, respectively. $CDCl_3$ was used as the solvent in all the NMR experiments. Residual $^1$H and $^{13}$C peaks of the solvent were used as internal standards to calculate chemical shifts referred to tetramethylsilane.

## RESULTS AND DISCUSSION

Acylglycerols purified by chromatographic techniques from the total lipid extracts of the various species of *Thapsia* were characterized by chromatographic, spectroscopic, and chemical means. Homogeneous results were obtained with all the analyzed plant species, therefore reported spectroscopic assignments from spectra related to a single acylglycerol sample from *Thapsia* apply to all of them as well.

Infrared (IR) spectra of *Thapsia* lipids showed typical absorptions at 3003 (C–H olefins), 2949, 2921, 2852 (C–H), 1744 (C=O), 1464 (C–H), 1179 (C–O) and 721 $cm^{-1}$.

More structural information came from their $^1$H and $^{13}$C spectral data (Fig. 1). 2D proton-proton and proton-carbon correlations also facilitated the assigning of relative signals (Fig. 2).

The $^1$H NMR signals at δ 4.11 (*dd*; glycerol α C$H_2$), 4.28 (*dd*; glycerol α C$H_2$), and 5.29 (*m*; glycerol β C*H*) indicated that the isolated lipids were triglycerides (Table 1). The observed chemical shifts in the $^{13}$C NMR spectra at δ 62.52 (glycerol α-carbon atoms) and 69.32 (glycerol β-carbon atom) and their relative size also confirmed that the isolated compounds were triglycerides (Table 1). This was further supported by the highest chemical shift signals at about δ

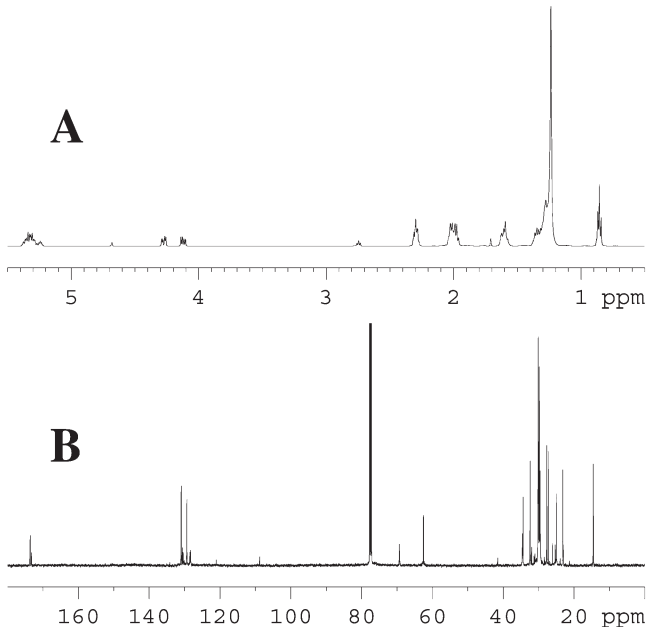


**FIG. 1.** $^1$H (A) and $^{13}$C (B) 500 MHz nuclear magnetic resonance (NMR) spectra of acylglycerol constituents from the fruits of *Thapsia* sp.

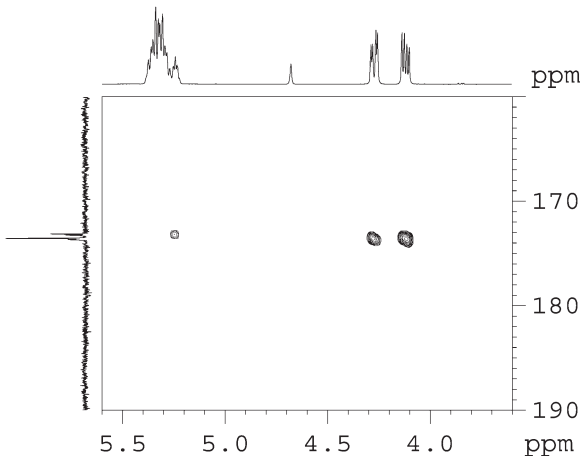


**FIG. 2.** Long-range two-dimensional heteronuclear NMR spectrum of acyglycerols from *Thapsia* sp. Selected region shows cross peaks between carbonyl resonances of C1 α and β chains and glycerol α and β hydrogens. For abbreviation see Figure 1.

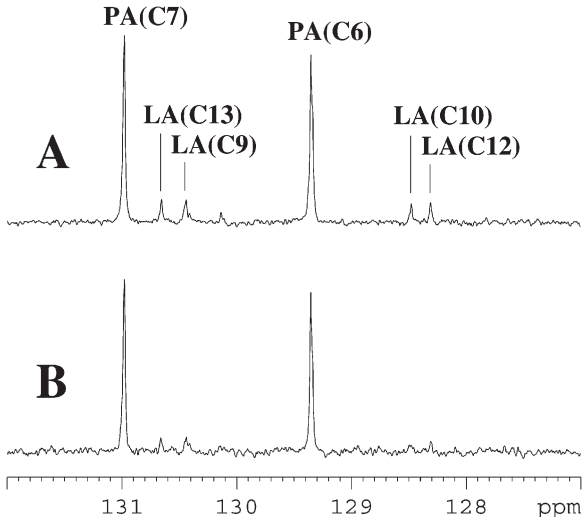


**FIG. 3.** Expansion of the olefin region of $^{13}$C NMR spectra of the acylglycerols from (A) *T. garganica* and (B) *T. villosa* (88-16). PA, petroselinic acid; LA, linoleic acid. For abbreviations see Figure 1.

173.11 (C1 β chains) and δ 173.52 (C1 α chains) in all the lipid samples from *Thapsia* species. The 2D NMR experiments were also useful to corroborate the 1,2,3 substitution pattern of the isolated esters. Cross peaks (Fig. 2) were in fact observed in the long-range $^{1}$H-$^{13}$C heteronuclear correlated spectra between the carbon resonance at δ 173.52 and 173.11 (C1 α and β chains) and proton resonances at δ 2.30 (C2) and δ 4.28, 4.11 and 5.29 (glycerol α and β hydrogens).

Moreover, the multiplet at δ 5.33 in the proton spectrum, together with the signals in the range between δ 128 and 131 in the $^{13}$C NMR spectra, suggested the presence of olefinic structural units. In addition, resonances of allylic and diallylic methylenes can be identified in the spectra (Table 1). The olefin region in the carbon NMR spectrum appears particularly important for the characterization of the oil composition (20–22). A closer inspection of this peak area, in fact, allowed us to distinguish in all the samples, on the basis of their chemical shifts, the following two unsaturated fatty acids esterified to the unit of glycerol: petroselinic acid, δ 130.95 (C7) and δ 129.33 (C6); linoleic acid (*cis*-9,12-octadecadienoic), δ 130.61 (C13), δ 130.37 (C9), δ 128.48 (C10), and δ 128.29 (C12) (Fig. 3).

Identity of the two fatty acids was further confirmed by comparison with NMR spectra of commercial tripetroselinin and trilinolein and with spectra obtained from constructed mixtures of saturated (tripalmitin) and unsaturated (tripetroselinin, trilinolein, and triolein) triacylglycerols. Literature data have also been evaluated for their identification (20–25).

Quantification of the relative proportions of petroselinic and linoleic acids in the NMR spectra according to Mallet *et al.* (22) indicated that petroselinic acid was by far the most abundant (≅90%) unsaturated fatty acid esterified to glycerol

**TABLE 1**
**Nuclear Magnetic Resonance Spectral Data of Fruit Triacylglycerols from *Thapsia* Species**

| $^{1}$H | δ (*J*) | $^{13}$C | δ |
|---|---|---|---|
| 2,2′ | 2.30 (*m*) | $CH_3$, ω1 | 14.51 |
| 3,4 | 1.61 (*m*) | $CH_2$, ω2[a] | 23.09 |
| Allylic C$H_2$ *(cis)* | 2.00(*m*) | $CH_2$, C–3 | 24.88 |
| Diallylic C$H_2$ *(cis)* | 2.75 (*m*) | –C=C–C$H_2$–C=C– | 26.04 (C11 LA) |
| Olefinic *(cis)* | 5.33 (*m*) | –C$H_2$–C=C–C$H_2$ *(cis)* | 27.21 (C5 PA) |
| | | | 27.66 (C8 PA) |
| Other (*n*-C$H_2$) | 1.34 (*m*) | *n*-$CH_2$ | 29.56–30.14 |
| C$H_3$, ω1 | 0.85 (*t, J* = 7 Hz) | $CH_2$, ω3[a] | 32.33 |
| Glycerol α C$H_2$ (1″ a, 3″ a) | 4.11 (*dd, J* = 18.6 Hz) | Glycerol $CH_2$ (α carbons) | 62.52 |
| Glycerol α C$H_2$ (1″ b, 3″ b) | 4.28 (*dd, J* = 17.5 Hz) | Glycerol CH (β carbons) | 69.32 |
| Glycerol β C*H* | 5.29 (*m*) | –C=CH | 128.29 (C12 LA) |
| | | | 128.48 (C10 LA) |
| | | | 129.33 (C6 PA) |
| | | | 130.37 (C9 LA) |
| | | | 130.61 (C13 LA) |
| | | | 130.95 (C7 PA) |
| | | C-1, C-1′ | 173.52, 173.11 |
| | | C-2, C-2′ | 33.90, 34.07 |

[a]Weak signals at 22.98 δ [ω2 linoleic acid (LA)] and at 31.93 δ (ω3 LA) were also observed. PA, petroselinic acid.

**TABLE 2**
**Relative Amount (%) of Fatty Acids Esterified to Glycerol in Fruit Oils from *Thapsia* Species**

| Species | Palmitic | Stearic | Petroselinic | Oleic | *cis*-Vaccenic | Linoleic |
|---|---|---|---|---|---|---|
| *T. garganica* | 4.9 | Trace | 82.8 | 4.4 | Trace | 7.9 |
| *T. villosa* (88-16) | 8.2 | 4.9 | 73.0 | 7.0 | 1.9 | 5.0 |
| *T. villosa* (88-9) | 4.7 | Trace | 72.3 | 6.1 | 0.6 | 16.3 |
| *T. laciniata* | 7.2 | 1.8 | 73.3 | 6.2 | 0.7 | 10.8 |
| *T. minor* | 8.2 | 1.7 | 69.7 | 4.0 | 0.7 | 15.7 |
| *T. maxima* I | 6.8 | 1.9 | 73.5 | 4.2 | 1.1 | 12.5 |
| *T. maxima* II | 5.0 | 0.8 | 74.8 | 4.6 | 0.4 | 14.4 |

in *Thapsia* fruit lipids. Petroselinic acid represents a positional isomer of oleic acid (18:1n-9*cis*). The two fatty acids often occur in combination as constituents of Apiaceae oils, and they are generally difficult to separate. According to our NMR results, *Thapsia* fruit oils should not contain oleic acid, or only in low amounts (Fig. 3). Oleic acid, as well as petroselinic and linoleic acids, shows well-defined signals related to the olefin region in the $^{13}C$ NMR spectra, namely, at around δ 129.7 (C9) and 130.0 (C10), the latter often overlapping with the C9 peak of linoleic acid (20–22). A weak signal at δ 130.1 attributable to oleic acid was sometimes detectable in our oil samples suggesting a trace quantity of oleic acid in *Thapsia* oils.

To achieve a good quantification of the fatty acids in their fruit oils, lipids from *Thapsia* plants were submitted to transmethylation and transbutylation reactions, and the methyl and butyl esters formed were subjected to GC analyses. Separation on a DB-23 column allowed the best determination and quantification of the fatty acid content in the acylglycerols from *Thapsia* oils (Table 2; Fig. 4). The column produced a good separation of the three isomers—oleic methyl ester (ME), petroselinic ME, and *cis*-vaccenic ME—as well as for the separation of their corresponding butyl esters (Fig. 4), which were used for the quantitative analysis. Neverthless, GC further supported NMR findings, that is, *Thapsia* triacylglycerols were mainly made up of petroselinic (69–82%) and

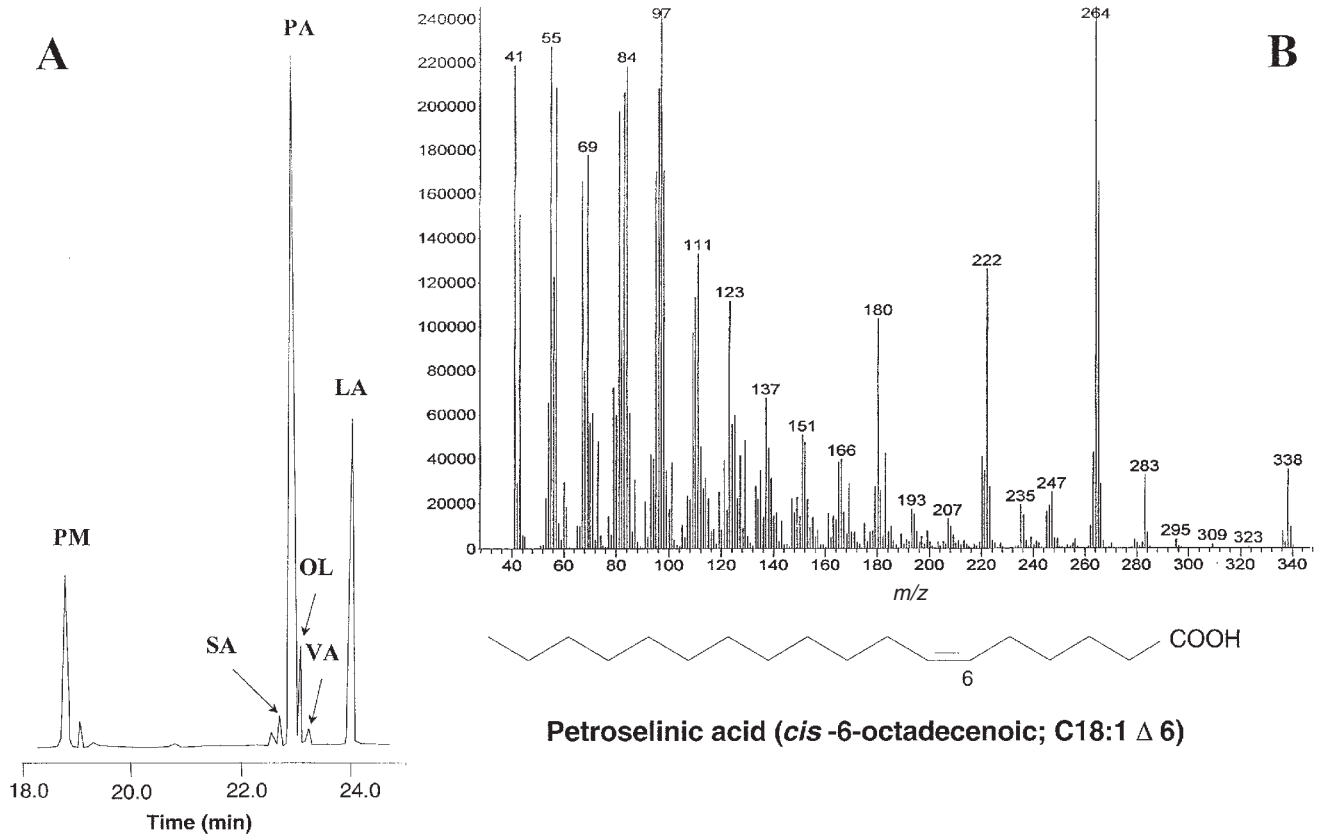


**FIG. 4.** Gas–liquid chromatographic analysis (A) of the butyl esters obtained from the acylglycerols of *T. minor,* and mass spectrum (B) of PA. PM, palmitic acid; SA, stearic acid; OL, oleic acid; VA, *cis*-vaccenic acid. For other abbreviations see Figure 3.

linoleic (trace–16%) acids. The amount of oleic acid generally ranged from 4 to 6%. GC analysis of the butyl ester derivatives from the oils also allowed the quantification of *cis*-vaccenic acid as minor component (from traces up to 1%). The two saturated palmitic (5–8%) and stearic (trace–8%) acids were also identified in the reaction mixtures (Table 2; Fig. 4). Their presence was not further investigated in the NMR spectra, however.

All the derivatized oil fractions were also analyzed by GC–MS; MS fragmentation of petroselinic acid butyl ester was as follows: *m/z* (relative intensity, r.i.), 338, [$M^+$, $C_{22}H_{42}O_2$ (14)], 264 (100) ($M^+ - 74$), 222 (51), 180 (42), 166 (16), 151 (21), 137 (25), 123 (45), 111 (54), 97 (99), 84 (89), 69 (72), 55 (92), 41 (89).

The following MS fragments were obtained for petroselinic (i) and linoleic (ii) ME derivatives, respectively, *m/z* (r.i.): (i), 296, [$M^+$, $C_{19}H_{36}O_2$ (100)], 264 (83) [$M^+ - 32$], 222 (49) [$M^+ - 74$], 180 (33), 123 (40), 110 (46), 96 (92), 84 (93), 74 (100), 55 (99), 41 (75); (2), 294, [$M^+$, $C_{19}H_{34}O_2$ (100)], 263 (15) [$M^+ - 31$], 220 (6) [$M^+ - 74$], 178 (8), 164 (10), 150 (14), 136 (15), 123 (18), 109 (35), 95 (63), 81 (77), 67 (100), 55 (54), 41 (44). Data were in agreement with mass spectra obtained with reference compounds.

Similarly, GC of the intact triglycerides allowed the separation of one main component (80–90%) coeluting with commercial tripetroselinin. Trilinolein resulted in a partially overlapping shoulder to the GC peak of tripetroselinin.

To our knowledge, this is the first extensive study on the distribution of tripetroselinate in the genus *Thapsia*. Petroselinic acid is one of the most common fatty acids in Apiaceae seed oils, thus its isolation from the genus *Thapsia* can be anticipated. Moreover, a previous report (26) showed that petroselinic acid, a component of the oil from *T. villosa*, amounted to 75%. Other studies showed that the different species of *Thapsia* can be distinguished by their phytochemical characters; in particular, the presence/absence of the typical sesquiterpene lactones and the constituents of the essential oils were of chemotaxonomic value in differentiating them (8,9). The most pronounced heterogeneity was found among plants identified as *T. villosa* species, which were divided into five types, corresponding to two distinctly different chemical, cytological and morphological groups (16,17). We cannot know what type of *T. villosa* has been investigated previously (26). However, according to the present investigation, in all the analyzed species belonging to the genus *Thapsia*, the acylglycerols have almost the same composition and tripetroselinate is equally abundant in all of them. Thus, in contrast to other chemical classes synthesized by *Thapsia* plants, this metabolite cannot further contribute to the chemotaxonomy of the genus. Nevertheless, the genus *Thapsia* should be regarded as an important source of petroselinic acid, an oleochemical raw material that is used in cosmetics, pharmaceuticals, or food (6,7,27,28).

## ACKNOWLEDGMENTS

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# Characterization of Lipoxygenase Oxidation Products by High-Performance Liquid Chromatography with Electron Impact-Mass Spectrometric Detection

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**ABSTRACT:** Lipoxygenase (LOX) is an enzyme that oxygenates polyunsaturated fatty acids to their corresponding hydroperoxy derivatives. For example, LOX found in plants produce the corresponding 13- and 9-hydroperoxide derivatives of linoleic acid (13-HPOD and 9-HPOD). Identification of the HPOD products is usually accomplished by using gas chromatography with mass spectrometric (MS) detection, which requires extensive derivatization of the thermally unstable hydroperoxy group. Here we report a high-performance liquid chromatographic method in combination with electron impact (EI)-MS detection that separates and characterizes the HPOD isomers generated by soybean LOX type I oxygenation of linoleic (LA) and linolenic acids as well as HPOD products produced by photosensitized oxidation of LA. The method does not required derivatization of the hydroxyperoxide group, and location of its position can be determined by the EI-MS fragmentation pattern. The method has been used for the analysis of HPOD produced by action of partially purified LOX from the micro-alga *Chlorella pyrenoidosa* on LA. The study suggests the presence of two LOX isozymes in the micro-alga that oxygenate LA to its 13-HPOD and 9-HPOD derivatives. Moreover, the 9-LOX isozyme under anaerobic conditions cleaves 13-HPOD to 13-oxo-tridecadienoic acid and pentane but does not cleave 9-HPOD.

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Lipoxygenase (LOX; EC 1.13.11.12) is a lipid dioxygenase enzyme that is widely distributed throughout the plant and animal kingdom. The enzyme catalyzes the regio- and steroselective peroxidation of polyenoic fatty acids, and often the characterization of a particular plant isozyme is made based on its specificity of reaction with linoleic (LA) and linolenic acid (LNA) (1,2). For example, when LOX oxygenates LA and LNA to produce 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13-HPOD) and 13-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13-HPOT), respectively, the enzyme is classified as 13-LOX, whereas when the products are 9-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HPOD) and 9-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid (9-HPOT), respectively, the enzyme is classified as 9-LOX (3). Depending on LOX source and reaction conditions, the amounts of the 9- and 13-HPOD isomers formed can vary. In addition, LOX activity in the fungi *Penicillium* sp. is reported to produce the 9-, 10-, 12-, and 13-hydroperoxy derivatives of LA. The HPOD products were racemic, which was attributed to either a low enantioselectivity of the LOX or the presence of isozymes showing complementary enantioselectivity, but no additional evidence for this unusual activity was reported (4).

Zimmerman and Vick (5) initially reported LOX-type activity in *Chlorella pyrenoidosa* in a partially purified $(NH_4)_2SO_4$ fraction, which produced both the 9- and 13-HPOD derivatives (ratio of 1:4) of LA. Later, these same authors reported that the molecular weight of this LOX enzyme was 100 kDa and that it had maximal activity at pH 7.5 (6). Bisakowski *et al.* (7) further investigated LOX present in *C. pyrenoidosa* and showed that the active fractions produced the 9-HPOD derivative together with the 10- and 13-HPOD isomers. In both studies, however, whether the reported activity resulted from the presence of one or more LOX isozymes was not established. In addition to its peroxidation activity, LOX can anaerobically cleave the oxidation products into short volatile fragments and an oxocarboxylic acid (8,9). For example, Nuñez *et al.* (10) recently reported that LOX present in *C. pyrenoidosa* was able to cleave 13-HPOD to an ω-oxo- fatty acid. It was not shown whether other HPOD isomers are substrates for this LOX oxidation reaction.

LOX activity is usually determined by measuring the ultraviolet (UV) absorption of the conjugated double bonds at 234 nm for the 9- and 13-HPOD isomers produced from LA or LNA. However, the method does not distinguish between them (11). The absence of conjugated double bonds in the 10- and 12-HPOD isomers [10-hydroperoxy 8(*E*),12(*Z*)-octadecadienoic acid and 12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid] limits their detection by UV. The position of the hydroperoxy group in HPOD isomers is determined by gas chromatography with mass spectrometric detection (GC/MS). Because of their thermal instability, the GC/MS technique requires reduction of the hydroperoxide group to an alcohol followed by silylation for better GC analysis (12). Finally, unambiguous assignment of

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Abbreviations: CI, chemical ionization; CID, collision-induced decomposition; EI, electron impact ionization; GC, gas chromatography; 13-HOD, 13-hydroxy-9(*Z*)-11(*E*)-octadecadienoic acid; HPLC, high-performance liquid chromatography; 9-HPOD, 9-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 10-HPOD, 10-hydroperoxy- 8(*E*),12(*Z*)-octadecadienoic acid; 12-HPOD, 12-hydroperoxy-9(*Z*),13(*E*)-octadecadienoic acid; 13-HPOD, 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 9-HPOT, 9-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid; 13-HPOT, 13-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; LA, linoleic acid; LC, liquid chromatography; LNA, linolenic acid; LOX, lipoxygenase; MS, mass spectrometric detection; PDA, photodiode array detector; TIC, total ion current; UV, ultraviolet.

the position of the hydroperoxide group requires saturation of the double bonds by hydrogenation (7,13).

Plattner and Gardner (14) attempted to analyze HPOD compounds directly without prior reduction by mass spectrometry by using a direct exposure probe with chemical ionization (CI) and collision-induced decomposition (CID) techniques. With ammonia gas as ionizing reagent, the CI-MS technique indicated the molecular mass of HPOD derivatives, whereas when isobutane was the ionizing reagent a series of fragmentation ions were produced, the formation of which was consistent with a mechanism proposed for the acid-catalyzed decomposition of HPOD derivatives. Determination of the position of the hydroperoxy group in the HPOD was made from an analysis of the daughter ions of higher-mass ions in the CID spectra (14). Burgess *et al.* (15) studied a series of alkyl hydroperoxides by using MS with electron impact (EI) ionization. The main features of the peroxide fragmentation pattern were inferred by analogy with the fragmentation pattern of the related alcohols. The hydroperoxides studied showed a characteristic elimination of peroxy radical $HO_2^{\cdot}$, since the formation of the alkyl cation radical occured at a lower ionization potential than that for $HO_2^{+}$. Additional characteristic losses from the molecular ion were $HO^{\cdot}$ and $H_2O$, which were explained through formation of a cyclic intermediate (15). However, EI-MS spectra for the 13- and 9-HPOD isomers have not been reported.

Recently we reported an analysis of LOX products using high-performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization-mass spectrometric detection (LC/APCI-MS). This MS technique, however, did not allow identification of the position of the hydroperoxide group in the products; rather, isomer assignments were made on the basis of elution order from a diol chromatographic column (10). In the present study, we developed an LC/EI-MS method that allows for the direct analysis of the methylated products of the HPOD and HPOT isomers and that provides evidence for the position of the hydroperoxide group without pretreatment of the sample. In using this method the HPOD products obtained using partially purified LOX fractions from *C. pyrenoidosa* were analyzed. In addition, anaerobic oxidative cleavage activity found in the same LOX fractions was assayed using pure 9- and 13-HPOD products as substrates.

## MATERIALS AND METHODS

*Materials.* LA and LNA acids were purchased from Sigma Chemical (St. Louis, MO). The 13-HPOD and -HPOT derivatives of LA and LNA acid were prepared as described previously (16). Reduction of the 13-HPOD isomer to its corresponding 13-alcohol was done by using $NaBH_4$ as reducing agent (7). All products were methylated before LC/EI-MS or GC/MS analysis using a hexane/diazomethane saturated solution. The hexane/diazomethane solution was obtained by decomposition of 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich, Milwaukee, WI) added to a bi-layer solvent system consisting of hexane (upper layer, 20 mL) and an aqueous solution of NaOH (20%) (lower layer, 3 mL). All other reagents were of the highest purity available. *Chlorella pyrenoidosa* was obtained from American Type Culture Collection (No. 11469) (Manassas, VA).

*Photosensitized oxidation of LA.* A rose bengal saturated methanol solution was added dropwise to a solution of LA (20 mg) in methanol (120 mL) until the reaction mixture had an absorption of 0.2–0.3 absorption units at 556 nm. The mixture was placed into a 10-cm diameter Pyrex reaction flask and oxygen was bubbled through the solution. The mixture was irradiated with a 500-watt halogen lamp for 10 h at 15°C. The methanol was removed by rotary evaporation and the residue redissolved in 20 mL of methylene chloride, which was washed several times with water until the rose bengal was removed. After removal of the solvent, the oxidation products were methylated and injected into the LC/MS for analysis. The yield of hydroperoxides varied between 30 and 40%.

*Protein extraction and purification. Chlorella pyrenoidosa* cells were grown and harvested as described previously (16). Typically, 3–4 g of wet cells were washed with distilled water, centrifuged at $1,000 \times g$ for 10 min, and resuspended in 30 mL potassium phosphate buffer (50 mM, pH 8.0). Homogenization of the cells was done with a bead-beater apparatus (Biospec Products, Bartlesville, OK) using 30–35 mL of 0.5 mm glass beads at 0°C for 30 s interspersed with 30 s cooling, for a total homogenization time of 2 min. The beads were separated by decantation. Cellular debris in the supernatant was removed by centrifugation at $12,000 \times g$ for 10 min. The supernatant was separated from the pellet and then centrifuged at $100,000 \times g$ for 65 min to remove the microsomal fraction. The supernatant was treated sequentially to 30, 45, and 80% $(NH_4)_2SO_4$ saturation, at 0°C for 30 min. After each $(NH_4)_2SO_4$ addition the precipitate was separated by centrifugation at $15,000 \times g$.

*LOX assay.* The precipitated fractions were resuspended in 5 mL of potassium phosphate buffer (100 mM, pH 8.0), the pH was adjusted at 8.0, and 4 mL were incubated with 50 mL solution of LA or LNA, 1 mM in potassium phosphate (100 mM, pH 8.0) and Tween 20 (0.1%) under oxygen. The oxidation reaction was followed spectrophotometrically at 234 nm. At the end of the reaction the oxidation products were extracted, methylated with diazomethane, and injected into the LC/EI-MS. The anaerobic cleavage activity of the LOX fractions was determined by analyzing the volatile products by GC/MS after incubation of the resuspended fractions (1 mL) with a solution in potassium phosphate (100 mM, pH 8.0) of 9-, 13-HPOD or 13-HPOT (1 mM, 5 mL) as previously described (10).

*LC/EI-MS.* LC was performed with a Waters HPLC 2690 Separation Module (Waters Co., Milford, MA) connected in series to a Waters 996 Photodiode Array Detector (PDA) and a Waters Thermabeam Mass Detector (Integrity System). The LC portion used a Valco LiChrosorb DIOL 5-µm column (2 × 250 mm) (Varian/Chrompack, Raritan, NJ). The linear gradient elution profile used was as follows: hexane (100%) hold for 5 min; to a final composition of hexane/isopropanol (99.5:0.5%, vol/vol) over 30 min, hold for 70 min at a flow rate of 0.3 mL/min. The PDA was set to scan from 200 to 400 nm, and the EI-MS detector was set to scan the mass range *m/z* 90–600 at 1 scan per second with an ionization energy of 70 eV. Ionization source temperature was 200°C, nebulizer

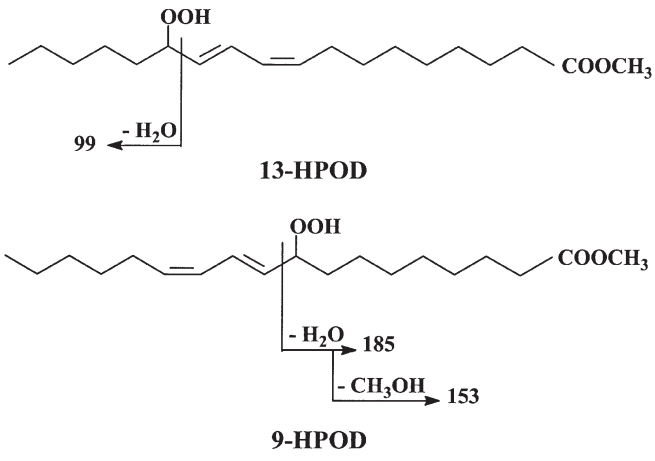
temperature was 42°C, and expansion region temperature was 75°C.

*GC/MS analysis of HPOD isomers.* Methylated HPOD products from soybean LOX and photo-oxidation were reduced with $NaBH_4$ (7) and silylated in pyridine with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce, Rockford, IL) and injected (1 μL) into a gas chromatograph (Hewlett-Packard, Wilmington, DE) model 5890 Series II Plus equipped with a capillary inlet and a Mass Selective Detector model 5972 (Hewlett-Packard) set to scan from *m/z* 45 to 600 at a scan rate of 1.2 scans per second. The capillary column used was an SP-2340 (60 m × 0.25 mm) (Supelco, Bellefonte, PA). The oven temperature was programmed from 130 to 230°C at 2°C/min. The temperature of the injector port was 230°C, and the detector transfer line temperature was 240°C. The carrier gas was He at a flow rate of 1 mL/min and a split ratio of 50:1.

## RESULTS AND DISCUSSION

Soybean LOX type I produces mainly 13-HPOD and minor amounts of 9-HPOD (Scheme 1) (1). The GC/MS spectra of the reduced and silylated LOX products were consistent with those reported by Wu and Robinson (12) with a molecular ion at *m/z* 382 and characteristic ions at *m/z* 311 (40%) for the 13-HPOD adduct and *m/z* 225 (60%) for the 9-HPOD adduct. However, the isomers can be differentiated only in the intensity of the characteristic ion, since both derivatives have ions at *m/z* 311 and 225 (12). When LOX type I products are analyzed on an HPLC diol column using the method described in the Materials and Methods section, the 13-HPOD isomer elutes at about 48 min and the 9-HPOD isomer at 55 min. Both peaks absorb in the UV at 234 nm owing to their conjugated double bonds. The EI spectra for the 9- and 13-HPOD isomers are shown in Figures 1A and B, respectively, together with the 13-alcohol [(13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid: 13-HOD] product obtained from 13-HPOD (Fig. 1C). The mass spectra of both HPOD isomers have an ion at *m/z* 310 [M – Oxygen], 308 [M – $H_2O$], 293 [M – $O_2H$], and 292 [M – $H_2O_2$], which are characteristic of hydroperoxide fragmentation (14,15). Although a molecular ion is not seen in the spectrum of the 13-HPOD isomer, the spectrum for the analogous 13-HOD isomer does show a molecular ion at *m/z* 310 and an ion at *m/z* 292 formed by loss of $H_2O$ from the parent molecule (Fig. 1C). Loss of a methoxy group from the 13-HOD molecular ion gives the ion at *m/z* 279 [M – $CH_3O$], while the ion at *m/z* 279 [M – 47] in the spectrum of the HPOD isomers suggests the elimination of oxygen and a methoxy group. The ions of the HPOD isomers at *m/z* 277 [M – $H_2O$ – $CH_3O$], and 261 [M – $H_2O_2$ – $CH_3O$] also establish a difference between 13-HOD and the 13-HPOD, but other portions of their spectra are similar.

Additional information on the location of the hydroperoxy group in the HPOD isomers can be obtained by examining the ions formed toward the saturated hydrocarbon portion of the molecule, which produces a series of acyl ions (14). Both 13-HOD and 13-HPOD have an ion at *m/z* 99 (100%), whereas 9-HPOD gives ions at *m/z* 185 (20.2%) and 153 (16.7%) (185 – MeOH) (Fig. 1A). Scheme 1 shows a fragmentation pattern of 13- and 9-HPOD that can account for the formation of these ions.




SCHEME 1

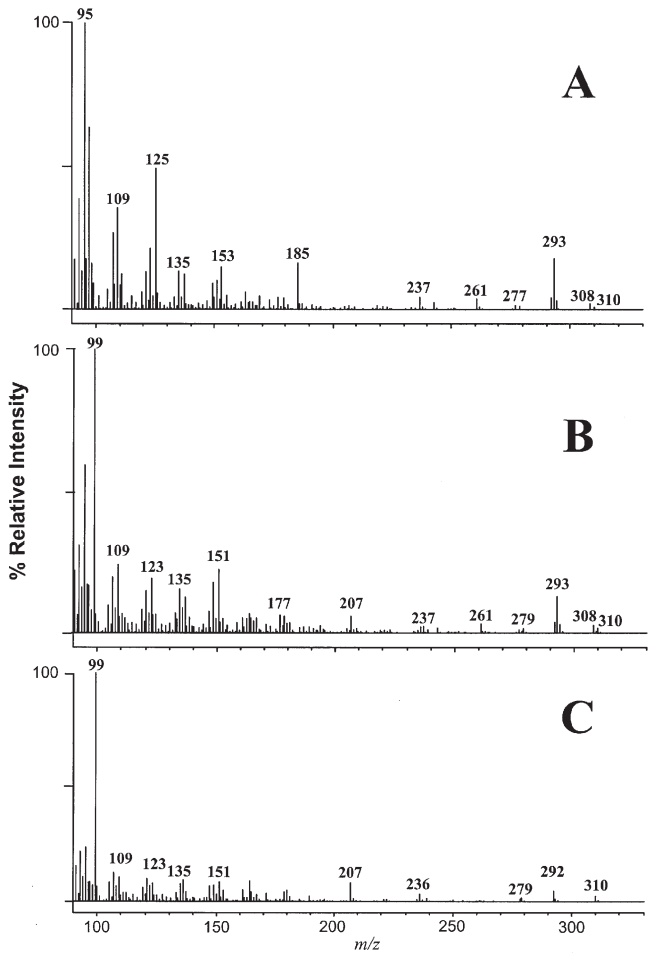



**FIG. 1.** High-performance liquid chromatography (HPLC)/electron impact-mass spectrometry (LC/EI-MS) spectra of the methyl esters of (A) 9-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HPOD); 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (B) (13-HPOD) produced by incubation of soybean lipoxygenase (LOX) type I with linoleic acid (LA) under oxygen; and (C) reduced and methylated 13-alcohol (13-HOD) of 13-HPOD.

When LNA is incubated with soybean LOX type I under an oxygen atmosphere, the 9- and 13-HPOT isomers are produced in a ratio similar to the HPOD isomers from LA. LC/EI-MS analysis of these products produced the spectra shown in Figure 2. Both EI spectra are characterized by ions at *m/z* 308 [M – Oxygen], 306 [M – $H_2O$], 291 [M – $O_2H$], 290 [M – $H_2O_2$], and 275 [M – $H_2O$ – $CH_3O$]. The presence of a third double bond in the HPOT isomers alters the fragmentation pattern. The location of the hydroperoxide group for the 13-HPOT isomer cannot be established by the presence of an acyl ion at *m/z* 97, which is found in 13-HPOD at *m/z* 99 (see Fig. 1B and Scheme 1). Instead, the 13-HPOT spectrum gives ions at *m/z* 255 (28.5%), 239 (9.1%), 238 (5.1%), and 237 (8.1%) suggesting the fragmentation pattern indicated in Scheme 2, each of which subsequently loses methanol to give the ions at *m/z* 223 (4.2%), 207 (19.3%), 206 (17.3%), and 205 (5.1%). The additional double bond in 9-HPOT also produced an EI spectrum different from that of 9-HPOD; however, the ions at *m/z* 185 (33.4%) and 153 (21.5%) [185 – MeOH] can be used to identify the location of the hydroperoxide group in 9-HPOT in an analogous fashion as for 9-HPOD (Scheme 1).

Rose bengal-photosensitized oxidation of LA inserts oxygen at the double bonds, leading to the formation of the 13-, 12-, 10-, and 9-HPOD isomers (17,18). The GC/MS spectra of reduced and silyated alcohols were consistent with the 9- and 13-HPOD adducts reported earlier (12), and the spectra of the 10- and 12-HPOD adducts after reduction showed a MW of 382, with characteristic ions at *m/z* 271 (100%) for the 10-HPOD adduct and *m/z* 185 (100%) for the 12-HPOD

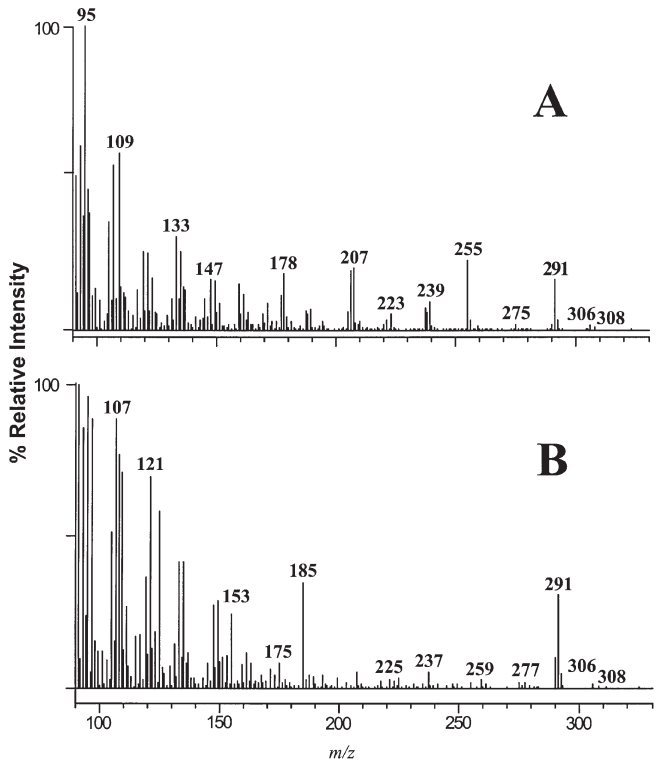
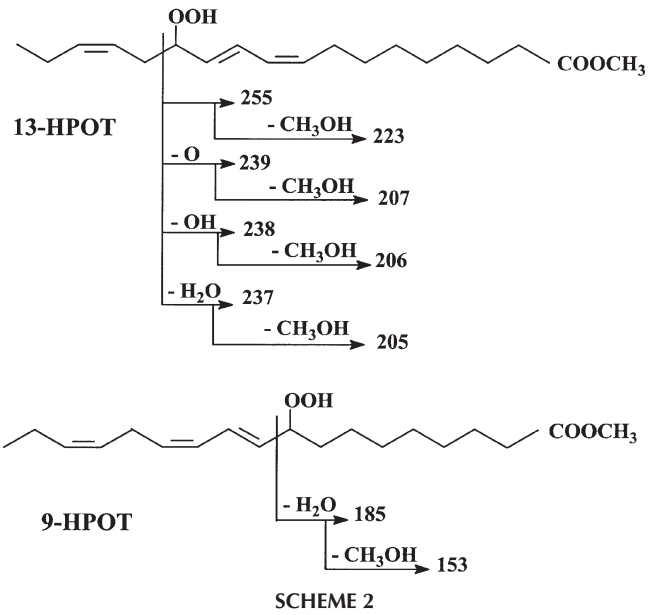



**FIG. 2.** LC/EI-MS spectra of the methyl esters of (A) 13-hydroperoxy-9(*Z*), 11(*E*),15(*Z*)-octadecatrienoic acid; and (B) 9-hydroperoxy-10(*E*),12(*Z*), 15(*Z*)-octadecatrienoic acid produced by incubation of soybean LOX type I with linolenic acid under oxygen. For abbreviations see Figure 1.




SCHEME 2

adduct as reported previously (18). The 9- and 13-HPOD isomers have *cis-trans* conjugated double bonds as in the LOX-produced products, but the reaction is not stereoselective and gives a racemic mixture of the *R* and *S* HPOD isomer products (18). Separation of the HPOD isomers on the DIOL column with UV and EI-MS detection produced the chromatograms shown in Figures 3A and 3B, respectively. The UV chromatogram shows three major peaks eluting at 48.1, 52.3, and 54.5 min, labeled 1a, 2a, and 3a, respectively, in Figure 3A. Peaks 1a and 3a have UV maxima at 234 nm, which is consistent with a conjugated *trans-cis* double bond, whereas peak 2a has a UV maximum at 228 nm, suggesting a conjugated *trans-trans* double bond (19). The total ion current (TIC) EI-MS chromatogram in Figure 3B shows significant differences from the UV chromatogram. Peaks 1b (48.3 min) and 4b (54.6 min) are not of equal size, which was also confirmed by using an evaporative light-scattering detector (result not shown), suggesting the elution of two products under peak 1b but with only one product absorbing at 234 nm. The mass spectrum of this peak showed a characteristic fragmentation pattern for 13-HPOD, i.e., a significant ion at *m/z* 99 and other characteristic ions at *m/z* 113 and 129. Extraction of the ion chromatograms at *m/z* 99 and 129 indicated two components with retention times of 48.2 and 48.5 min, respectively as shown in the insert of Figure 3B. The EI spectrum of the early portion of the 48.2 min peak is completely consistent with 13-HPOD. The EI spectrum of the later portion of the 48.5 min peak (Fig. 4A) shows ions at *m/z* 310 (>1%), 308 (1%), 293 (10.3%), 279 (2.3%), and 261 (4.6%) that are characteristic for an HPOD isomer. These ions and the lack of UV absorption at 234 nm indicate an HPOD isomer with nonconjugated double bonds. The UV and EI-MS data for this peak and its elution order from the DIOL column suggest this peak is the 12-HPOD isomer. The ions at *m/z* 129 (65.4%), 113 (30%), 112 (23.9%), and 111 (22.4%) (Fig. 4A) also are consistent with a 12-HPOD isomer, as indicated by

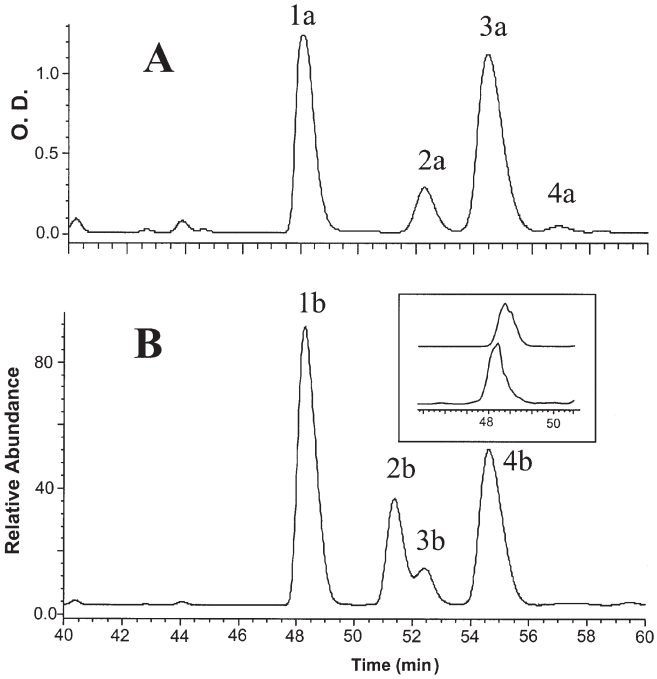



**FIG. 3.** HPLC DIOL column separation of the methylated HPOD products obtained by photosensitized oxidation of LA (see Materials and Methods section). (A) Ultraviolet chromatogram at 234 nm; (B) MS total ion chromatogram. Insert: extracted ion chromatograms for 129 amu (top); and 99 amu (bottom). See Results and Discussion section for peak labeling. O.D., optical density; for other abbreviations see Figure 1.

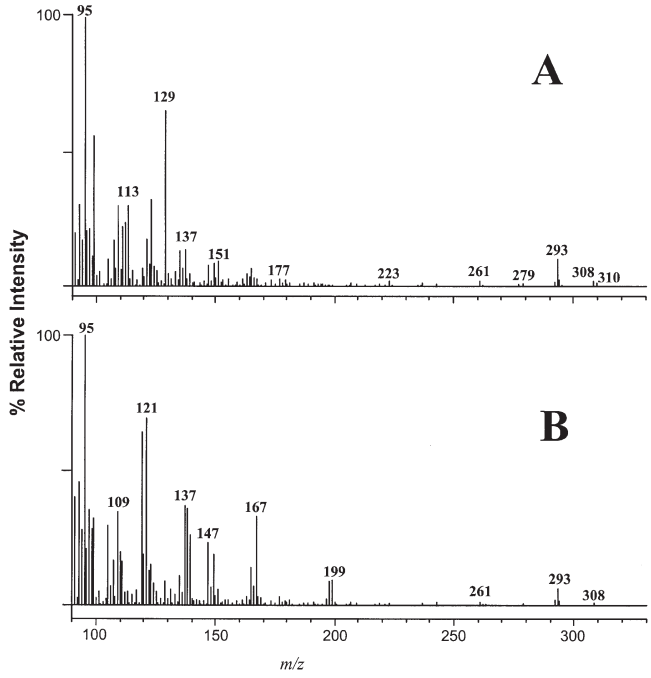


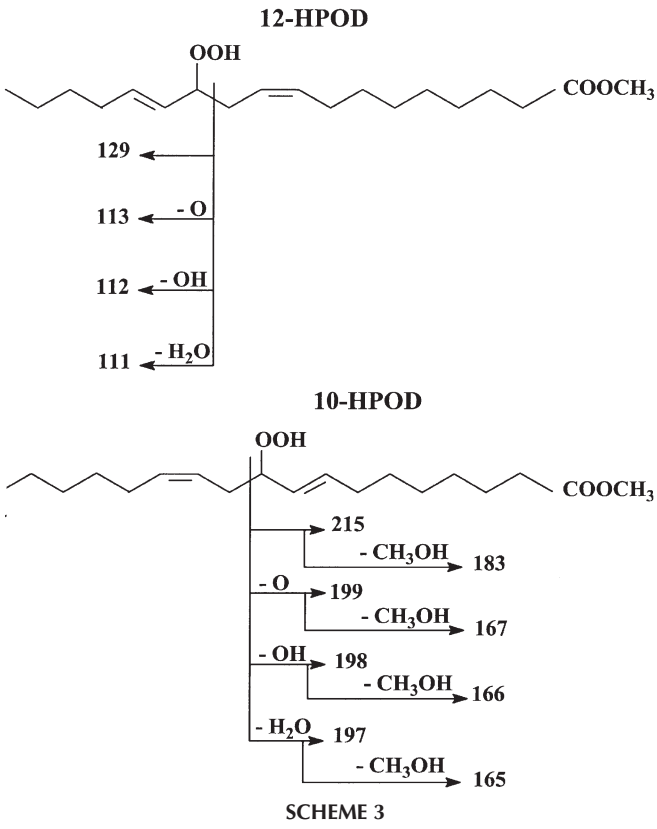

**FIG. 4.** LC/EI-MS spectra of the methyl esters of (A) 12-hydroperoxy-9(*Z*), 13(*E*)-octadecadienoic acid and (B) 10-hydroperoxy-8(*E*),12(*Z*)-octadecadienoic acid produced by photosensitized oxidation of LA. For abbreviations see Figure 1.

the fragmentation pattern shown in Scheme 3. These ions present a fragmentation pattern similar to that observed for 13-HPOT, in which cleavage toward the proximal *trans* double bond generates the observed ions (Scheme 2). Thus, the EI spectrum for the peak at 48.3 min (insert Fig. 3B) indicates co-elution of the 13- and 12-HPOD isomers.

Peak 2b (51.3 min) in the TIC chromatogram in Figure 3B also does not have UV absorption at 234 nm. However, its EI spectrum (Fig. 4B) has the higher mass ions (*m/z* 308, 293, and 261) that are consistent with an HPOD isomer. Additional ions for this isomer are at *m/z* 199 (14.5%), 198 (12.9%), 197 (2.9%), along with ions at *m/z* 167 (42.2%), 166 (10.9%), and 165 (20.4%). Elution of this isomer after the 12- and 13-HPOD isomers and the absence of UV absorption at 234 nm suggests it is a 10-HPOD isomer, as indicated from the fragmentation pattern shown in Scheme 3.

The unresolved TIC peak 3b (52.3 min, Fig. 3B) had a spectrum consistent with 13-HPOD (Fig. 1A). The elution order of this peak from the column along with its UV maximum at 228 nm supports its assignment as *trans-trans* 13-HPOD (19). The TIC for peak 4b (54.6 min, Fig. 3b) had an EI spectrum and UV maxima consistent with *cis-trans* 9-HPOD. The minor companion peak 4a (57 min, Fig. 3A) had a UV maximum and mass spectrum consistent with the *trans-trans* 9-HPOD isomer (19).

Chlorella pyrenoidosa *HPOD products.* LOX activity present in *C. pyrenoidosa* was isolated by $(NH_4)_2SO_4$ precipitation (45 and 80% saturation). The active fractions were subsequently used to oxygenate LA to HPOD isomers at pH 8.0; after methylation with diazomethane they were separated and analyzed by LC/EI-MS. The chromatogram of the product obtained with the LOX fraction obtained at 45% $(NH_4)_2SO_4$ saturation showed two main peaks with retention times of 48 and 55 min. Both




**SCHEME 3**

peaks had UV maxima at 234 nm, which indicates the presence of conjugated double bonds. The EI spectra of both peaks had ions at *m/z*: 310 [M – Oxygen], 308 [M – $H_2O$], and 293 [M – $O_2H$], which are ions characteristic for HPOD derivatives. The first-eluting peak (>60% of the product) had an EI spectrum with an ion at *m/z* 99 (100%), which is characteristic for the 13-HPOD isomer. The EI spectrum of the second-eluting peak (<45%) had ions at *m/z* 185 (24%) and 153 (18%), which is indicative for 9-HPOD (14). Other minor peaks in the chromatogram were identified as the corresponding 9- and 13-HPOD *trans, trans* isomers. Incubation of LA under oxygen with the LOX fraction obtained at 80% $(NH_4)_2SO_4$ saturation produced the 9-HPOD isomer as the main product (>85%) as determined from its UV and EI spectra.

Previously it was reported that *C. pyrenoidosa* produced 9-, 10-, and 13-HPOD isomers (7). However, the LC/EI-MS analysis presented here did not detect the 10-HPOD isomer. The 45 and 80% $(NH_4)_2SO_4$ LOX fractions produced different HPOD isomers, which suggested the presence of two LOX isozymes in *C. pyrenoidosa*. Moreover, the 45% $(NH_4)_2SO_4$ LOX fraction oxygenated both LA and LNA, whereas the 80% $(NH_4)_2SO_4$ LOX fraction oxygenated only LA (yield near 50%).

We reported (10) that *C. pyrenoidosa* LOX has a cleavage activity on 13-HPOD and 13-HPOT under anaerobic conditions. Both substrates produced a mixture of volatile $C_5$ monomers as well as $C_5$ dimers when 13-HPOT was the substrate. These results are consistent with the LOX anaerobic cleavage mechanisms proposed by Salch *et al.* (9). The 45 and 80% $(NH_4)_2SO_4$ saturated LOX fractions were assayed for their cleavage activity under anaerobic conditions with 13-HPOD and 13-HPOT by using a GC/MS method previously described (20). The 45% $(NH_4)_2SO_4$ LOX fraction showed low cleavage activity, whereas the 80% $(NH_4)_2SO_4$ LOX fraction showed high cleavage activity. However, other factors such as the concentration of free fatty acids in both fractions could play a role, and studies with more highly purified enzyme fractions are planned. In addition, when the 80% $(NH_4)_2SO_4$ LOX fraction was incubated under anaerobic conditions with its own product, 9-HPOD, no cleavage products were detected by GC/MS analysis and no decrease in UV absorption at 234 nm was observed, suggesting that the LOX that produces 9-HPOD does not promote anaerobic cleavage of 9-HPOD.

*Conclusion.* The LC/EI-MS method presented here can effectively identify the 9-, 10-, 12-, and 13-HPOD isomers of LA and the analogous HPOT isomers of LNA. EI-MS fragmentation patterns for the HPOD isomers provide distinctive MS peaks that are characteristics for each HPOD isomers. Co-elution of peaks such as the 12- and 13-HPOD isomers can be detected by analysis of ion chromatogram. Hence LC/EI-MS is a powerful tool for the analysis of mixtures of HPOD isomers. The method is particularly useful for analyzing HPOD isomers produced by LOX isozymes. For example, the assay of partially purified LOX from *C. pyrenoidosa* showed the presence of two LOX isozymes, the characterization of which is currently under study in our laboratory.

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# The Role of Docosahexaenoic Acid in Retinal Function

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**ABSTRACT:** An important role for docosahexaenoic acid (DHA) within the retina is suggested by its high levels and active conservation in this tissue. Animals raised on n-3–deficient diets have large reductions in retinal DHA levels that are associated with altered retinal function as assessed by the electroretinogram (ERG). Despite two decades of research in this field, little is known about the mechanisms underlying altered retinal function in n-3–deficient animals. The focus of this review is on recent research that has sought to elucidate the role of DHA in retinal function, particularly within the rod photoreceptor outer segments where DHA is found at its highest concentration. An overview is also given of human infant studies that have examined whether a neonatal dietary supply of DHA is required for the normal development of retinal function.

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Docosahexaenoic acid (DHA, 22:6n-3) is found in very high concentration in the retina (1,2). DHA may be obtained directly from the diet or synthesized from one of its n-3 precursors (3). The retina possesses an efficient conservation and recycling mechanism that helps preserve retinal DHA concentrations even during prolonged periods of low n-3 dietary intake (4–6). The high concentration of DHA in the retina and existence of the conservation and recycling mechanisms suggest that DHA may be important to retinal function. The growing body of evidence to suggest that DHA performs several important roles within the retina is the subject of this review. We have chosen to focus on recent research that has provided new information regarding the role of DHA in the retina, particularly within the photoreceptors. Key results from early studies that have contributed to our understanding of the role of DHA in the retina are also discussed. A comprehensive review of earlier studies in this field was published previously (7).

A major portion of this review is dedicated to animal studies that have investigated the effect of large reductions in retinal DHA levels on retinal function. An overview is also given of studies with human infants that have sought to determine whether a supply of preformed DHA is required in the diet of human infants to achieve optimal retinal function. A description of the basic structure and physiology of the retina and how its function may be assessed with the electroretinogram (ERG) will preface the review of the human and animal studies.

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; E, cGMP phosphodiesterase; EPA, eicosapentaenoic acid; ERG, electroretinogram; IPR, isolated probe response; IRBP, interphotoreceptor retinal binding proteins; ISI, interstimulus interval; LC-PUFA, long-chain polyunsaturated fatty acids; PE, phosphatidylethanolamine; ROS, rod outer segment; RPE, retinal pigment epithelium.

## THE RETINA

The following sections provide a brief overview of basic retinal structure and physiology; more detailed information on these subjects may be found elsewhere (for review, see Refs. 8–10). The process of vision begins with light being focused through the cornea and lens onto the retina. The retina contains the cells responsible for light capture and transduction, the rod and cone photoreceptors. The rod system has high sensitivity at low light levels to provide animals with "night vision," but does not provide color vision or high spatial resolution. The retinas of nocturnal mammals such as rats are rod dominant and have few cones. Cones provide the basis for color vision and the ability to see over a wide range of light intensities in daylight. Higher primates, including humans, apes and old world monkeys, have three classes of cones that respond optimally to either long, medium, or short wavelengths (i.e., red, green, or blue) (11). Cones are tightly packed within the fovea to provide the basis for high visual acuity.

Photoreceptor cells contain two distinct compartments, the inner and outer segments (Fig. 1). The inner segments contain the components necessary for cell metabolism, whereas the outer segments contain the photopigments that absorb photons of light. The rod outer segments (ROS) contain thousands of vertically stacked free-floating disks that are rich in DHA and the photopigment rhodopsin (Fig. 1). The cone photoreceptors also contain disks within the outer segment, but they are not free floating and remain contiguous with the outer plasma membrane.

In the dark, a continuous current flows into the rod photoreceptor outer segments through cation channels kept open by a high cytosolic concentration of cGMP (see Fig. 2). After photon capture, rhodopsin rapidly undergoes a conformational change to form metarhodopsin II (R*), the activated form of rhodopsin. R* activates the trimeric G protein, transducin, by catalyzing the exchange of GDP for GTP, which binds to the α subunit of transducin (Fig. 2). The activated

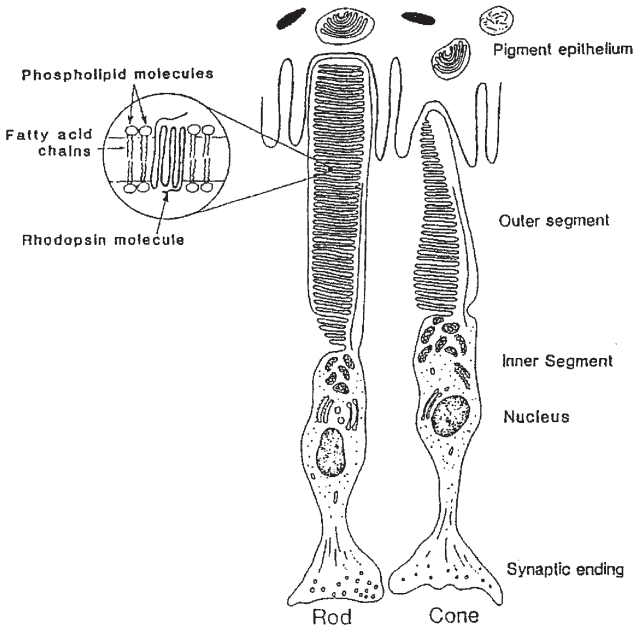


**FIG. 1.** Rods contain thousands of vertically stacked free-floating disks that are rich in docosahexaenoic acid (DHA) and the photopigment, rhodopsin. The cone photoreceptors also contain disks within the outer segment, but they are not free floating and remain contiguous with the outer membrane. Each cone photoreceptor contains one of three photopigments that respond optimally to short, medium or long (red, green or blue) wavelength light. The inner segments contain the components necessary for cell metabolism. The pigment epithelium has several important roles including regulation of the exchange of nutrients and by-products between the photoreceptors and the choroidal blood supply. Inset: The photopigments are surrounded by the DHA-rich phospholipids. Reproduced with permission from Reference 7.

transducin (Gα*) binds to the tetrameric cGMP phosphodiesterase (E), thus removing the influence of one of its inhibitory γ subunits. The activated E (E*) hydrolyzes cGMP to GMP. The resulting fall in cytosolic cGMP after rhodopsin activation closes cation channels causing the photoreceptor to hyperpolarize. The phototransduction cascade has a high gain, so the activation of a single rhodopsin molecule results in the hydrolysis of some $10^5$ cGMP molecules (12).

In the dark, photoreceptors continually release the neurotransmitter glutamate into their synaptic junctions with the bipolar and horizontal cells. Light-induced hyperpolarization of the photoreceptors causes a graded reduction in the release of glutamate, which in turn causes the bipolar cells to either depolarize (ON-bipolars) or hyperpolarize (OFF-bipolars).

## THE ERG AS A MEASURE OF RETINAL FUNCTION

The ERG is a record of the voltage change that occurs across the retina in response to a brief flash of light (for reviews, see Refs. 10,13). The ERG may be recorded using a wide variety of electrodes, but a contact lens electrode placed on the cornea provides the largest and most stable response (14). The following section gives a brief overview of the ERG with particular reference to the components most commonly recorded in studies that have assessed the role of DHA in retinal function. A typical ERG and its characteristic parameters are shown in Figure 3.

*Origin of the ERG.* The leading edge of the ERG a-wave recorded to a bright saturating flash, i.e., one that closes all cation channels, reflects the hyperpolarization of the massed photoreceptor response (15,16). The rod-dominated ERG b-wave recorded in the dark reflects depolarization of the rod bipolar cells (17,18). The cone-dominated ERG b-wave recorded with a bright background is shaped by both ON and OFF bipolar cells and horizontal cells (19). The oscillatory potentials are high-frequency oscillations superimposed upon the b-wave (Fig. 3), and are thought to originate from different levels within the proximal retina, including the amacrine, interplexiform, and ganglion cells (20).

*Factors affecting ERG morphology.* One important determinant of ERG amplitudes and implicit times is the retinal illuminance produced by the flash. Retinal illuminance refers to the number of photons arriving at the retina measured in trolands (Td) (21). Retinal illuminance takes into account not only the intensity and spectral content (color) of the flash but also a number of other factors, including dark adaptation, the length of the eye, and the size of the pupil (21). It has been estimated for dark-adapted humans that a troland of light induces, on average, 8.6 photoisomerizations per rod (22). For the average human rod with 70 million rhodopsin molecules (9), a flash that produces a retinal illuminance of 1.0 log scotopic troland-seconds (scot-Td-sec) will therefore bleach ~0.0001% of the available rhodopsin.

Another important determinant of ERG morphology is the background luminance. Scotopic ERG (rod dominant) are recorded in the dark, whereas photopic ERG (a measure of the massed cone response) are recorded against a background light sufficient to saturate the rods. Photopic ERG are typically smaller and faster than scotopic ERG (23).

*Conventional analysis of the ERG.* The "conventional" method of ERG assessment involves measurement of ERG b-wave amplitude over a range of flash intensities (Fig. 4). The change in amplitude with flash intensity may be described in terms of the Naka-Rushton function (Fig. 5), an adaptation of the Michaelis-Menten equation (24,25):

$$V/V_{max} = I^n/(I^n + K^n) \quad [1]$$

where $V$ is the amplitude (μV), $I$ is the flash intensity, $n$ is the slope of the curve, $V_{max}$ is the maximum amplitude (μV), and $K$ is the intensity that elicits half-maximal response. $V_{max}$ has been interpreted as an index of both the number of rods responding and the gain (μV/quantum captured) for each b-wave generator (26). The parameter $K$ has been interpreted as an index of retinal sensitivity that represents quantal capture (26). Rod threshold, defined as the flash intensity at which an ERG b-wave is just detectable, may also be determined from the Naka-Rushton equation (27).

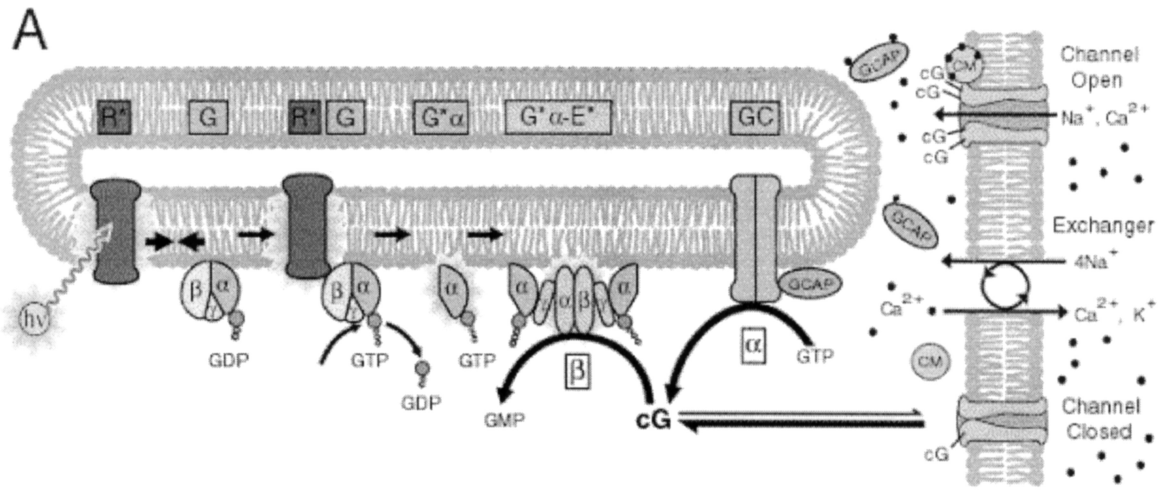


**FIG. 2.** Upon capture of a photon of light (hν), rhodopsin is activated (R*). R* activates the G protein by catalyzing the exchange of GDP for GTP. Upon activation, the G protein separates into two parts; one is the isolated subunit to which GTP is now attached (G*α). G*α binds to cyclic guanosine monophosphate phosphodiesterase (E), releasing the inhibitory influence of one of the E γ subunits. The activated E begins to hydrolyze cGMP (cG) to GMP. The resulting fall in cytosolic cGMP concentration causes cGMP molecules to dissociate from the ion channel, which now closes. Rod recovery occurs in part due to an increase in cGMP concentration, which is synthesized from GTP by guanylate cyclase (GC). Reproduced with permission from Reference 78.

One limitation of conventional ERG analysis is that the ERG waveforms are formed by a composite of generators and thus are nonspecific in terms of cellular origin. For example, the ERG b-wave contains not only bipolar responses but also contributions from the photoreceptor a-wave and oscillatory potentials from the proximal retinal layers (18). The b-wave also includes a substantial corneal negative scotopic threshold response generated by the proximal retina at low-to-moderate flash intensities (18,28). In most of the studies investigating the role of fatty acids in retinal function, the ERG has typically been measured up to flash intensities sufficient to produce maximal ERG b-wave amplitude ($V_{max}$). At this flash intensity, the ERG a-wave does not provide an accurate description of the massed hyperpolarization response of the photoreceptors (16).

*Analysis of phototransduction.* Recent developments in ERG recording and analysis techniques have enabled better isolation of the massed responses from the photoreceptor and bipolar cells. *In vitro* experiments with isolated photoreceptors have enabled the development of a quantitative model describing the G-protein cascade of phototransduction (29). The same quantitative model or slight variants of it (see Eq. 2) have been used successfully to describe the leading edge of the ERG a-wave in response to a high intensity flash that causes a-wave saturation (22,30–32). Figure 6 shows an example of ERG recorded to three different flash intensities (solid lines) and the fit of the model given in Equation 2 to the leading edges of the ERG a-waves (dashed lines). The phototransduction model is given by

$$P3(i, t) \approx \{1 - \exp[-i \cdot S \cdot (t - t_d)^2]\} \cdot R_{maxP3} \qquad t > t_d \qquad [2]$$

where $P3$ is the voltage of the ERG a-wave (μV), at time $t$ seconds, in response to a flash with a retinal illuminance of $i$ scot Td-sec. The $P3$ term describing the ERG a-wave is so named after Granit's classic analysis, in which the ERG was formed by the addition of two cellular responses, $P2$, a single postreceptoral response and $P3$ the response from the pho-

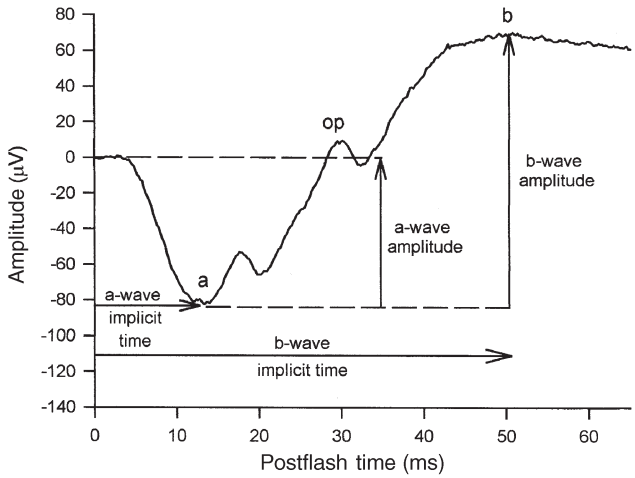


**FIG. 3.** The electroretinogram (ERG) a-wave represents the hyperpolarization of the massed photoreceptor response, whereas the ERG b-wave represents the depolarization of the rod-bipolar cells. Small oscillatory potentials shown superimposed on the rising b-wave originate from interactions among the amacrine, interplexiform, and ganglion cells. ERG a-wave amplitude is measured from the baseline to the trough of the ERG a-wave, and ERG b-wave amplitude is measured from the trough of the a-wave to the peak of the ERG b-wave. Implicit times are measured from flash onset (time 0) to the trough of the ERG a-wave and to the peak of the ERG b-wave.

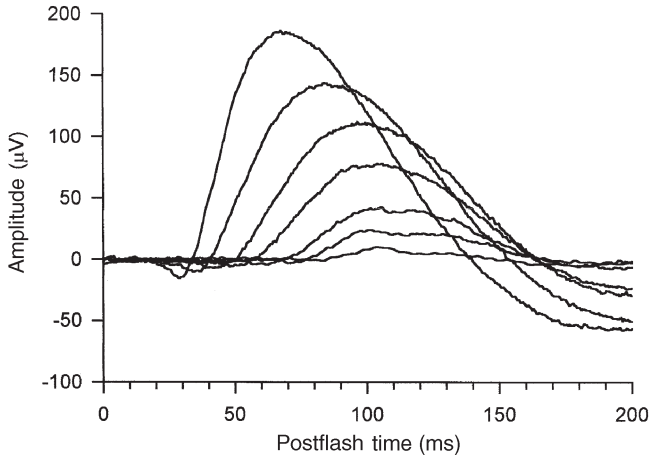


**FIG. 4.** Variation in ERG b-wave amplitude and implicit time over a 4 log unit range of retinal illuminance. With increasing retinal illuminance (bottom to top), ERG b-wave amplitude increases and implicit time decreases. For abbreviation see Figure 3.

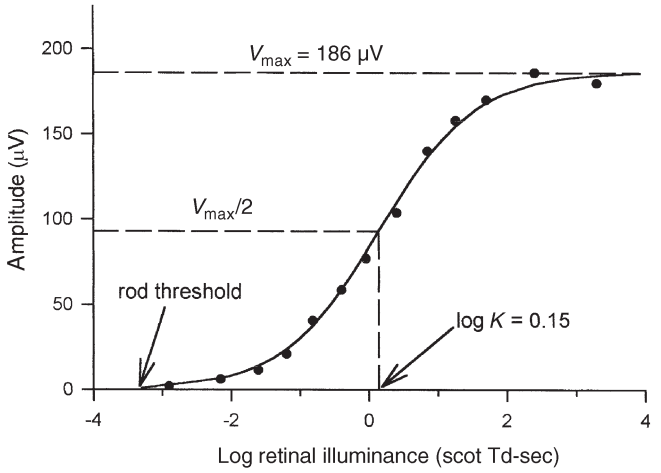


**FIG. 5.** Variation in ERG b-wave amplitude with retinal illuminance from an adult rhesus monkey. The solid line shows the fit of the Naka-Rushton equation (Eq. 1 in text) to the data. The parameters derived from the fit of the Naka-Rushton equation are as follows: $V_{max}$, the maximum amplitude (μV), and *K*, the intensity that elicits half-maximal response. Rod threshold, defined as the flash intensity at which an ERG b-wave is just detectable, may also be derived from the Naka-Rushton equation. For abbreviation see Figure 3.

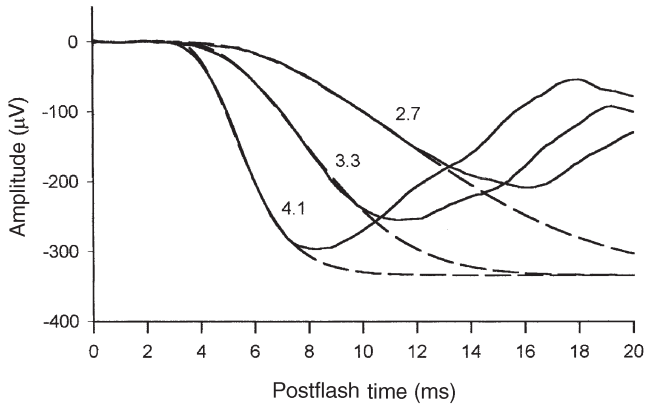


**FIG. 6.** The *P*3 model given by Equation 2 (see main text) provides an excellent fit (dashed lines) to the leading edges of the rod-isolated ERG a = waves (solid lines). The numbers above each ERG a-wave indicate the retinal illuminance in log scot Td-sec used to generate the ERG.

toreceptors (33,34). The parameters of the model that are adjusted to provide the best description of the leading edge of the rod isolated ERG a-wave are as follows: *S*, a sensitivity parameter that scales retinal illuminance [(scot Td-sec)$^{-1}$ sec$^{-2}$]; $t_d$, a delay (sec), and $R_{maxP3}$, the maximum amplitude response (μV) (35). The parameter *S* has been interpreted to represent the gain of the phototransduction cascade, i.e., it is proportional to the number of cation channels closed per molecule of rhodopsin activated (22,30). Alternatively, *S* may also be altered by a change in the local rhodopsin density on the disk membrane (22,30). $R_{maxP3}$ is the change in voltage corresponding to the maximum number of cation channels that can be closed. A reduction in the number of photoreceptors or shortening in the length of the ROS would cause a proportional reduction in $R_{maxP3}$ (22,30). The parameter $t_d$ is a nonphysiologic delay due to instrumentation filtering and finite duration of the flash. One variation to the model given in Equation 2 is to include a term that accounts for membrane capacitance (32,36). Membrane capacitance becomes an important factor when describing the cone photoreceptor ERG a-wave or the rod ERG a-wave recorded to extremely bright flash intensities (31,32,36). Employing the model given by Equation 2 to describe the ERG a-wave thereby provides an *in vivo* method of quantifying the phototransduction process.

To derive the phototransduction parameters, *S*, $R_{maxP3}$, and, $t_d$ Equation 2 is typically fitted simultaneously to multiple ERG a-waves recorded to a series of bright flashes (ensemble fit) (Fig. 6). The highest retinal illuminance used in Figure 6 is ~500 times brighter than the illuminance required to produce ERG b-wave saturation, as used in conventional ERG analysis.

*Assessment of rod recovery.* After light capture, the phototransduction proteins (R*, G*, and E*) are activated, and cytosolic cGMP concentration falls, closing a portion of the cGMP-gated ion channels that are open (Fig. 2). Before the rod can fully respond to subsequent photons of light, the rod must be returned to its dark-adapted resting state. The steps necessary for rod recovery include deactivation of R*, G*, and E* and the return of cGMP concentration to its preflash level. The latter step reopens the same proportion of cGMP-gated ion channels that were closed by the flash. The time course of rod recovery can be assessed using a paired-flash ERG method (35,37). With the paired-flash method, a high intensity "test" flash that forces the rod into saturation is followed by a second "probe" flash at varying interstimulus intervals (Fig. 7). If the probe flash is delivered during the period of rod saturation when all cation channels are closed, there will be no measurable ERG response (Fig. 7, ERG at 6-sec interstimulus in-

terval). Once cation channels begin to reopen, a subsequent probe flash will close all newly opened cation channels, driving the rod back into saturation. The amplitude of the ERG a-wave to the probe flash is proportional to the number of cation channels closed and therefore, the amount of recovery that has occurred since the test flash (35,38). Figure 7 illustrates the recovery of ERG amplitude (thin solid lines) from an adult rhesus monkey at various interstimulus intervals after a bright test flash (5.4 log scot Td-sec). At the end of the recovery experiment, the ERG is recorded in response to a probe flash presented in isolation without a preceding test flash ("Probe only," Fig. 7). The dashed lines in Figure 7 show the fits of the *P*3 model (Eq. 2) to the leading edges of each ERG a-wave. The maximal response derived at each interstimulus interval (ISI), $R_{maxISI}$, is normalized with respect to the maximal response derived from the isolated probe response (IPR), $R_{maxIPR}$. Figure 8 shows the plot of the normalized responses $R_{maxISI}/R_{maxIPR}$ against log interstimulus interval. The solid line is the best fit of the sum of two exponentials to the data. Also shown are two characteristic parameters derived from the analysis, i.e., $T_c$, the time the rod remains saturated after the test flash and $T_{50}$, the time required from $T_c$ to reach 50% of full recovery.

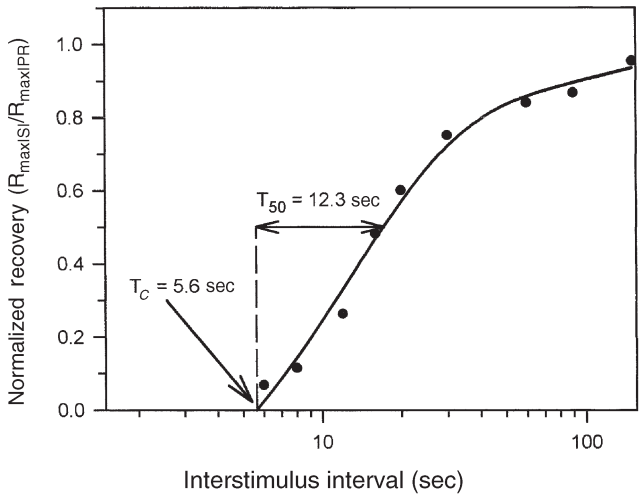


**FIG. 8.** Normalized ERG a-wave recovery plotted against interstimulus interval. The amplitudes of the probe flash ERG at each interstimulus interval (ISI) ($R_{maxISI}$) are normalized with respect to the amplitude of the isolated probe response (IPR) flash ERG recorded at the end of the sequence ($R_{maxIPR}$). The solid line is the best fit of the sum of two exponentials to the data. Also shown are two characteristic parameters derived from the analysis, $T_c$, and $T_{50}$. For abbreviation see Figure 3.

## DHA IN THE RETINA

Within the retina, DHA is incorporated primarily into structural glycerophospholipids of the cell membrane lipid bilayer (39). DHA accounts for 8–20% of total retinal fatty acids in humans and 38–92% of total polyunsaturates within the mammalian retina (2,40–45). DHA is particularly concentrated within the disk membranes of the ROS where it accounts for up to 30% of total fatty acids and 54% of phosphatidylethanolamine (PE) fatty acids (2,42,46,47). The retina is unique in that it contains phospholipids with polyunsaturates at both the *sn*-1 and *sn*-2 positions (dipolyenes). In the monkey, dipolyenes with long-chain polyunsaturated fatty acids (LC-PUFA) in both the *sn*-1 and *sn*-2 positions constitute 16% of the diacyl ethanolamine phosphoglycerides, and 15% of these have DHA in both positions (48).

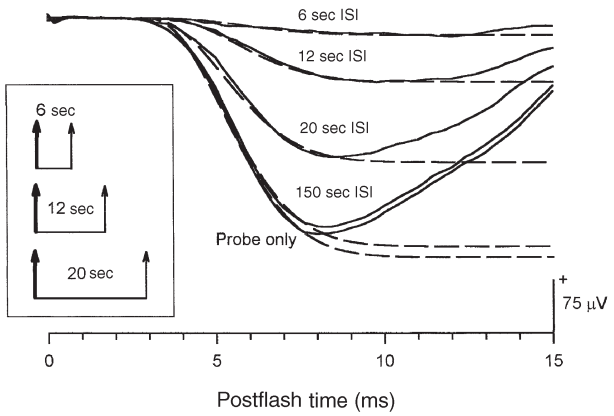


**FIG. 7.** Recovery of the rod ERG a-wave after a saturating flash. The solid lines show the rod isolated ERG recorded to the second (probe) flash (4.1 log scot Td-sec) at a given interstimulus interval (ISI) after a test flash (5.45 log scot Td-sec). The bottom-most response (probe only) is the ERG recorded to the probe flash presented without a preceding test flash 150 sec after the last double flash pair. The dashed curves show the best fits of Equation 2 to the leading edges of the ERG a-waves. Inset: Graphic representation of the separation between the saturating test flash (thick arrow) followed by the probe flash (thin solid arrow) at a given interstimulus interval. For abbreviation see Figure 3.

## LC-PUFA SUPPLEMENTATION IN HUMAN AND NONHUMAN PRIMATES

Birch *et al*. (27) reported an elevation in log *K* and a reduction in $V_{max}$ at 36 wk postconception (~6 wk postnatal age) in preterm infants fed a corn oil-based formula with a low level (0.5%) of α-linolenic acid (ALA) compared with infants fed a fish oil-supplemented formula [0.35% DHA and 0.65% eicosapentaenoic acid (EPA)]. Analysis of the Naka-Rushton function from these infants also revealed an elevation in rod threshold in the corn oil group, which likely reflects the elevation in log *K*. Infants fed a soybean oil-based diet (2.7% ALA) had parameter values that were in between those of the corn and fish oil diet groups. In a follow-up study of term infants by the same group, only log *K* was elevated at 6 wk postnatal age in infants fed a formula with 1.7% ALA as the sole n-3 fatty acid, compared with infants fed a formula supplemented with LC-PUFA [0.36% DHA, 0.72% arachidonic acid (AA)] (49). In both studies, the early differences noted in the ERG between the diet groups were no longer present at 4 mon of age (cor-

rected age for premature infants). However, in the preterm study, infants in the corn oil group had longer latencies for light-adapted oscillatory potentials at 4 mon corrected age (27).

In another study, term infants were randomized to either a control formula containing 2.1% ALA as the sole n-3 fatty acid or one of two formulas supplemented with LC-PUFA, either 0.12% DHA and 0.43% AA, or solely 0.23% DHA. There were similarly no ERG differences between the diet groups at the sole test age of 4 mon (50).

Owing to limitations in ERG methodology used, results from a fourth study are difficult to interpret. Faldella *et al*. (51) recorded ERG from a skin electrode placed on the bridge of the nose. ERG recorded from this position exhibit high variability, are extremely small, and lack the sensitivity even to distinguish different forms of retinal pathology (52). The failure to dilate the pupil or dark-adapt the infants in this study would have further reduced ERG amplitudes and increased variability of the recordings. Given these limitations, it is difficult to draw any conclusions about the effects on retinal function of the diets used in this study.

The preterm infants in the study of Birch *et al*. (27) were born at 28–33 wk gestation. Over the last trimester of pregnancy, there is a 35% increase in retinal DHA level (40), and infants born prematurely are likely to be more susceptible to any reduction in the availability of DHA for accretion by the retina. This greater susceptibility in infants born prematurely may explain the more marked ERG alterations at 6 wk postnatal age in the preterm infants compared with the term infants. Other differences between the term and premature infant studies, notably the level of dietary ALA used, may also account for the greater ERG alterations in the premature infants. The results from both preterm and term infants suggest that retinal function may be altered by the level of n-3 fatty acids supplied in the diet for at least the first six postnatal weeks.

Owing to the difficulty of recording ERG with a contact lens in older infants and young children, the evaluation of retinal function after varying dietary n-3 fatty acid content has been limited to a maximum of 4 mon postterm in humans. The rhesus monkey provides an ideal animal model of the human for the long-term study of the effect of diet on retinal function. The similarities between retinal structure, function, and development in macaque monkeys (e.g., rhesus) and humans are well described (11,53–56). Additionally, rhesus monkeys, like humans, are capable of desaturating and elongating the n-3 and n-6 essential fatty acids to obtain their respective long-chain polyunsaturates (57–60). Adjustments necessary for comparison of humans and macaque monkeys are the different rates of visual development (1 wk for monkeys is equivalent to ~4 wk in humans) and the greater neural and retinal maturity of the monkey at birth.

In a recent study, infant monkeys were raised for the first six postnatal months on either a formula containing 1.7% ALA as the sole n-3 fatty acid or a diet supplemented with 0.8% DHA and 0.8% AA. At 4 and 13 mon of age, there were no significant differences in rod or cone ERG between the two diet groups (62).

In a separate study, retinal function was assessed in monkeys raised their entire lives on either a diet with 8% ALA as the sole n-3 fatty acid or a diet supplemented with 0.6% DHA and 0.2% AA. The mothers of these monkeys consumed diets identical to those of their offspring throughout pregnancy. There were no significant differences in the ERG between the two groups of monkeys when tested as adults (4–6 yr) (62). However, several alterations were found in the ERG of monkeys fed very low ALA diets, and these are described in the following section.

The results from the two monkey studies suggest that a diet with 1.7–8% ALA as the sole n-3 fatty acid does not adversely affect the long-term development of retinal function in higher primates, compared with a diet containing at least 0.6% DHA. The similarity of the rhesus monkey retina to that of humans, together with the data for human infants at 4 mon, suggests that this finding is likely valid for humans. It has not been tested in humans whether a diet with a level of ALA >1.7% would eliminate the alterations in the ERG noted at 6 wk postnatal age compared with infants fed a LC-PUFA-supplemented diet containing 0.35% DHA.

The combined results from the human and monkey studies suggest that the period of vulnerability of the retina to a low supply of dietary n-3 fatty acids is short. If a lowering of retinal DHA level underlies the ERG alterations reported in the human infants, what are the possible mechanisms that result in this early vulnerability? In both rhesus monkeys and baboons, DHA is readily accreted by the retina from the circulating blood supply during the latter stages of fetal development and during the neonatal period (57–59). The retina also has a sophisticated recycling system that ensures conservation of retinal DHA levels even during periods of prolonged dietary n-3 deficiency (4–6). Both term and preterm human infants are capable of synthesizing some DHA from ALA (63,64). However, the above results suggest that infants, particularly preterm infants, fed formulas with <1.7% ALA may not be able to synthesize sufficient DHA to meet their retinal requirements over the early postnatal weeks. The lack of ERG differences at 4 mon of age suggests that any such limitation is transient.

## THE EFFECT OF DHA DEFICIENCY ON RETINAL FUNCTION IN ANIMALS

*Rats and guinea pigs.* Reductions in conventional ERG a- and b-wave amplitudes have been consistently reported in rats and guinea pigs fed n-3-deficient diets in comparison with control animals fed high ALA, n-3-sufficient diets (46,65–70).

The newer methods of ERG recording and analysis have been used in only a few studies. Weisinger *et al*. (70) reported that phototransduction sensitivity, *S*, varies as a saturating function of retinal DHA level at 16 wk of age in guinea pigs (Fig. 9, lower graph). In the same guinea pigs, the maximal rod response $R_{\max P3}$ was reduced by ~0.16 log units when retinal DHA fell below 16% of total fatty acids (Fig. 9, upper graph). Further reductions in retinal DHA did not produce any additional loss in $R_{\max P3}$. The pattern of variation in *S* with

retinal DHA level differs from that of $R_{maxP3,}$ suggesting that two separate mechanisms may be affected in n-3-deficient guinea pigs. In a subsequent study in rats from the same laboratory, a reduction in retinal DHA from 34.1 to 25.5% of total fatty acids was associated with a 0.28 log unit reduction in both $R_{maxP3}$ and $S$ at 35 wk of age (71).

The mechanisms by which lowering retinal DHA levels causes reductions in ERG amplitudes and phototransduction sensitivity in rats and guinea pigs are not yet fully understood. The rate of ROS disk synthesis (42), the number of photoreceptors (46,72), ROS length (42,72), and the width of the outer nuclear layer (42) remain unaltered in n-3-deficient rats. The number of retinal pigment epithelium (RPE) phagosomes is reduced in n-3-deficient rats (66,73), but such changes seem unlikely to alter ERG components generated by the photoreceptors and bipolar cells within the first 70 msec after flash onset. Alteration in the local concentration of rhodopsin within the ROS would likely affect phototransduction sensitivity (30), and the rate of rhodopsin regeneration after a 100% bleach is slowed in n-3-deficient rats (72). However, slower rhodopsin regeneration is unlikely to have caused the ERG alterations in n-3-deficient rats and guinea pigs, given that all studies, with the exception of Watanabe *et al.* (66), allowed sufficient time to achieve full dark adaptation. There are conflicting reports as to the effect of altering the level of dietary n-3 fatty acids on absolute rhodopsin content in the retina after complete dark adaptation. Higher levels of rhodopsin have been reported in rats fed either an n-3-deficient diet (72) or a high DHA/EPA fish oil-supplemented diet (74) compared with rats fed a diet containing ALA as the sole n-3 fatty acid. Further experiments are required to determine whether altered rhodopsin levels underlie the sensitivity changes reported in rats and guinea pigs. Higher rhodopsin levels in n-3-deficient rats might be expected to lead to higher rather than lower sensitivity. However, low DHA in the membrane lipid environment of rhodopsin may reduce its photochemical activity and thus lower sensitivity. This possibility is supported by *in vitro* model membrane studies showing lower metarhodopsin II formation in membranes low in DHA (75–77).

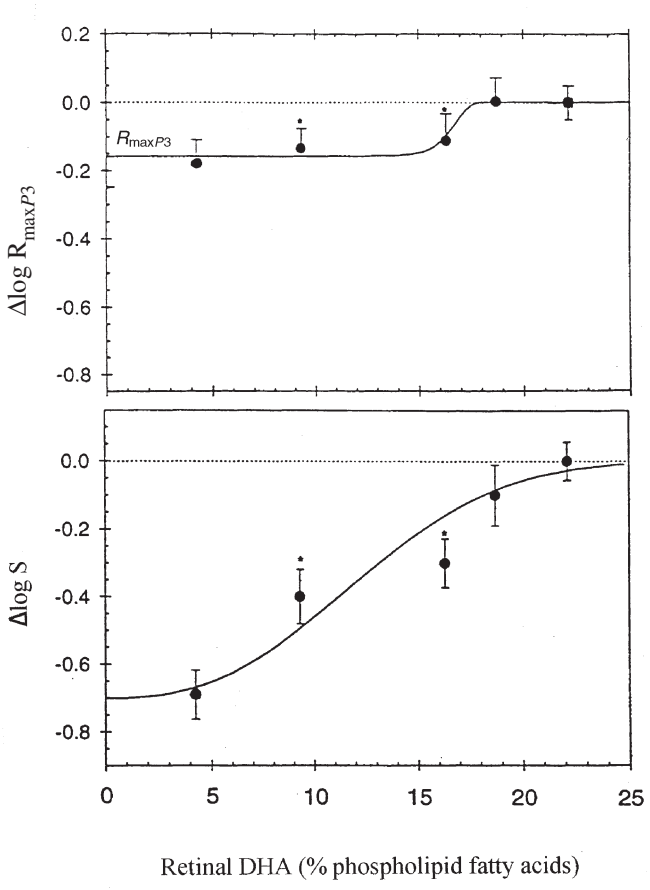


**FIG. 9.** Variation in $S$ (lower graph) and $R_{maxP3}$ (upper graph) with retinal docosahexaenoic acid (DHA) in 16-wk-old guinea pigs. Values are expressed as the change (Δlog value ± SEM) from control guinea pigs fed a canola oil-based diet. *Significantly different from the canola oil group, $P < 0.05$. Data were fit by a Weibell function to emphasize the transitions. Reproduced with permission from Reference 70.

Another possibility is that low retinal DHA affects the ERG through changes in ion channels. $R_{maxP3}$ is proportional to the magnitude of the circulating photocurrent, i.e., the number of open cGMP-gated ion channels within the retina (22). The number of cGMP-gated ion channels open at any given time is regulated by intracellular cGMP concentration, which in turn depends on the rates of cGMP hydrolysis and synthesis. A number of the mechanisms involved in determining the rates of cGMP hydrolysis and synthesis are $Ca^{2+}$ dependent (78). On the basis of the results from experiments in monkeys described below, it is speculated that a reduction in retinal DHA may alter the calcium current flowing into the photoreceptor outer segments through the cGMP-gated ion channels. Whether alteration in cytosolic $Ca^{2+}$ concentration underlies the reduction in $R_{maxP3}$ in n-3-deficient rats and guinea pigs remains to be determined.

No significant alterations in ERG a-wave and b-wave implicit times have been reported in n-3-deficient guinea pigs compared with those fed a high ALA diet (68,70). Rats fed a diet with DHA enriched at the *sn*-2 position of triglycerides had a 3.5–5.5% delay in ERG b-wave implicit times compared with rats fed either rat milk or a diet with DHA distributed equally across the *sn*-1, -2, and -3 positions (79). A caveat in interpreting this study is that the delay in ERG implicit times was present for only one of three flash intensities. Absolute retinal fatty acid levels were not significantly different between the two groups fed the experimental diets, but it would be of interest to know whether the experimental diets altered the dipolyene composition of the retinas. The retina is unique in containing a high level of dipolyenes, and these are known to increase the rate of rhodopsin activation (metarhodopsin II formation) compared with monoenes in recombinant membranes (76,77,80). Delays in metarhodopsin II formation or the rate of activation of the phototransduction proteins could slow the ERG response.

*Cats.* The effects of reducing retinal DHA levels in a single study of cats were quite different from those reported in

rats and guinea pigs. In 12-wk-old cats, ERG a-wave implicit times were delayed by 10% in those fed diets with differing levels of ALA (0.2–1.3%) compared with cats fed diets that contained LC-PUFA (>0.3% DHA, >0.5% AA) (81). There were no significant differences in ERG amplitudes between the groups. Delays in ERG b-wave implicit times in this study were reported, but are difficult to interpret because the ERG was recorded to a red flash that elicited a mixed rod/cone response. The mean ERG b-wave implicit time for the LC-PUFA-supplemented cats was 48.6 msec, consistent with the peak of the cone response (23), whereas the mean ERG b-wave implicit time for the ALA cats was 70.7 msec, consistent with the peak of the rod response (23). These results suggest that different ERG b-wave components were measured for the two diet groups, or that the relative amplitudes of these components were altered.

*Rhesus monkeys.* Neuringer and co-workers (82–85) fed two cohorts of rhesus monkeys diets that contained either 0.3% ALA (Low ALA) or 8% ALA (High ALA) as the sole n-3 fatty acid. The second cohort also included a third dietary group supplemented with LC-PUFA (0.6% DHA, 0.2% AA, and 0.2% EPA). ERG were recorded from the monkeys as infants (3–4 mon), juveniles (1–2 yr), and for the second cohort as young adults (4–6 yr). In the first cohort, Low ALA monkeys had reduced rod and cone ERG a-wave amplitudes as infants compared with the High ALA monkeys (84). This result was not repeated in the second cohort when they were tested at the same age or in either cohort when tested as juveniles or adults (62,84,86). In addition, there was a 5–10% delay in rod and cone ERG b-wave implicit times in the Low ALA monkeys when tested as juveniles and adults compared with monkeys fed the High ALA and LC-PUFA-supplemented diets (62,84,86).

The most marked and consistent alteration in retinal function in monkeys fed the Low ALA diet was a delay in the time required for the rod photoreceptors to recover after a light flash. When moderately intense flashes (2.6 log scot Td-sec) were separated by intervals of 20 sec, ERG amplitudes were not significantly different between the rhesus monkeys fed the Low ALA diet and those fed the High ALA diet. When the interval between flashes was reduced to 3.2 sec, ERG a- and b-wave amplitudes were reduced in both groups of monkeys, but there was a significantly greater reduction in the Low ALA monkeys compared with the High ALA monkeys (83–85). This effect was present in both cohorts of monkeys when tested as infants and juveniles. The reduction in ERG amplitude at short intervals between flashes suggested a delay in recovery of the rod photoreceptors, a conclusion recently confirmed in adult rhesus monkeys by measuring rod recovery with the paired flash method (62,86). The Low ALA monkeys were delayed by ~30% in reaching 50% recovery ($T_{50}$) compared with the 5% delay in ERG implicit times in the same monkeys (62). There was no effect of diet groups on the duration of complete rod saturation, $T_c$ (62).

Results from *in vitro* experiments using recombinant membranes and isolated cells suggest a number of possible mechanisms that could account for the delays in ERG b-wave implicit times and rod recovery in the Low ALA monkeys. Activation of the G-protein cascade involves contact between the activated proteins, R*, G*, and E* as the result of two-dimensional diffusion through the disk membrane lipid bilayer. It has been proposed that the high concentration of DHA and dipolyenes in the disk membrane imparts a number of biophysical properties (77) that should facilitate the diffusion of the phototransduction proteins through the disk membrane. Recent results demonstrate that the diffusion of transducin is faster in model membranes containing DHA at the *sn*-2 position than in membranes with oleic acid at the *sn*-2 position (87). Thus, slower diffusion of the phototransduction proteins through the lipid bilayer of the disk membrane may account for the small delays in ERG implicit times in n-3-deficient animals. The same principle could also apply to proteins such as rhodopsin kinase and recoverin, which are involved in the deactivation of R* and thus could help to explain part of the delay in rod recovery as well. However, the delay in rod recovery in the Low ALA adult rhesus monkeys was ~6 times greater than the delay in ERG implicit times, and this discrepancy suggests a separate mechanism in the recovery process that is altered in the Low ALA monkeys. One possibility is that a larger $Ca^{2+}$ current flows through the cGMP-gated cation channels of the Low ALA monkeys. There are numerous processes required for rod recovery, including the deactivation of the phototransduction proteins and the return of cytosolic cGMP concentration to preflash levels. Higher intracellular $Ca^{2+}$ slows the deactivation of metarhodopsin II and the synthesis of cGMP (88); the net effect of both is to slow the rate of rod recovery. DHA as a free fatty acid suppresses voltage-gated L type $Na^+$ and $Ca^{2+}$ channel currents in isolated neonatal and adult rat cardiomyocytes and in CA1 neurons from the rat hippocampus (89,90). Whether DHA similarly affects the cGMP-gated ion channel of the rod photoreceptor is unknown. A larger transient $Ca^{2+}$ influx through the cGMP-gated ion channels in the Low ALA monkeys could explain the observed delay in rod recovery.

*Comparison of ERG results among animal species.* The studies in rats, guinea pigs, cats, and rhesus monkeys highlight important species differences with respect to the effect of reducing retinal DHA levels on retinal function. It is unclear why these species should exhibit such contrasting ERG alterations in response to an n-3-deficient diet. In the ERG studies, rats and guinea pigs fed n-3-deficient diets had retinal DHA levels reduced by 30–65% in comparison with n-3-sufficient control animals. In the rhesus monkeys, retinal DHA was reduced by 50% at birth and by 80% at 2 yr of age compared with the High ALA control group. In each of the animal studies described, the fall in retinal DHA was largely compensated for by an increase in retinal 22:5n-6 and, to a lesser extent, 22:4n-6. These comparisons highlight the fact that the n-3-deficient diets used in each species induced similar changes in retinal fatty acids.

The timing of retinal development and accretion of DHA to the retina also appear to be unable to explain the different

ERG results between monkeys and guinea pigs. There is substantial growth and differentiation of retinal cells *in utero* in both monkeys and guinea pigs (53,91), and mothers were fed the same n-3-deficient diets in studies using both species. The structure of the monkey retina is quite different from that of rats and guinea pigs. In higher primates, cones account for ~5% of the total photoreceptors and interact with rods in a complex manner (92). The primate retina also has a macula, a central area containing the pigments, lutein, and zeaxanthin (93). At the center of the macula lies the fovea, a region with very high cone density that enables primates to achieve high visual acuity (94). The retinas of rats and guinea pigs by comparison are specialized for nocturnal vision, are dominated by rod photoreceptors, and have no macula or fovea. Therefore, rats and guinea pigs have excellent sensitivity in the dark but poor visual acuity. It is possible that these differences in retinal structure among species may account for the different effects on retinal function of lowering retinal DHA.

## CRITICAL PERIODS FOR ACCRETION OF DHA TO THE RETINA

A number of repletion studies have provided evidence for a "critical period" during retinal development when an inadequate supply of DHA to the retina will result in permanent retinal dysfunction that cannot be normalized even when retinal DHA is returned to normal.

A subset of the monkeys from cohort 1, described previously, was fed the Low ALA diet for 10–22 mon after birth before being switched to a fish oil-based diet containing 9% DHA and 13% EPA (wt% of total fatty acids) (60). After 9 mon of dietary repletion, when DHA levels had increased to above normal, repleted monkeys still had delayed ERG b-wave implicit times and a greater reduction in ERG b-wave amplitude at short interflash intervals compared with control monkeys (84). In this study, repletion was initiated at ages when rhesus monkeys have an adult-like retina (53,95). In one monkey repleted with 1.6% DHA ethyl ester from 4 mon of age, when the retina is not fully developed, the ERG normalized by 2 yr of age (96).

In a study by Armitage *et al*. (71), rats were fed either a safflower oil-based diet with 1% ALA and a LA/ALA ratio of 72:1 or a canola oil-based diet with 8% ALA and a LA/ALA ratio of 2.5:1. At 8 wk of age, half of the rats fed the safflower diet were switched to the canola oil diet for 25 wk. By 33 wk, there were no significant differences in retinal DHA levels between the repleted rats and those fed the canola oil diet. Nevertheless, the repleted rats had a 60% reduction in $R_{\max P3}$ compared with the those fed the canola oil diet. In a similar repletion study using the same diets, no ERG alterations were found in guinea pigs after retinal DHA levels were returned to within 85% of normal after repletion from 5 to 16 wk. In both species, repletion was initiated at an age when the retina has functionally reached adult levels (97).

The above results provide strong support for the existence of a critical period in both monkeys and rats in which DHA must be accreted to the retina to achieve normal development of retinal function. The length of these critical periods with regard to either age or the stage of retinal development has yet to be determined.

## A ROLE FOR DHA IN THE REGENERATION OF RHODOPSIN

Rhodopsin is formed in the disk membrane of the retinal ROS when opsin binds its chromophore, 11-*cis* retinal. The capture of light by 11-*cis* retinal results in its isomerization to all-*trans* retinal and leads to the formation of metarhodopsin II. After deactivation of metarhodopsin II, some of the all-*trans* retinal separates from opsin and is converted to all-*trans* retinol. Regeneration of rhodopsin from these components is a two-step process. First, all-*trans* retinol must be removed from the ROS and transferred to the RPE. Second, a new 11-*cis* retinal molecule manufactured within the RPE must be transferred to the ROS, where it binds opsin to form a new rhodopsin molecule. The mechanism whereby the highly insoluble retinoids, 11-*cis* retinal and all-*trans* retinal, are transferred across the aqueous interphotoreceptor matrix between the RPE and the ROS is known to involve the interphotoreceptor retinal binding proteins (IRBP). The IBRP are the major soluble proteins within the interphotoreceptor matrix, and bind both retinoids and fatty acids (98).

In a single fluorescence study, DHA had the highest affinity for IRBP, twice that of AA and three times that of ALA (99). A subsequent fluorescence study found that DHA hindered the interaction of 11-*cis* retinal at a hydrophilic binding site on IRBP and also facilitated the dissociation of 11-*cis* retinal from this binding site (100). In contrast, DHA did not alter the interaction or dissociation of all-*trans* retinol with IRBP. Preliminary data indicate that AA does not affect the interaction of 11-*cis*-retinal with IRBP (100).

Chen *et al*. (100) reported that the concentration of DHA noncovalently bound to IRBP represented 8.6% of total bound fatty acids in bovine retina. In the same retinas, DHA accounted for 20 and 3.5% of lipids in ROS and RPE, respectively. Thus, there is a DHA concentration gradient between the ROS and RPE. On the basis of the presence of this concentration gradient and the above fluorescence studies, Chen *et al*. (100) proposed the following model for how DHA modulates the transfer of retinoids between the RPE and ROS. When IRBP is located near the RPE, it binds a saturated fatty acid, resulting in high affinity for 11-*cis* retinal at the IRBP hydrophilic binding site. When the IRBP approaches the ROS, DHA will displace the saturated fatty acid from the IRBP because of its higher affinity. The binding of DHA to IRBP will in turn cause a rapid dissociation of 11-*cis* retinal, which moves into the ROS. DHA does not affect the binding affinity of all-*trans* retinol, which moves from the ROS into the hydrophilic binding site on IRBP that was previously occupied by 11-*cis* retinal. When IRBP moves back near the RPE, the process is reversed as DHA is swapped for a saturated fatty acid and the retinoid binding site regains its affinity for 11-*cis* retinal.

There is strong evidence from a single study to suggest that DHA plays an important role in the regeneration of rhodopsin. An 80% reduction in retinal DHA level in n-3-deficient rats was associated with a significantly slower rate of rhodopsin regeneration after a 100% bleach (72). The 11-*cis* retinal necessary for forming rhodopsin is synthesized in the RPE from all-*trans* retinol. The RPE obtains all-*trans* retinol from the choroidal blood supply, and in many mammals, the RPE acts as a secondary store for all-*trans* retinol (9). In rats, the RPE is a relatively weak store for all-*trans* retinol (9), which may make rats more susceptible than other mammals to the effect of lower DHA levels on the rate of rhodopsin regeneration.

## SUMMARY AND CONCLUSIONS

Early studies in this field provided conclusive evidence that a reduction in retinal DHA level was associated with altered retinal function as assessed with the ERG. Recent studies have extended these findings to include specific alterations in photoreceptor function in n-3-deficient animals, including slower rod recovery in monkeys and reductions in the maximal response and phototransduction sensitivity in guinea pigs and rats. On the basis of the experiments in n-3–deficient animals, it has been proposed that DHA serves a number of important roles in photoreceptor function, including regulation of retinoid transport between the RPE and ROS and regulation of the $Ca^{2+}$ photocurrent through the cGMP-gated ion channels. It was also proposed that DHA provides the membrane properties that allow the phototransduction proteins to diffuse rapidly through the lipid bilayer of the disk membranes.

The alterations in phototransduction and rod recovery mechanisms in the retina of n-3-deficient animals may be applicable to similar mechanisms in other tissues with high DHA concentration. DHA is also found at high levels within the brain (83,101), which lacks the same efficient mechanism for DHA conservation present within the retina. Several of the mechanisms studied within the photoreceptor are found elsewhere in the body. For example, the phototransduction cascade has a high degree of commonality with the many other signal transduction pathways mediated by G proteins (12,102). Although the ligands may include light, neurotransmitters or hormones, once initiated, the respective G-protein cascades are remarkably similar (12,102). DHA is also found in high concentration within synapses (103,104) where calcium also plays an important role in the modulation of neurotransmitter release (105). The results from ERG experiments with n-3-deficient animals may therefore be relevant for many other neural and physiologic processes. They may also have implications for human infant nutrition despite the lack of ERG alterations beyond 6 wk of age in human infants fed a diet without DHA supplementation.

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# Randomized Trials with Polyunsaturated Fatty Acid Interventions in Preterm and Term Infants: Functional and Clinical Outcomes

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**ABSTRACT:** The role of polyunsaturated fatty acids (PUFA) in infant nutrition has now been well studied through many randomized controlled trials (RCT) that provide us with high-quality evidence, particularly in relation to efficacy. As a result of a systematic search of the literature for RCT of supplementation of formulas of term and preterm infants with long-chain polyunsaturated fatty acids (LC-PUFA), we have identified 21 studies that have physiological responses or growth as outcomes. There have been 11 RCT involving preterm infants, and many of these claim a beneficial effect on visual, neural, or developmental outcomes. There are some reports of negative effects on growth in relation to the addition of n-3 LC-PUFA to preterm formulas but not when AA is added with n-3 LC-PUFA. Small studies have shown no differences in prostanoid formation or peroxidative stress between n-3 LC-PUFA-supplemented and unsupplemented infants. There have been 10 RCT involving term infants; whereas some studies report an effect on visual/neural/developmental outcomes, an equal number report no effect. There have been no reports of negative effects of n-3 LC-PUFA on growth in term infants. In summary, there appear to be few safety concerns relating to the use of LC-PUFA in infant nutrition. The potential medium- and long-term effects of including these compounds in the early diet of infants remain to be assessed.

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Unlike the roles of many other nutrients in infant nutrition, the role of polyunsaturated fatty acids (PUFA) has been well studied through numerous randomized controlled trials (RCT) that provide us with high-quality evidence. For this reason, primary consideration in this review is given to the results of RCT because these studies limit the bias and confounding influences such as those associated with comparisons between breast-fed and formula-fed infants. The aim of this paper is to bring together all the fully published RCT testing the effects of supplementation with PUFA on growth or other physiological measures reported to date. Observational studies, comparisons of breast-fed and formula-fed infants, or controlled trials with only biochemical outcomes have not been included in this review.

A systematic literature search was undertaken using the Medline database. In most cases, the complete database was searched and the date of the last search was June 28, 2001. Search terms used included: infant food, formula or milk, infant, docosahexaenoic acid, arachidonic acid, alpha-linolenic acid, n-3 (omega-3) fatty acid, linoleic acid, n-6 (omega-6) fatty acid, growth, visual acuity, neural development, and randomized controlled trial. The names of authors who had published relevant studies were also used as search terms to identify previous or more recent work that may be appropriate. Search limits included human and English language.

To the nonexpert, reviewing this area can be confusing because many trials have been reported in more than one paper. For example, results from the very first trial with clinical outcomes involving long-chain polyunsaturated fatty acid (LC-PUFA) supplementation to preterm infants have been reported in at least five separate papers (1–5).

## TRIALS INCLUDING LC-PUFA IN PRETERM INFANTS

Our literature search identified 11 RCT reported in 22 separate papers (1–22). These trials were designed to test the efficacy and safety of varying levels of eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and arachidonic acid (AA, 20:4n-6) in the diets of preterm infants. These trials are summarized in Table 1.

All trials involved healthy preterm infants fed preterm formula, and 8 of 11 trials had a breast-fed reference group (1,11–13,15,19–21). Only one (15,16) of these trials reported adequate concealment of allocation, and 9 of 11 trials were either unclear regarding losses to follow-up or failed to follow up ≥20% of infants randomly allocated to treatment groups. Not all trials were analyzed on an intention-to-treat basis, with infants developing complications after randomization either being excluded or withdrawn from the trials. Only one study (9) attempted to specifically address the effect of n-3 LC-PUFA supplementation in preterm infants with bronchopulmonnary dysplasia (BPD), a major cause of medical complication in preterm infants.

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; BAEP, brainstem auditory-evoked potential; BPD, bronchopulmonary dysplasia; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERG, electroretinogram; LA, linoleic acid; LC-PUFA, long-chain polyunsaturated fatty acid; PUFA, polyunsaturated fatty acids; RCT, randomized controlled trials; VEP, visual-evoked potential.

**TABLE 1**
**Intervention Trials Involving Supplementation with Long-Chain Polyunsaturated Fatty Acids (LCPUFA) from a Variety of Sources in Preterm Infants[a]**

| Trial results reported by: | Age and characteristics at start of study | Type of intervention | How were the groups allocated? | Number of infants enrolled per group | Blinded outcome assessment (yes/no) | Outcome measures and results | Percentage of dropouts (loss to follow-up) | Other comments | Power calculation (yes/no) |
|---|---|---|---|---|---|---|---|---|---|
| Birch *et al.* (1,2), Uauy *et al.* (3), Uauy *et al.* (4), Hoffman and Uauy (5), plus other not listed | Healthy preterm infants | FC1 = LA/ALA, 24:0.5; FC2 = LA/ALA 21:2.7; FFO = LA/ALA, 20:1.4 + FO (0.65% EPA, 0.35% DHA, 0.1% AA); BM. | Random, no details regarding assignment. | 83 enrolled; initial distribution among groups unclear. | Yes | SS VEP acuity and visual acuity cards at 36 and 57 wk PMA: BM and FFO better than FC1; FC2 had intermediate value.<br>Rod ERG at 36 wk PMA:<br>Rod threshold: BM, FFO shorter than FC1; FC2 not diff. from other groups.<br>$V_{max}$: BM; FFO higher than FC1; FC2 not diff. from other groups.<br>Log *K*: BM, FFO shorter than FC1; FC2 not diff. from other groups.<br>Rod ERG at 57 wk: Not diff. among groups for any parameters.<br>Cone ERG: At 36 and 57 wk PMA not diff. among groups.<br>Oscillatory potentials: At 36 wk PMA, not detectable. At 57 wk PMA, dark adapted: not diff. among groups; light adapted: generally implicit times of BM shorter than FC1.<br>Growth: No effect of diet.<br>Bleeding times: All within normal range but FFO longer compared with FC1.<br>Vitamin A and E: No diff. with diet.<br>RBC membrane fluidity: No diff. with diet. | 16% at 40 wk PMA<br>37% at 57 wk PMA | When insufficient breast milk available for BM groups, infants were fed FFO. | Yes for some outcomes |
| Carlson *et al.* (6,7, 22), Werkman and Carlson (8) | Healthy preterm infants exclusively fed formula | FC = LA/ALA, 19:3 up to 1800 g, then LA/ALA; 33:4.8; FFO = FC + FO (0.36% EPA, 0.2% DHA). | Random, no details regarding assignment. | 79 enrolled; unclear distribution between groups at assignment; completed; FC = 34, FFO = 33. | Partial | Visual acuity cards: At 2 and 4 mon CA, FFO better than FC; no diff. between groups at 6.5, 9, and 12 mon CA.<br>Growth (z-scores for weight, length, head circumference): FFO infants lower z-scores at 2, 4, 6.5, 9, 12 mon CA than FC.<br>Fagan test: No diff. in novelty preference at 6.5, 9, and 12 mon CA; FFO compared with FC infants had more and shorter duration looks.<br>Bayley's MDI and PDI: No diff. at 12 mon CA. | Unclear, 10/79 infants replaced post-randomization | 3 of the 67 infants were diagnosed with BPD; size at term was interpolated from 38 and 48 wk PMA data. | Yes |
| Carlson *et al.* (9, 22), Carlson and Werkman (10) | Preterm infants exclusively fed formula | FC = LA/ALA, 21:2.4; FFO = FC + FO (0.06% EPA, 0.2% DHA); these formulas fed to 2 mon CA and then both groups fed LA/ALA = 34:5. | Random, no details regarding assignment. | 94 enrolled; 35 excluded or lost to follow-up; completed to 2 mon CA: FC = 33, FFO = 26. | Yes | Visual acuity cards: at 2 mon CA, FFO better than FC; interaction between BPD and diet. At 2 mon, infants without BPD and fed FFO had better acuity than all other infants.<br>No other acuity differences due to diet at other ages.<br>Growth: Compared with FC, FFO had lower weight z-score at 6 and 9 mon CA, smaller head circumference z-score at 9 mon CA and lower weight-for-length at 2, 6, 9, and 12 mon CA.<br>Fagan test (subgroup): FFO compared with FC had more and shorter duration looks at 12 mon CA; | Unclear, infants replaced post-randomization | 15 BPD in FC and 8 BPD in FFO; more FC infants than FFO infants lost to follow-up. | Yes |

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| | | | | | | no diff. between groups on novelty preference at 12 mon CA. Bayley's MDI: FFO > FC at 12 mon CA. Bayley's PDI: No diff. between groups at 12 mon CA. | | | |
| Faldella *et al.* (11) | Healthy preterm infants fed formula or breast milk | FC = LA/ALA, 19:0.9; FEPL = LA/ALA, 11:0.7 + 0.3% DHA, 0.05% EPA, 0.44% AA; BM (fed FEPL when necessary). | Random, method of assignment not stated. | FC = 26, FEPL = 23, BM = 17. | Yes | Flash VEP latency for $N_4$ and $P_4$: BM FEPL shorter than FC at 52 wk PMA. BAEP: No diff. between groups. Flash ERG: No diff. between groups. Growth: No diff. between groups. | FC = 4%, FEPL = 10%, BM = 30% | ERG methdology not according to International Standards. Formula-fed infants received <25% intake from BM. | No |
| Bougle *et al.* (12) | Healthy preterm infants fed formula or breast milk | FC = LA/ALA, 14:1.3; FFO = LA/ALA, 18:1.2 + 0.1% EPA, 0.6% DHA, 0.1% AA; BM. All diets fed for 30 d. | Random, double blind, no other details. | FC = 11, FFO = 14, BM = 15. | Yes | VEP latency: No diff. between groups 30 d post-treatment. BAEP: No diff. between groups 30 d post-treatment. Nerve conduction: FFO slower than BM; FC intermediate. Growth: Weight gain greater in FFO than BM or FC. | FC = 18%, FFO = 7%, BM = 27% | Mean weight not diff. between groups. Dietary intake of infants in FFO group > intake of FC and BM groups. | No |
| Clandinin *et al.* (13,14) | Healthy preterm infants fed formula or breast milk | FC = LA/ALA, 13:1.4; FAF1 = FC + 0.24% DHA, 0.32% AA; FAF2 = FC + 0.35% DHA, 0.49 AA; FAF3 = FC + 0.76% DHA, 1.1% AA; BM. All diets fed for 6 wk. | Controlled clinical trial; details of allocation not clear. | FC = 22, FAF1 = 22, FAF2 = 22, FAF3 = 18, BM = 33. | Not stated | Growth: Weight gain and length. gain over the 6-wk study period not diff. between formula groups; some differences noted between breast-fed and formula-fed infants. | FC = 18%, FAF1 = 18%, FAF2 = 18%, FAF3 = 33%, BM = 24% | Comparison of growth: No adjustment for baseline variables. | No |
| Vanderhoof *et al.* (15,16); Field *et al.* (17) | Healthy preterm infants fed formula or breast milk | FC = LA/ALA, 13:1.4; FAF = LA/ALA, 12:1.5 + 0.35% DHA, 0.50% AA; BM. All diets fed to 48 wk PMA. | Formula groups assigned by "a central computerized system of automatic randomization." Stratified by birth weight. Breast-fed reference group was not randomized. | FC = 78, FAF = 77, BM = 33. Subgroup tested for immune outcomes: FC = 12, FAF = 15, BM = 17. | Yes (formula groups only) | Growth: No diff. in weight, length, head circumference or mid-arm circumference between formula groups at full feeds 40, 48, and 92 wk PMA; some differences noted between breast- and formula-fed infants at all ages. Subgroup tested for immune markers: At 42 d, total T cells T-CD4, and CD4/CD8 ratio in BM and FSCO diff. in FC. Mononuclear cell phenotypes: More mature in BM and FSCO compared with FC. | Until 48 wk PMA; FC = 36%, FSCO = 38%, BM = 60%; until 92 wk unclear. | Many withdrawn and excluded infants to ensure healthy study groups. Small sample sizes for immune outcomes. | Yes, for growth at 40 wk PMA only |
| Ryan *et al.* (18) | Healthy low-birth-weight infants (750–2250 g) exclusively fed formula prior to | FC = LA/ALA, 17:2.5 to 43 wk PMA, then LA/ALA, 22.4:2.4 to 59 wk PMA; | Blinded random assignment; each study center had | FC = 45, FFO = 45. | Yes | Growth: Between enrollment and 59 wk PMA, males fed FFO had lower weight, shorter lengths, and smaller head circumferences than FC; no diff. in female infants. | FC = 27%, FFO = 33% | Many withdrawn and excluded infants. 4 SIDS deaths | Yes, for weight gain |

*(Continued p. 876)*

**TABLE 1**
**(Continued)**

| Trial results reported by: | Age and characteristics at start of study | Type of intervention | How were the groups allocated? | Number of infants enrolled per group | Blinded outcome assessment (yes/no) | Outcome measures and results | Percentage of dropouts (loss to follow-up) | Other comments | Power calculation (yes/no) |
|---|---|---|---|---|---|---|---|---|---|
| | enrollment; enrollment was 7–10 d before discharge | FFO = LA/ALA, 16:2.3 + 0.2% DHA, 0.04% EPA to 43 wk PMA, then LA/ALA 21:2.0 + 0.2% DHA, 0.07% EPA to 59 wk PMA. | own randomization schedule; all schedules were prepared in a central location. | | | Male infants in FFO drank less formula than male infants in FC. | | in the FFO group and 0 deaths in the FC group. Investigators and ethics review considered each death unrelated to formula assignment. | |
| Diersen-Schade *et al.* (19) | Healthy very-low-birth-weight infants (846–1560 g) exclusively fed formula; healthy term infants breast-fed for at least 4 mon (85% of nutrition from breast milk) | FC = LA/ALA, 22:3.1; FA = FC + 0.34% DHA; FAF = FC + 0.33% DHA, 0.60% AA. All formulas fed for at least 28 d and then graded onto standard, non-LC-PUFA term formula to 57 wk PMA. BM (term infants). | Randomized study; no details of allocation procedures. | 194 enrolled; unclear about group distributions at enrollment. At 40 wk PMA, FC = 52, FA = 54, FAF = 59, BM = 90. | Yes | Weight: No diff. between formula groups at 40 and 57 wk PMA, but at 48 wk PMA FAF > FA, FC. Length: FAF > FA at 40 and 48 wk PMA but not at 57 wk PMA; no diff. between FC and FAF. Head circumference: No diff. between formula groups at any time. Weight and length of BM group generally greater than all preterm groups. | Unclear over whole study duration; study formula phase, 9% | Number of infants in study groups at each assessment not stated. | Stated for weight, but no details given |
| Stier *et al.* (20) | Healthy preterm infants (<2000 g) fed formula or breast milk | FC = LA/ALA, 19:0.9; FEPL = LA/ALA, 12:0.6 + 0.2% DHA, 0.35% AA; BM. All diets fed for a 3-wk period while infants in hospitals. | Randomized study; no details of allocation procedure given. | FC = 10, FEPL = 10, BM = 10. | Yes | Urinary prostanoid excretion: No diff. between groups at end of diet treatment. Growth: No diff. between groups at end of diet treatment. | Unclear | Small sample sizes. | No |
| Jacobs *et al.* (21) | Healthy preterm infants (30–35 wk gestation) fed formula or breast milk | FC = LA/ALA, 17:1.6; FFO = LA/ALA, 11:1.8 + 0.5% DHA, 0.5% EPA: BM. Diets fed for 6 wk. | Randomly assigned for formula groups; no other details regarding allocation. | Total enrolled unclear. Included in the analysis: FC = 15, FFO = 13, BM = 7. | Yes for formula groups | Growth: Gains in weight, length, and head circumference not diff. between FC and FFO. Weight and head circumference gains were greater in formula-fed infants than BM group. No. diff. between groups for plasma vitamin E and C levels, plasma TBARS, or reduced/oxidized glutathione ratio. FFO group had reduced susceptibility of red blood cells to $H_2O_2$ oxidative stress compared with FC and BM groups. | Unclear | Small sample sizes. | No |

[a]Abbreviations: BM, breast milk fed; FC, control (standard) infant formula; FFO, formula supplemented with fish oil; FEPL, formula supplemented with egg phospholipid; FO, fish oil; FA, formula supplemented with algal oil; FAF, formula supplemented with algal and fungal oils; FSCO, formula supplemented with single-cell oils; ALA, α-linolenic acid; LA, linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; BAEP, brainstem auditory-evoked potential; ERG, electroretinogram; BPD, bronchopulmonary dysplasia; VEP, visual-evoked potential; SS, steady-state; TBARS, thiobarbituric acid-reactive substances; CA, corrected age; PMA, postmenstrual age; MDI, Mental Developmental Index; PDI, Psychomotor Developmental Index; RBC, red blood cell; d, days; mon, months; wk, weeks.

Up until 1996, fish oil of the MaxEPA type containing more EPA than DHA (15 vs. 11% of total fatty acids) was used as a source of DHA, whereas in breast milk the proportion of EPA rarely exceeds that of DHA. EPA is the precursor to 3-series eicosanoids which may suppress some effects of the 2-series eicosanoids originating from AA. Some studies (9,18) have avoided the addition of excessive EPA by using fish oils high in DHA, such as tuna oil. Other studies used other sources of LC-PUFA such as fractions of egg phospholipids (11,20) or fungal and algal oil (15,16,19). It is doubtful whether the source of LC-PUFA has much effect on LC-PUFA status of the infant (23).

Most workers investigating the potential benefits of LC-PUFA supplementation of preterm infants have assessed infant sensory modalities. Most trials have assessed aspects of visual function, including the sensitivity of the retina to light (2,11), visual-evoked potential (VEP) latency (11,12), or visual acuity measured by VEP or tested by behavioral means (cards) (1,6,9). More complex visual behaviors such as novelty preference and infant looking behavior to paired comparisons have also been assessed (8,10). Other efficacy assessments have included brainstem auditory-evoked potential (BAEP) (11,12), nerve conduction studies (12), and the Bayley Scales of Infant Development (22). In most trials, infant growth has been employed as a measure of safety, although some trials have used other surrogate markers of safety such as vitamin A and E levels (4), bleeding times (4), prostanoid formation (20), and measures of oxidative stress (4,5,21).

Benefits of adding n-3 LC-PUFA to preterm infant formulas (with or without AA) on electroretinographic responses and VEP acuity have been reported in one study (1,2). Benefits have also been reported on visual acuity assessed *via* cards (Teller) in all trials that used this outcome (1,6,9). One study (11) detected a benefit of LC-PUFA supplementation on VEP (flash) latency, whereas another study (12) showed no benefit of LC-PUFA supplementation on VEP (transient) latency. Visual recognition memory (novelty preference) and visual attention (number and duration of discrete looks) were determined with the Fagan Test of Infant Intelligence by two trials (8,10) from one center, which reported that the DHA-supplemented group compared with the control group had more and shorter-duration looks in comparisons of familiar and novel stimuli.

Studies investigating other sensory modalities have reported no effect of dietary LC-PUFA on BAEP (11,12). The only potential negative finding relating to LC-PUFA–supplementation of preterm infants has come from somatosensory assessments where preterm infants fed n-3 LC-PUFA–supplemented formula had slower peripheral nerve conduction than infants in the human milk group (12).

The immune consequences of adding AA and DHA to preterm infant formula have been reported in a small subgroup of one trial (17), which found that adding AA and DHA to a preterm infant formula resulted in lymphocyte populations, cytokine production, and antigen maturity that were more consistent with those in human milk-fed infants. This may affect the ability of the infant to respond to immune challenges.

Two initial trials (7,9) and one more recent study (18) that supplemented infant formula with fish oil suggested negative effects on growth, although other studies (4,12,21) reported no effect of fish oil supplementation on growth. The possibility that fish oil treatment had negative effects on growth through a lowering in infant AA status remains a significant cause for concern, although all the studies assessing fish oil supplementation and growth of preterm infants had flaws that could have influenced growth outcomes. Nevertheless, most recent trials have attempted to add AA with n-3 LC-PUFA to avoid the decline in plasma and erythrocyte AA caused by fish oil supplementation. The five trials (11,13,15,19,20) that added both n-3 and n-6 LC-PUFA reported no adverse effects on growth. In fact, one of these five trials claimed benefits of both AA and DHA supplementation on growth, although shortcomings in the design and analysis may have contributed to these findings.

Stier *et al.* reported that neither conventional formula nor supplementation of a preterm formula with LC-PUFA for a period of 3 wk substantially influenced prostanoid formation in healthy infants (20). In addition, other trials found that supplementation of n-3 LC-PUFA had no effect on vitamin A, E, or C levels, plasma thiobarbituric acid-reactive substances (TBARS), and reduced/oxidized glutathione ratio but found reduced susceptibility of erythrocyte membranes to $H_2O_2$ treatment (4,5,21). All three trials had relatively small sample sizes and thus had limited power (4,5,20,21).

Although there are still some concerns on safety issues regarding the addition of LC-PUFA to preterm infant formula, the evidence in support of a beneficial effect of such supplementation on visual function is relatively compelling. Moreover, it seems that the possible negative effects of n-3 LC-PUFA on growth of preterm infants have been overcome through improved study design and/or the addition of a balance of n-6 and n-3 LC-PUFA.

## TRIALS INVOLVING LC-PUFA SUPPLEMENTATION OF TERM INFANTS

Our literature search identified 10 RCT (24–38) that were designed to test the efficacy and safety of adding either n-3 LC-PUFA or a combination of n-3 LC-PUFA and AA to formulas for term infants. These trials are summarized in Table 2.

All trials involved healthy term infants fed formulas from near birth, and all but one had a breast-fed reference group (Table 2). Most trials appeared to have adequate randomization and masking procedures, and most presented power calculations for their primary outcome measurements. Five of 10 trials reported less than 20% loss to follow-up among the randomly allocated formula-fed infants.

VEP acuity was an outcome measure in five studies, while acuity was tested through the use of cards in three studies. Various standardized and unstandardized developmental tests were used as outcome measures in six studies. Growth was assessed in most trials.

There have been no negative findings in relation to growth in term infants regarding LC-PUFA supplementation of in-

**TABLE 2**
**Intervention Trials Involving Supplementation with LC-PUFA from a Variety of Sources in Term Infants[a]**

| Trial results reported by: | Age and characteristics at start of study | Type of intervention | How were the groups allocated? | Number of infants enrolled per group | Blinded outcome assessment (yes/no) | Outcome measures and results | Percentage of dropouts (loss to follow-up) | Other comments | Power calculation (yes/no) |
|---|---|---|---|---|---|---|---|---|---|
| Makrides *et al.* (24) | Healthy term infants exclusively fed formula or breast milk | FC = LA/ALA, 17:1.6; FFO + EPO = LA/ALA, 17:1.5 + 0.58% EPA, 0.36% DHA, 0.27% GLA; BM. Diets fed from <5 d to 30 wk. | Random; method not stated. | 89 enrolled; completed: FC = 19, FFO + EPO = 13, BM = 23, partial BM = 24 | Yes | VEP acuity: FFO + EPO, BM better than FC at 16 and 30 wk.<br>Growth: No effect of diet. | Overall dropout = 11% | Uneven randomized groups due to unavailability of test formula | Yes |
| Auestad *et al.* (25); Scott *et al.* (26) | Healthy term infants exclusively fed formula or breast milk | FC = LA/ALA, 22:2.2; FEPL = LA/ALA, 22:1.9 + 0.12% DHA, 0.43% AA; FFO = LA/ALA, 21:1.9 + 0.23% DHA, 0.07% EPA; BM ≥ mon. Diets fed from <7 d to 12 mon. | Central preparation of randomization schedules; separate randomization at each of 3 centers; details of allocation not stated. | FC = 65, FEPL = 68, FFO = 65, BM = 76 | Yes | Sweep VEP and acuity cards: No diff. with diet at 2, 4, 9, and 12 mon.<br>Growth: No diff. with diet at 2, 4, 6, 9, and 12 mon.<br>Bayley's MDI and PDI: No effect of diet at 12 mon.<br>McArthur CDI: In one sub-scale, FFO < FC, FEPL, and BM at 14 mon. | FC = 31%, FEPL = 32%, FFO = 34%, BM = 17% | Each of the 3 sites had different visual acuity protocols | No |
| Carlson *et al.* (27) | Healthy term infants exclusively fed formulas or breast milk | FC = LA/ALA, 22:2.2; FEPL = LA/ALA, 22:2.0 + 0.1% DHA, 0.43% AA; BM ≥ 3 mon. | Random, double-blind study; details of allocation not stated. | FC = 31, FEPL = 28, BM = 35 | Yes | Card visual acuity: BM, FEPL groups better than FC at 2 mon but no diff. between groups at 4, 6, 9, and 12 mon. | FC = 35%, FEPL = 32%, BM = 46% | Well-balanced groups; well-designed study | Yes for card acuity |
| Agostoni *et al.* (28, 29) | Healthy term infants exclusively fed formula or breast milk | FC = LA/ALA, 11:0.7; FEPL + EPO = FC + 0.3% DHA, 0.44% AA, 0.05% EPA, 0.3% GLA; BM. Diets fed from 3 d to 4 mon. | Random—no details on method of allocation. | FC = 31, FEPL + EPO = 29, BM = 30 | Yes | Brunet–Lezine DQ: FEPL + EPO and BM groups higher scores at 4 mon. No diff. between groups at 24 mon. | At 4 mon: No losses to follow-up.<br>At 24 mon: FC = 3%, FEPL + EPO = 10%, BM = 17% | Well-balanced groups; well-designed study | Yes for DQ at 4 mon |
| Makrides *et al.* (30,31) | Healthy term infants fed formula or breast milk | FC = LA/ALA, 17:1.5; FFO = LA/ALA, 17:1.2 + 0.35% DHA, 0.1% EPA; FEPL = LA/ALA, 17:1.0 + 0.34% DHA, 0.34% AA; BM. Diets fed from <7 d to 12 mon. | Randomization and allocation adequately concealed. | FC = 28, FFO = 27, FEPL = 28, BM = 63 | Yes | VEP acuity: No diff. in formula groups at 16 and 34 wk; BM better than formula groups at 34 wk.<br>Bayley's MDI and PDI: No diff. between formula groups at 1 or 2 yr; BM better at 2 yr.<br>Growth: No diff. between formula groups in weight, length, or head circumference at 6, 16, and 34 wk, 1, 2 yr. BM group had lower weight and length at 34 wk and 1 yr than formula groups. | FC = 25% FFO = 15%, FEPL = 14%, BM = 27% | Well-designed study | Yes for VEP acuity and growth |

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|---|---|---|---|---|---|---|---|---|---|
| Lucas *et al.* (32) | Healthy term infants fed formula or breast milk from birth | FC = LA/ALA, 12:1.1; FEPL = LA/ALA, 16:1.4 + 0.32% DHA, 0.30% AA for 6 mon, then formula of choice; BM ≥ 6 wk. | Random permuted block design stratified by site and gender; concealed assignment. | FC = 155; FEPL = 154, BM = 138 | Yes | Bayley's MDI and PDI: No effect of diet at 18 mon. Language, motor, and social scales: No effect of diet at 9 mon. Growth: No diff. between formula groups. Infection and atopy to 9 mon: No diff. between formula groups. | FC = 19%, FEPL = 19%, BM = 25% | | Yes for Bayley's MDI and PDI |
| Jorgenensen *et al.* (33) | Healthy term infants, fully formula feeding at 1 mon, 2 BM groups, one cross-sectional and one prospective | FC = LA/ALA, 12:1.2; FFO = FC + FO, 0.32% DHA, 0.39% EPA, 0.60% AA; FFO + BO = LA/ALA, 13:1.2 + 0.32% DHA, 0.37% EPA, 0.06% AA, 0.54% GLA; BM (2 groups). | Random and masked assignment. | FC = 11, FFO = 12, FFO + BO = 14, BM1 = 17 (prospective for growth), BM2 = 25 (cross-sectional for sweep VEP reference) | Yes | Sweep VEP acuity: No diff. between formula groups at 4 mon, BM2 better acuity than FC group. Growth: No diff. in weight length, or head circumference between groups at 1, 2, and 4 mon. | Unclear | Successful VEP tests: 18/26 (combined FFO and FFO + BO) and 8 (FC) | Yes for sweep VEP acuity; did not reach estimated sample size in all groups |
| Willatts *et al.* (34, 35) | Healthy term infants exclusively fed formula from birth | FC = LA/ALA 11:0.7; FEPL = LA/ALA, 12:0.63 + 0.2% DHA, 0.35% AA. Diets fed to 4 mon. | Randomized using computer-generated schedule; no details about concealment at allocation. | FC = 38, FEPL = 34; some inconsistency between the two reports | Yes | Means–end problem solving: No diff. between groups at 9 mon; more solutions in FEPL group compared with FC at 10 mon. Growth: No diff. in weight length, head circumference, skinfolds at 3 mon. | Unclear as inconsistent between the two reports | Developmental test unvalidated | Yes for problem-solving task |
| Birch *et al.* (36,37) | Healthy term infants fed formula or breast milk from birth | FC = LA/ALA, 15:1.5; FA = FC + 0.35% DHA; FAF = FC + 0.36% DHA, 0.72% AA; BM. Diets fed from <5 d to 17 wk. | Randomized from single center; masked assignment. | FC = 26, FA = 26, FAF = 27, BM = 29 | Yes | Sweep VEP acuity: FA, FAF, BM better scores at 6, 17, and 52 wk but not 26 wk compared with FC. FPL acuity: No effect of diet. Growth: No effect of diet. Bayley's test: FAF better MDI than FC at 18 mon; FA had intermediate value; no effect of diet on PDI. | At 52 wk: FC = 19%, FA = 23%, FAF = 29%, BM = 31% | Small sample size for MDI and PDI, but small SD allowed a result | Stated for VEP and card acuity and MDI |
| Gibson *et al.* (38) | Healthy term infants exclusively fed formulas or breast milk from birth | FC = LA/ALA, 18:1.4; FAF1 = FC + 0.2% DHA, 0.2% AA; FAF2 = FC + 0.2% DHA, 0.32% AA; FAF3 = FC + 0.25% DHA, 0.4% AA; BM. All diets fed for 6 wk. | Randomization and allocation were concealed; allocation from central location. | FC = 29, FAF1 = 31, FAF2 = 29, FAF3 = 29, BM = 40 | Yes | Growth: No diff. between groups at 6 wk. | FC = 24%, FAF1 = 32%, FAF2 = 24%. FAF3 = 28%, BM = 35% | | Yes for plasma DHA and AA |

[a]Abbreviations: BO, borage oil; EPO, evening primrose oil; GLA, γ-linolenic acid; DQ, Developmental Quotient; CDI, Communicative Developmental Inventory; FPL, Forced Preferential Looking. See Table 1 for other abbreviations.

fant formulas. This is despite the fact that four trials have supplemented formulas with DHA alone (tuna oil or MaxEPA) without added AA, resulting in the AA, status of infants being depleted (24,25,30,33). It should also be noted that, in most of these trials, infants were fed the test formulas for one year. Thus, there is little evidence for n-3 LC-PUFA supplementation of term infant formulas causing perturbations in growth. The safety of LC-PUFA–supplemented formulas for term infants has been further highlighted by the trial of Lucas *et al*., who reported similar rates of infection and atopy in infants fed either standard or LC-PUFA–supplemented formulas (32).

Benefits of adding DHA to formulas (with or without AA) on VEP acuity have been reported in some studies (24,36), whereas other studies have failed to detect a benefit of LC-PUFA supplementation (25,31,33). Benefits have also been reported on visual acuity assessed *via* cards (Teller) in one study (27) but not in others (25,36). There is also mixed evidence for the support of an effect of dietary LC-PUFA on more global measures of development (Bayley's Scales of Infant Development or Brunet–Lezine test). Agostoni *et al.* (28) and Birch *et al.* (37) both reported benefits of dietary LC-PUFA, as did Willatts *et al.* (35) using an unstandardized test of infant problem-solving ability. However, the larger studies conducted by Auestad *et al.* (25), Lucas *et al.* (32), and Makrides *et al.* (31) all failed to detect effects of LC-PUFA supplementation on Bayley's Scales of Infant Development. Possible interpretations of these data include a small individual effect (type 2 error) or that only a proportion of infants will benefit or the presence of confounding variables. Further studies are needed to elucidate this issue.

## TRIALS INVOLVING LINOLEIC ACID AND α-LINOLENIC ACID IN TERM AND PRETERM INFANTS

Our literature search identified three RCT involving term infants (39–41) and one involving preterm infants (1–5) designed to test the efficacy and safety of varying levels of linoleic acid (LA) and α-linolenic acid (ALA) and thus varying LA/ALA ratios. The trials with term infants are summarized in Table 3; the trial involving preterm infants is included in Table 1 because this study involved both PUFA and LC-PUFA interventions.

All three RCT involved healthy term infants fed infant formulas from near birth and had a breast-fed reference group (Table 3). Two trials appeared to have adequate randomization and masking procedures, and both presented power calculations for their primary outcome measurement (40,41). The study by Ponder *et al*. (39) did not state the method of randomization or power calculations. Makrides *et al.* (40) attempted to test the benefit of increasing ALA intake (or lowering the LA/ALA ratio) by measuring VEP acuity, whereas Jensen *et al.* (41) used VEP latency, which cannot yield an acuity determination. All studies measured growth using standard procedures.

The level of essential fatty acids tested in term infants ranged from 11 to 34% LA and 0.4 to 4.8% ALA (note that all fatty acid data are expressed as percent total fats) with LA/ALA ratios varying from 5:1 to 44:1. The data indicate that LA/ALA ratios as low as 5:1 and as high as 44:1 have no clinically relevant effect on growth. Although Jensen *et al.* (41) reported a lower weight in infants fed a 5:1 ratio formula compared with higher LA:ALA ratio formulas at 120 d of age, this group had excessive losses to follow-up at this age that prevented adequate interpretation. Thus, there appear to be few safety concerns in regard to the addition of oils rich in LA or ALA to infant formulas.

Whereas infants fed formulas with the lowest LA/ALA ratios (5:1) have higher plasma DHA levels than those fed formulas with higher LA/ALA ratios (10:1 and above), no study has demonnstrated a clear clinical benefit of one LA/ALA ratio over another with respect to effects on either VEP acuity, latency, or amplitude. No LA/ALA ratio raises the plasma or erythrocyte level of DHA to that of breast-fed infants.

Claims have been made by Heird *et al.* (42) that beneficial effects of supplementing formulas with LC-PUFA have only been seen when the level of ALA is less than 2% of total fats. The underlying hypothesis is that ALA levels of <2% total fatty acids do not allow adequate synthesis of n-3 LC-PUFA, and hence infants fed such formulas are more likely to benefit from dietary LC-PUFA. However, this hypothesis cannot be entirely substantiated. For example, the reference formula used in the large study by Lucas *et al.* (32) contained 1.1% ALA, and they reported no effect of LC-PUFA supplementation on Bayley's Developmental Indices at 18 months or on growth, infection, or atopy rates. Justification of an exact level of ALA in the dietary fats of term infants must await further studies. Although breast milk contains about 1% ALA, it must be remembered that breast milk also contains n-3 LC-PUFA.

There has been only one preterm trial (1–5) designed to test the efficacy and safety of varying levels of LA and ALA. The levels of essential fatty acids in different groups were 24% LA and 0.5% ALA in one group and 21% LA and 2.7% ALA in the other (Table 1). The ratios of LA/ALA were 48:1 and 8:1, respectively. These investigators reported that LA/ALA levels as low as 8:1 and as high as 48:1 have no effect on growth. The preterm infants fed 8:1 ratio formula had levels of visual acuity and electroretinogram (ERG) responses intermediate between those of infants fed a 48:1 ratio formula and those of infants fed a 14:1 ratio formula supplemented with n-3 LC-PUFA. This preterm infant trial (1–5) did not adequately report the randomization and allocation procedure, although investigators and participants were blinded to the treatment group. Furthermore, there were significant losses to follow-up (37%) by the time infants were 57 wk postmenstral age (PMA). Although an important trial, the quality of reporting does not allow firm conclusions to be drawn regarding LA/ALA ratios in infants formulas and their effects on preterm infants.

The strategy of improving the PUFA blends of infant formula by adding LC-PUFA is probably the preferential method to meet the requirement of n-3 fatty acids for preterm infants.

## SUMMARY AND CONCLUSIONS

*Preterm infants.* Four of the five trials assessing retinal sensitivity and visual acuity in response to dietary LC-PUFA

**TABLE 3**
**Intervention Trials of Changes in the Balance of n-6 and n-3 Essential Fatty Acids, Linolenic Acid, and α-Linolenic Acid in Term Infants[a]**

| Trial results reported by: | Age and characteristics at start of study | Type of intervention | How were the groups allocated? | Number of infants enrolled per group | Blinded outcome assessment (yes/no) | Outcome measures and results | Percentage of dropouts (loss to follow-up) | Other comments | Power calculation (yes/no) |
|---|---|---|---|---|---|---|---|---|---|
| Ponder *et al.* (39) | Healthy term infants exclusively fed formula or breast milk | F7:1 = LA/ALA, 34:4.8; F39:1 = LA/ALA, 31:0.8; BM. Diets fed from 3 d to 8 wk. | Randomly assigned; no details regarding allocation | Not stated; number completed per group: F7:1 = 11, F39:1 = 14, BM = 18 | Not stated | Growth: No diff. between groups at 4 and 8 wk. | Not reported | | No |
| Makrides *et al.* (40) | Healthy term infants fed formula or breast milk | F10:1 = LA/ALA, 17:1.7; F5:1 = LA/ALA, 17:3.3; BM. Diets fed from <5 d to 34 wk. | Randomization and allocation adequately concealed | F10:1 = 36, F5:1 = 37, BM = 103 | Yes | VEP acuity: No diff. between dietary groups at 16 and 34 wk. Growth: No diff. between formula groups at 6, 16, 34 wk, after adjustment for birth size; BF infants less weight and length gain at 16 and 34 wk than formula groups. | F10:1 = 17%, F5:1 = 24%, BM = 17% | Well-designed study. Birth weight and birth length of F5:1 group was greater than F10:1 group. | Yes |
| Jensen *et al.* (41) | Healthy term infants fed formula or breast milk from birth | F44:1 = LA/ALA, 18:0.4; F18:1 = LA/ALA, 17:0.95; F10:1 = LA/ALA, 17:1.7; F5:1 = LA/ALA, 16:3.2; BM. Diets fed from 1 to 120 d. | Random and masked assignment | F44:1 = 20, F18:1 = 20, F10:1 = 20, F5:1 = 20, BM = 19 | Yes | VEP latency and amplitude: No diff. between groups at 120 and 240 d. Growth: Weight of F5:1 group less than other formula groups at 120 d; no other diff. in weight; no diff. in length, head circumference, and skinfolds at 21, 60, 120, and 240 d. | At 120 d: F44:1 = 15%, F18:1 = 15%, F10:1 = 20%, F5:1 = 35%. At 240 d: F44:1 = 35%, F18:1 = 15%, F10:1 = 25%, F5:1 = 40% | Difficult to assess growth effects due to small numbers in F5:1 group. At 120 and 240 d, numbers were less than estimated sample size. | Yes for growth, VEP latency, and amplitude |

[a]See Table 1 for abbreviations.

supplementation of preterm infants reported a measurable benefit, whereas the fifth reported no effect.

Two trials also reported a measurable benefit in visual attention in n-3 LC-PUFA–supplemented infants compared with controls, while a subgroup of infants from one trial showed more mature immune markers compared with unsupplemented infants.

There are some reports of negative effects on growth in relation to the addition of n-3 LC-PUFA to preterm formulas but not when AA is added with n-3 LC-PUFA. Small studies have shown no differences in prostanoid formation or peroxidative stress between n-3 LC-PUFA–supplemented and unsupplemented infants.

*Term infants.* There appear to be few safety issues regarding the addition of LC-PUFA to term formulas, but there is controversy about their efficacy.

Five of nine trials with at least one efficacy outcome reported a measurable benefit in either visual or global development as a result of adding LC-PUFA, while four trials reported no effect in either visual or global development.

While a proof of essentiality may be lacking, there is some evidence of benefit of LC-PUFA to term infants. There is no evidence from RCT that AA must be added along with DHA in term infant formula. The fact that AA and DHA are in a balance of about 2:1 in the breast milk of most Western countries is due to the nature of the Western diets rather than a reflection of any requirement of the infant. We note that breast milk of fish-eating communities (such as most Asian countries) has AA/DHA ratios of 1:1 or lower.

*LA/ALA ratio.* While there appear to be few functional benefits of one LA/ALA ratio over another, there also appear to be few safety issues in relation to the LA/ALA ratio.

While infants fed low LA/ALA ratios have higher DHA levels, no LA/ALA ratio raises the level of DHA to that of breast-fed infants.

Justification of an exact level of ALA in the dietary fats of term infants and preterm infants must await further studies.

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# Essential Fatty Acids in Visual and Brain Development

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**ABSTRACT:** Essential fatty acids are structural components of all tissues and are indispensable for cell membrane synthesis; the brain, retina and other neural tissues are particularly rich in long-chain polyunsaturated fatty acids (LC-PUFA). These fatty acids serve as specific precursors for eicosanoids, which regulate numerous cell and organ functions. Recent human studies support the essential nature of n-3 fatty acids in addition to the well-established role of n-6 essential fatty acids in humans, particularly in early life. The main findings are that light sensitivity of retinal rod photoreceptors is significantly reduced in newborns with n-3 fatty acid deficiency, and that docosahexaenoic acid (DHA) significantly enhances visual acuity maturation and cognitive functions. DHA is a conditionally essential nutrient for adequate neurodevelopment in humans. Comprehensive clinical studies have shown that dietary supplementation with marine oil or single-cell oil sources of LC-PUFA results in increased blood levels of DHA and arachidonic acid, as well as an associated improvement in visual function in formula-fed infants matching that of human breast-fed infants. The effect is mediated not only by the known effects on membrane biophysical properties, neurotransmitter content, and the corresponding electrophysiological correlates but also by a modulating gene expression of the developing retina and brain. Intracellular fatty acids or their metabolites regulate transcriptional activation of gene expression during adipocyte differentiation and retinal and nervous system development. Regulation of gene expression by LC-PUFA occurs at the transcriptional level and may be mediated by nuclear transcription factors activated by fatty acids. These nuclear receptors are part of the family of steroid hormone receptors. DHA also has significant effects on photoreceptor membranes and neurotransmitters involved in the signal transduction process; rhodopsin activation, rod and cone development, neuronal dendritic connectivity, and functional maturation of the central nervous system.

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Studies over the past five decades have evaluated the effects of nutrition on central nervous system (CNS) development in experimental animals and humans. The results reveal that a reduction in the supply of energy and/or several essential nutrients during the first stages of life has profound effects on the structural and functional development of the nervous system. Malnutrition impairs brain growth and development by decreasing the number of cell replication cycles, reducing total brain DNA, and restricting dendritic arborization, thus reducing neuronal connectivity. Intrauterine and early postnatal malnutrition affect cell number and hyperplastic growth as measured by DNA content (1). Development of the cerebellum is most affected by nutritional deprivation around the time of birth in humans. Synaptic connectivity is particularly affected if malnutrition occurs after birth but before year 3 of life. Alterations in dietary precursors may affect tissue levels of neurotransmitters (serotonin, norepinephrine, dopamine, and acetylcholine) in specific brain regions (2). The supply of essential fatty acids (EFA) affects the structural composition of the brain and myelin sheaths in particular. The functional correlates of these biochemical changes induced by malnutrition include alterations in the waking electroencephalographic activity, visual- and auditory-evoked responses, motor and cognitive development, and social abilities. Sleep-wake cycle organization as well as autonomic nervous system functioning during sleep are perturbed by early human malnutrition (3,4). Most of these effects are potentiated by other environmental factors that interact with poor diet in defining the adverse consequences.

The traditional point of view, that protein and energy deficits *per se* directly affect brain structural development and cognitive performance, has been challenged because protein energy malnutrition coexists with multiple micronutrient deficiencies and with psychosocial deprivation that interact to disrupt brain development. This makes it particularly difficult to tease out the role of specific nutrients such as EFA from multiple deprivations that act in tandem, contributing to the final outcome in terms of growth and mental developmental (5). The major effects on growth and brain development associated with EFA deficiency are explained by the role of fatty acids (FA) as basic components of biological membranes, precursors of eicosanoids, and regulators of gene expression. The effect on growth is most likely linked to the role of EFA in energy balance and eicosanoid-mediated growth factors. The effects on CNS development are likely mediated by the role of EFA on gene expression, membrane structures, and electrophysiologic responses. The purpose of this review is to examine the effects of dietary EFA on the development of the brain and vision and the potential mechanisms by which modulation occurs.

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Abbreviations: AA, arachidonic acid (20:4n-6); ABER, auditory brainstem-evoked response; ALA, α-linolenic acid (18:3n-3); CNS, central nervous system; DHA, docosahexaenoic acid (22:6n-3); EFA, essential fatty acid; ERG, electroretinogram; FA, fatty acids; FPL, forced-choice preferential looking; GLA, γ-linolenic acid (18:3n-6); LA, linoleic acid (18:2n-6); LC-PUFA, long-chain polyunsaturated fatty acids; M I, metarhodopsin I; M II, metarhodopsin II; MDI, Mental Development Index; PC, phosphatidylcholine; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; RBC, red blood cell; RxR, retinoic acid receptor; TR, thyroxine receptor; VEP, visual-evoked potential.

**TABLE 1**
**Controlled Studies of Long-Chain Polyunsaturated Fatty Acid (LC-PUFA) Supplementation and Visual/Neurodevelopment of Preterm Infants[a]**

| Reference | Study groups (*n*) | LC-PUFA level in formulas (% total lipids) | Evaluation, outcome, age | Main results |
|---|---|---|---|---|
| 6,7 | F1 (12)<br>F2 (10)<br>F3 (10)<br>HM (10) | ALA 1.4, DHA 0.35<br>EPA 0.65<br>LA 20.8, ALA 2.7<br>LA 24.2, ALA 0.5 | ERG at 36, 57 wk<br>PCA | F3, lower rod amplitude and higher rod threshold at 36 wk. n-3 LC-PUFA related to rod threshold and inversely to amplitude. No difference at 57 wk. |
| 8 | F1 (13)<br>F2 (16)<br>F3 (12)<br>HM (9) | ALA 1.4, DHA 0.35<br>EPA 0.65<br>LA 20.8, ALA 2.7<br>LA 24.2, ALA 0.5 | VEP at 36, 57 wk<br>FPL at 36, 57 wk<br>PCA | F3, poorer visual acuity by VEP and FPL.<br>F1 and HM similar results |
| 9 | F1 (33)<br>F2 (34) | ALA 3.1, DHA 0.2–0.3<br>ALA 3–4.8 | Teller acuity cards, 38, 48, 57, 68, 79, and 92 wk PCA | F1, better acuity at 48 wk.<br>DHA level related to visual acuity at 48 wk. |
| 10 | F1 (27)<br>F2 (27) | ALA 3.1, DHA 0.2–0.3<br>ALA 3–4.8 | Bayley Mental and Psychomotor Developmental Index, 92 wk PCA | F1, lower PDI |
| 11 | F1 (33)<br>F2 (34) | ALA 3, DHA 0.2, EPA 0.3<br>ALA 3–4.8 | Fagan's NP, 68, 79, 92 wk PCA | F1, less % novel look time |
| 12 | F1 (26)<br>F2 (33) | ALA 3.1, DHA 0.2<br>ALA 4.8 | Teller acuity cards, 39, 48, 57, 68,79, 92 wk PCA | F1, better acuity at 48 wk in nonbronchopulmonary dysplasia infants |
| 13 | F1 (15)<br>F2 (12) | ALA 3.1, DHA 0.2<br>ALA 4.8 | Fagan's NP, 92 wk PCA | F1, shorter look duration<br>F2, more % novel time |
| 14 | F1 (21)<br>F2 (25)<br>HM (12) | DHA 0.3, AA 0.44<br>ALA 0.9, LA 19.4<br>DHA 0.16, AA 0.4 | Flash VEP<br>ERG<br>ABER at 52 wk PCA | F1 and HM, better acuity<br>Not different<br>Not different |
| 15 | F1 (140)<br>F2 (143)<br>F3 (144)<br>HM | DHA 0.25–0.15[b], AA 0.4<br>DHA 0.25–0.15[b], AA 0.4<br>ALA 2.4<br>F1 and F2 supplemented with two AA/DHA LCP sources<br>Egg/marine<br>Fungal/marine<br>DHA 0.25 until 40 wk then 0.15 DHA for 12 mon | Teller acuity cards, 2, 4, 6 mon PCA<br>Fagan's NP, 6, 9 mon<br>MacArthur, 9, 14 mon<br>Bayley II Mental and Psychomotor Development Index, 18 mon<br>Sweep VEP, 2, 4, 6 mon corrected age | Not different<br>Not different<br>Not different<br>F1, F2, 8 point higher PDI for infants with birth weight <1250 g<br>F1, F2, and HM, better visual acuity at 2, 4, 6 mon |

[a]Abbreviations: ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERG, electroretinogram; PCA, postconceptional age; LA, linoleic acid; HM, human milk; VEP, visual evoked potential; FPL, forced-choice preferential looking; PDI, Psychomotor Development Index; NP, novelty preference; AA, arachidonic acid; ABER, auditory brainstem evoked response. F1, F2, and F3 correspond to different formulas.
[b]First value refers to content during the period up to the gestational age (40 wk); second value is the content after 40 wk.

## EFFECT OF EFA ON BRAIN DEVELOPMENT

*Preterm infants (randomized controlled trials).* Very-low-birth-weight infants born with a birth weight of <1500 g are considered particularly vulnerable to EFA deficiency given the virtual absence of adipose tissue at birth, the possible insufficiency of FA elongation/desaturation enzymatic pathways, and the inadequate intake of long-chain polyunsaturated fatty acids (LC-PUFA) provided by formula. Randomized controlled clinical trials that have included formula feeding with or without LC-PUFA and functional assessment of visual/neurodevelopment in preterm infants are summarized in Table 1.

Over the past decade, we and others have conducted studies to evaluate the effect of n-3 FA in preterm infant development. These studies examined the effects of α-linolenic acid (ALA), ALA plus docosahexaenoic acid (DHA), or ALA plus DHA and arachidonic acid (AA) supplementation on plasma and tissue lipid composition, retinal electrophysiologic function, maturation of the visual cortex, and on other measures of infant growth and development (6–14). In our initial work, we showed that retinal function, as determined by the electroretinogram (ERG), is affected by n-3 supply in preterm human infants. Infants fed an n-3–deficient corn oil diet (standard formula at the initiation of the study in 1987) had significantly higher rod ERG thresholds (i.e., more light was necessary to elicit a threshold rod ERG response, resulting in a less mature ERG response) than infants receiving either human milk or n-3–supplemented formula. An analysis of the amplitude–intensity behavior of the responses (Naka-Rushton functions) from the infants in the study revealed that the threshold elevation in the n-3–deficient infants was due primarily to a shift in log *k* (i.e., the sensitivity of the rod system was lower). In addition there was a modest but significant de-

crease in the amplitude in the maximal amplitude ($V_{max}$) that could be elicited from infants in the deficient group. An analysis of the leading edge of the a-wave showed that the decreased sensitivity in the infants fed n-3–deficient formula originated in the rod photoreceptors (7). The results of dietary FA modification on the function of the visual cortex, as measured by pattern reversal visual-evoked potential (VEP) acuity and behaviorally by the forced-choice preferential looking (FPL) visual acuity response, demonstrated that infants in the human milk and marine oil groups (both receiving DHA) had improved maturation of VEP relative to infants fed formulas devoid of DHA (corn and soy oil groups) at the 4-mon adjusted-age followup (8). No differences were found between soybean- and marine oil-containing formula if acuity was assessed by FPL. Visual acuity tests measure the integrity of the neural pathway from the retina to the occipital cortex and provide a surrogate measure of CNS function. The long-term significance of the improved retinal and visual function on later neurodevelopment is presently being explored.

Carlson's first randomized clinical study in preterm infants supplemented with LC-PUFA demonstrated better visual acuity in infants up to 4 mon of age using FPL measurements. After this time, control infants "caught up" in visual function measures. These investigators report evidence of more rapid visual processing as measured by shorter look duration in the Fagan test of visual recognition at 6 and 12 mon of age in LC-PUFA–supplemented infants (9–11). The reduction in AA in blood lipids, when marine oil was provided as a source of n-3 fatty acids, was associated with reduced weight and linear growth (10–13). In a second preterm infant study using low eicosapentaenoic acid (20:5n-3) marine oil for up to 2 mon corrected age, Carlson demonstrated improved visual development at the 2-mon followup; at 12 mon of age, there was a 10-point Bayley Mental Development Index (MDI) difference favoring the DHA-supplemented group (11,12). Furthermore, the DHA-supplemented group had shorter look times in the novelty preference test at 9 mon, suggesting better visual processing (12).

In our recent studies, conducted in Chile, no LC-PUFA effect on auditory brain stem-evoked responses (ABER) was demonstrated; this coincides with the results of a preterm infant study by Faldella (14). One of our centers (INTA) recently participated in a collaborative multicenter study of a large group of preterm infants. This study included 470 preterm infants fed LC-PUFA formula supplemented with different AA and DHA sources, i.e., marine oil and egg phospholipids or fungal oil. The level of DHA was 0.25% of total fat in preterm formulas and 0.15% in follow-up formula; both formulas contained 0.4% AA (15). Significant differences were found in sweep VEP at 6 mon favoring the LC-PUFA–supplemented formula group compared with the control formula group. There were no diet-induced differences in behavioral tests of visual acuity (determined using Teller acuity cards). Mean novelty preference was significantly better at 6 mon in the LC-PUFA–supplemented group. No differences in MacArthur vocabulary tests were found, but an improved 14-mon score was noted in the supplemented group when non-English-speaking subjects were excluded from the analysis. For infants <1250 g at birth, an advantage of 8.8 points in the LC-PUFA–supplemented group in the Bayley II Psychomotor Development Index was observed at 12 mon (15).

*Significance of LC-PUFA for term infants (randomized controlled trials).* The question of whether healthy full-term infants require LC-PUFA in their formula has received considerable attention over the past decade. The finding of lower plasma DHA and red blood cell (RBC) concentrations in infants fed formula compared with breast-fed infants suggests that formulas provide insufficient ALA or that chain elongation–desaturation enzymes are not sufficiently active during early life to support optimal tissue accretion of DHA. Full-term infants also may be dependent on dietary DHA for optimal functional maturation of the retina and visual cortex (16–38). Controlled trials that have included formula feeding with or without LC-PUFA and functional assessment of visual and neural development are summarized in Table 2.

Makrides *et al.* (25) conducted the first controlled randomized study in term infants using a formula supplemented with 0.36% DHA and 0.27% γ-linolenic acid (GLA) compared with a formula providing ample ALA (1.6%) but no DHA as well as a breast-fed reference group. They demonstrated delayed visual acuity at 4 and 6 mon of age in the formula group lacking DHA. Infants who received breast milk for >16 wk had better VEP acuity than those breast-fed for shorter times or given formula without LC-PUFA. A separate study showed better visual acuity at 2 mon but no benefits after 6 mon of life in breast-fed or 0.1% DHA formula-fed infants relative to control formula (26). Biochemical indices of DHA status and visual acuity maturation of infants receiving only ALA were delayed relative to those receiving formula supplemented with ALA + DHA; these latter infants had maturational indices similar to those of the breast-fed group (27).

Auestad *et al.* (28) compared infants fed standard formula, formula supplemented with 0.2% DHA (from marine oil), or formula with 0.12% DHA and 0.43% AA. No significant differences could be detected among the three diet groups using ERG at 4 mon, visual acuity, or the Bayley Scales of Infant Development test at 12 mon. Furthermore, negative correlations were found between RBC and DHA at 4 mon and language development assessed by the MacArthur Communicative Development Inventory given at 14 mon ($r = -0.20$ to $-0.37$ depending on the specific item, $P < 0.05$) (29). A beneficial effect of DHA, AA, and GLA supplementation on psychomotor development assessed by the Brunet-Lezine method was reported at 4 and 12 mon but not at 24 mon of age (30,31). This study reported a strong association between the erythrocyte phosphatidylcholine (PC) AA/linoleic acid (LA) ratio and the developmental quotient at 24 mon, but there was no relation to the dietary intervention in the first 4 mon of life (31). In an attempt to control for confounding variables that could affect visual and neural development, Gibson *et al.* (32) supplemented mothers with LC-PUFA to produce DHA-enriched breast milk with concentrations ranging from 0.1 to 1.7% of total FA. The plasma and erythrocyte-phospholipid DHA level

**TABLE 2**
**Controlled Studies of Long-Chain Polyunsaturated Fatty Acid (LC-PUFA) Supplementation and Visual/Neurodevelopment of Term Infants[a]**

| Reference | Study groups (*n*) | LC-PUFA level in formulas (% total lipids) | Evaluation, outcome, age | Main results |
|---|---|---|---|---|
| 25 | F1 (13) | DHA 0.36, EPA 0.58 GLA 0.27 | VEP at 16, 30 wk | F1 and HM, better visual acuity |
| | F2 (19) | LA 16.8, ALA 1.6 | | |
| | HM (23) | | | |
| 26 | F1 (19) | DHA 0.35, AA 0.43 | Teller acuity cards, 2, 4, 6, 9, 12 mon | F1, better visual acuity only at 2 mon |
| | F2 (20) | LA 21.9, ALA 2.2 | | |
| | HM (19) | | | |
| 30 | F1 (27) | DHA 0.3, AA 0.44 | Brunet-Lezine's developmental quotient at 4 mon | F1 and HM, better developmental quotient than F2 |
| | F2 (29) | LA 11.1, ALA 0.7 | | |
| | HM (30) | | | |
| 31 | F1 (26) | DHA 0.3, AA 0.44 | Brunet-Lezine's developmental quotient at 24 mon | Not different, developmental quotient related to 24-mon plasma DHA level, but not to 4-mon plasma DHA |
| | F2 (30) | LA 11.1, ALA 0.7 | | |
| | HM (25) | | | |
| 35 | F1 (24) | DHA 0.2, AA 0.35 | Infant habituation at 4 mon | F1, shorter fixation times |
| | F2 (24) | LA 11.4, ALA 0.7 | | Mean scores on habituation not different |
| | HM (27) | | | |
| 36 | F1 (21) | DHA 0.2, AA 0.35 | Problem solving at 10 mon | F1, better |
| | F2 (22) | LA 11.4, ALA 0.7 | | |
| 28 | F1 (26) | DHA 0.12, AA 0.43 | Sweep VEP acuity 2, 4, 6, 9, 12 mon | Not different |
| | F2 (28) | DHA 0.23 | | |
| | F3 (28) | LA 21.9, ALA 2.2 | FPL acuity 2, 4, 6, 9, 12 mon | Not different |
| | HM (38) | | | |
| 29 | F1 (38) | DHA 0.12, AA 0.43 | MacArthur's developmental index at 14 mon | F3, more vocabulary comprehension/ production than DHA group |
| | F2 (33) | DHA 0.23 | | |
| | F3 (42) | LA 21.9, ALA 2.2 | | |
| | HM (60) | | Bayley I Mental and Psychomotor Developmental Index at 12 mon | Not different |
| 27 | F1 (14) | DHA 0.3, GLA 0.5 | Sweep VEP at 4 mon | HM better than formulas F1 and F2 |
| | F2 (12) | DHA 0.3 | | |
| | F3 (11) | LA 12, ALA 1.2 | | Nonsignificantly better visual acuity than F3 |
| | HM (25) | DHA 0.38, AA 0.4 | | |
| 33 | F1 (23) | DHA 0.36, AA 0.72 | FPL and Sweep VEP at 6, 17, 26, 52 wk | Not different |
| | F2 (22) | DHA 0.35 | | F1, F2, HM better visual acuity at 6, 17, and 52 wk, but not at 26 wk |
| | F3 (23) | LA 14.6, ALA 1.49 | | |
| | HM (21) | DHA 0.29, AA 0.56 | | |
| 34 | F1 (19) | DHA 0.36, AA 0.72 | Bayley II Mental and Psychomotor Developmental Index at 18 mon | F3, worse in MDI |
| | F2 (17) | DHA 0.35 | | MDI related to plasma and RBC DHA at 4 mon |
| | F3 (20) | LA 14.6, ALA 1.49 | | |
| 38 | F1(154) | DHA 0.32, AA 0.3 | Bayley I Mental and Psychomotor Developmental Index at 18 mon | Not different |
| | F2 (155) | LA 12.4, ALA 1.1 | | |
| | HM (138) | | | |
| 40 | F1 (24) | DHA 0.34, AA 0.34 | Flash VEP, 16, 34 wk | Not different |
| | F2 (23) | DHA 0.35 | | |
| | F3 (21) | LA 16.8, ALA 0.13 | Bayley Mental and Psychomotor Developmental Index at 12, 18 mon | Not different and not related to plasma DHA levels |
| | HM (46) | DHA 0.2, AA 0.4 | | |

[a]Abbreviations: GLA, γ-linolenic acid; MDI, Mental Development Index; RBC, red blood cell; for other abbreviations see Table 1.

of these infants was related to breast milk DHA in a saturable manner; no significant increases were noted in blood DHA levels when the breast milk content of DHA was >0.6%. Infant VEP acuity had no relationship to DHA content of breast milk; the developmental quotient at 12 mon was significantly but weakly correlated with breast milk DHA. At 24 mon, this effect was no longer evident.

Recently, Birch *et al.* (33) showed a persistent benefit in visual acuity development for year 1 of life in DHA-supplemented formula-fed infants compared with infants fed formula with ample ALA but devoid of LC-PUFA. The formula given for the first 17 wk of life contained 0.35% DHA, with or without 0.72% of AA; both LC-PUFA were derived from single-cell oils in the supplemented groups. The dietary effects on vi-

sual acuity development were evident using sweep VEP acuities but absent if the FPL behavioral measure of acuity was used. Supplemented groups receiving DHA or DHA + AA and the breast milk group had better acuity. The differences were significant during the periods of rapid change in development of VEP acuity, i.e., in the first 20 wk and near 12 mon of life. The developmental outcome of these infants was reported recently (34). Scores on the Bayley MDI II at 18 mon of age for the DHA + AA group were significantly better than those observed in the non–LC-PUFA formula-fed infants. A 7-point normalized MDI score difference was highly significant despite the relatively small sample size ($n$ = 20/group). The small variability in developmental score obtained was likely due to the highly homogeneous population studied and the fact that one observer evaluated mental development in all subjects. The DHA-only group had marginally higher MDI scores than controls. This is the first randomized controlled study that reports an LC-PUFA effect on mental development at 18 mon of age. Moreover, positive significant correlations between blood DHA levels at 4 mon with measures of visual acuity at 1 yr and mental development at 18 mon were noted (34). The existence of a relationship between early biochemical and later functional data suggests that visual function and neurodevelopmental phenomena may be related but do not constitute proof of a causal relationship.

A behavioral study of 44 term infants fed a combined DHA- and AA-supplemented formula or a control formula during the first 4 mon demonstrated that visual habituation performance scores of infants at 4 mon of age were better in the LC-PUFA–supplemented formula group (35). Infant cognitive behavior was assessed at 10 mon of age by a means-end problem-solving test (36). The LC-PUFA–supplemented group had significantly more intentional solutions and scored higher than infants fed the non–LC-PUFA–containing control formula. Higher problem-solving scores in infancy have been shown to relate to higher childhood intelligence quotient scores (37). These studies provide a solid indication of efficacy but are limited in their extrinsic validity because of small sample size and rather homogeneous infant populations.

Lucas *et al.* (38), in the largest controlled term-infant study to date, did not find a beneficial effect of LC-PUFA supplementation in a group of 309 infants randomized to formula diets with or without LC-PUFA. A reference group of 138 infants included for comparative purposes were breast-fed. No biochemical data on FA composition of plasma or tissues were obtained in this study, limiting the assessment of compliance to the test formula. Follow-up studies at 18 mon revealed no benefit in cognitive or motor development. No adverse effects of LC-PUFA supplementation were noted in terms of infection, atopy, or formula tolerance. However, the interpretation of these data is limited by the fact that study formulas differed not only by the presence or absence of LC-PUFA but also by several other fatty acids. The expected higher MDI score in breast-fed infants compared with standard formula-fed infants was not apparent in this study, and because there was no plasma or tissue biochemical evaluation of infant EFA status, a relationship between LC-PUFA status and neurodevelopment could not be established (38). In addition, the maternal population studied had a low educational level, i.e., ~20% had no formal education at all and ~70% had not completed a high school education. Mean MDI values for all groups of infants were 4–6 points below the norm, suggesting that these groups may not be representative of the normal population in the United Kingdom but rather a group subjected to an environmental factor that restricted their neurodevelopment. The possible effect of maternal education or ethnicity is raised from the analysis of confounding variables (39). Using the same LC-PUFA–enriched and control formulas, Makrides *et al.* (40) also did not find diet-induced differences in VEP acuity or Bayley MDI scores during year 1 of life. In this case, breast-fed infants had higher MDI scores than formula-fed infants at 2 yr, even after adjusting for environmental variables. No correlations between plasma LC-PUFA levels and neurodevelopment indices were found by these investigators.

Other studies have attempted to optimize ALA to DHA conversion by providing sufficient ALA (>0.7% of total energy) as well as lowering the LA to ALA ratio in the formula (<10:1). This alternative strategy for the formulation of infant diets would be simpler and less expensive to produce than the addition of n-3 LC-PUFA (21,24). Unfortunately, results from infants fed formula with a ratio of 4.8:1 demonstrated poorer growth, possibly due to the lower AA levels found with this dietary regimen (24).

*Recent systematic reviews of LC-PUFA supplementation.* The results of two recent meta-analyses conducted on the effect of LC-PUFA on visual acuity maturation in preterm and term infants are summarized in Figures 1 and 2. As can be observed in the figures, most studies demonstrated a positive effect for assessments conducted during the first 2–4 mon of life; the results at 6 mon were equivocal and again, at 12 mon, some studies were positive. The conclusions by San Giovanni *et al.* (41,42), in their two reports, is that there is a significant overall advantage for the LC-PUFA–supplemented infants in terms of visual acuity. The main differences in experimental design, subject selection, dietary supplementation, other nutrition-related factors, and the primary outcomes of these studies are summarized in Table 3. Most studies have chosen the prospective, randomized, controlled, double-blind design, but some studies included in the systematic reviews compared human milk-fed with formula-fed infants. This comparison has limited validity because infants of breast-feeding mothers and formula-fed infants usually vary in terms of maternal education, birth weight, home environment, socioeconomic level, and other confounders. To evaluate efficacy, most studies have used "healthy" subjects controlling for birth weight, gestational age, socioeconomic status, and maternal characteristics. These well-controlled studies have strong intrinsic validity. However, these studies cannot be generalized for all infants because the study groups in the controlled studies may not be representative of the population at large. The diets studied have provided 0.1–0.35% DHA in both preterm and term infant studies. These values are in the middle-to-lower

range of mean DHA content found in breast milk as derived from combined data of omnivorous women around the world. No formal controlled DHA dose-response studies evaluating visual maturation and/or mental development at varying levels of supplementation have been conducted in preterm (8–15) or term infants (25–31,33–40).

The duration and reversibility of diet-induced effects is another important consideration in assessing outcome. In evaluating diet-induced changes in visual/neural development, the selection of outcomes is crucial. The sensitivity and variability of the measurements are vital to detect an effect given the presence of multiple known and unknown confounders. The timing of the measurement is also of great importance; most LC-PUFA effects are evident only over the period of rapid development and do not persist once functional maturation has been achieved. The visual acuity results suggest that transient effects reflect the acceleration or the slowing of a maturational process with a fully normal final outcome. Several studies have demonstrated significant effects of dietary LC-PUFA on visual maturation in the first 4 mon of life, but in most studies, visual acuity normalized by 6 mon or at most by 1 yr of age (41,42). However, we need to consider whether this phenomenon can truly be dismissed as transitory and of limited significance and instead consider that we may have failed to detect a significant change later in life. This may be explained by the limitations in our tools, which may not be sufficiently sensitive; moreover, other related functions may indeed have been affected. As an example of this problem, in term infants, we failed to detect differences in visual acuity of breast-fed compared with formula-fed infants at 6 mon but stereo-acuity responses were different at 3 yr of age (16). In this study, most breast-fed infants (92%) had mature operant preferential looking stereo acuity, whereas only 35% of the infants in the formula-fed group met the maturity criteria. Visual recognition in the breast-fed group was also better; only 61% of the formula-fed infants had a perfect score, whereas 93% of the breast-fed group had a perfect score (16). This illustrates the need to select sensitive outcome measures and provide for sufficient follow-up of cohorts in these randomized clinical trials. It is impossible to fully discard the possible long-term consequences of early developmental delays in function unless followup is conducted over a sufficient period of time.

## MECHANISMS FOR EFA EFFECTS ON GROWTH AND BRAIN DEVELOPMENT

*Molecular regulation of gene expression by nutrients*. Regulation of gene expression by nutrients can occur at multiple levels (43,44). For example, FA can bind to specific or nonspecific ligands that interact with response elements in specific DNA motifs usually present in the promoter region of the gene, affecting gene transcription. DHA and other PUFA interact with nuclear factors modulating the activation of specific nuclear proteins such as peroxisome proliferator-activated receptors (PPAR), which in turn act as transcription regulators (45). In this case, the nutrient effect is indirect because it is the activated transcription factor that binds *cis*-regulatory elements of DNA found in target genes. Another form of nutrient ligand interaction includes changes in phosphorylation mediated by the nutrient. At the posttranscriptional level, once mRNA are formed, nutrients may act by modifying native RNA processing, mRNA transport and stability, and breakdown rates. Nutrients may also modify the rate of mRNA translation by the activation of protein synthesis in the polyribosomal complex. Nutrients such as vitamin K can affect gene expression at the posttranslational level beyond the synthesis of protein by modifying the gene products formed. Vitamin K-dependent amino acid carboxylation, which is necessary for active prothrombin synthesis, represents an example of nutrient-induced posttranslational modification of gene products. Finally, nutrients can modify the turnover rates of proteins including enzymes, thus affecting their activity level. In many cases, the nutrient-induced changes in gene expression are part of the adaptive response to a given level of nutrient exposure (43,44). Thus, nutrients may affect the uptake, metabolism, storage, or excretion of the nutrient that triggered the gene regulatory response.

LC-PUFA are involved in the regulation of cell growth and differentiation by modulation of gene expression. For example, the effect of DHA on the functional maturation of the retina observed in several animal species including primates may now be potentially related to a direct effect on photoreceptor differentiation. Studies using primary culture of retinal neuronal cells have revealed a potential mechanism by which this conditionally essential nutrient affects gene expression critical to retinal function and survival (46). In cultured cells from rats, DHA significantly increased the differentiation of apical processes in rod outer segments, the locus for rhodopsin and opsin-dependent light transduction. This was paralleled by an increase in opsin expression and content in the rod photoreceptor apical processes (46). The molecular mechanisms underlying these effects have not been fully clarified, but these data suggest an effect of DHA on opsin gene expression and possibly on other proteins required for the assembly of disc membranes. Recent studies from Bazan's group (47) have demonstrated that the transport of opsin and rhodopsin to the apical process *via* post-Golgi membranes is coupled to DHA transport. The close molecular interaction between these key photoreceptor proteins and DHA suggests that DHA influences retinal photoreceptor structural development as well as function.

Regulation of gene expression by LC-PUFA also occurs at the transcriptional level and is mediated by transcription factors that bind *cis*-regulatory elements found in target genes. These transcription factors, which are activated by FA, have a structure similar to that of the steroid-thyroid supergene family of nuclear receptors, which includes the steroid hormone receptors, glucocorticoid receptor, vitamin D receptor, thyroxine receptor (TR), and the retinoic acid receptor (RxR) (48–50). PUFA-responsive transcription factors recently have been characterized; for example, PPAR can be activated by clofibric acid and other peroxisome proliferators (45,48,50). Recent studies have identified a number of proteins and co-activators that in-

teract with nuclear receptors involved in the regulation of transcriptional activity. The formation of the binding site for the co-activator in the nuclear receptor is ligand dependent.

The activation of PPAR by FA was first characterized in *Xenopus laevis*; α-, β-, and γ-isoforms were able to respond to FA with overlapping specificity (45). However, few studies have systematically explored the differential activation of PPAR by FA of different chain length or unsaturation. Yu *et al.* (49) compared the ability of FA to activate the different PPAR isoforms using chimeric constructs. The tetR/PPARα chimeric receptor was activated to almost the same extent by LA and DHA, whereas the γ-isoform was activated by DHA but not by LA and the β-isoform was responsive to DHA > LA. PPARα is apparently also activated by medium- and long-chain unsaturated FA. This evidence has been used to support the notion that reduction of hepatic expression of lipogenic enzymes induced by dietary n-3 and n-6 PUFA is mediated by PUFA-activated PPARα (45,48,50). In this chimeric PPAR expression model, DHA was the most potent activator, whereas saturated myristic acid (14:0) was a considerably poorer inducing agent (48). The net effects of PPAR on cellular processes and metabolism include enhanced peroxisomal proliferation, increased FA oxidation, decreased FA synthesis, and enhanced glucose oxidation (50). Additional work will be necessary to better characterize the intracellular FA metabolites that regulate transcriptional activation and responsiveness of target genes critical for retinal and brain development.

*EFA effects on neural structures and functional properties.* Lipids, such as various phospholipids and cholesterol, serve as components of specialized cell membranes and organelles. The overall quantity and relative composition of these lipid species may affect membrane fluidity and protein/lipid interactions, resulting in changes in overall cell function. The FA composition of structural membrane lipids can affect membrane function by modifying overall membrane fluidity (order parameter), membrane thickness, lipid phase properties, membrane microenvironment, or by interaction of FA with membrane proteins (51–53). These effects may modulate receptor activity, transport of metabolites in and out of cells, and hormonal or other signal transduction processes. Most dietary n-3 FA–induced membrane changes are not reflected by an overall change in membrane fluidity, but result in selective changes in specific domains of membrane microenvironment (54). The replacement of DHA by docosapentaenoic acid (22:5n-6) observed in n-3 deficiency results in a very similar overall lipid unsaturation level because only one double bond has been lost. Thus, on average, membrane fluidity as measured by fluorescent probe polarization remains unchanged. Furthermore, the major changes in the physical state induced by the FA composition of lipid bilayers occur when mono- or diunsaturated moieties are introduced; namely, when a saturated FA such as stearic acid (18:0) is replaced by oleic acid (18:1n-9) or by LA (18:2n-6) (52,53). Others have suggested that the DHA supply modifies the phospholipid molecular species present in neural tissues, thus affecting overall function (55).

One of the most significant membrane effects of DHA is its role in photoreceptor signal transduction processing. Recently, Litman and Mitchell (56) reported that LC-PUFA present in membrane phospholipid molecular species have profound effects on rhodopsin activation and related structural modifications. Rhodopsin is a membrane protein present in rod outer segment disk membranes, accounting for 90% of the protein content. It functions as a photon receptor coupled to a G protein. The light-induced conformational change of rhodopsin triggers a biochemical cascade finally leading to an increase in phosphodiesterase activity and a decrease in cGMP that closes sodium ion channels in the photoreceptor disk membrane. The result is a hyperpolarization that is reflected in the ERG as the leading edge of the a-wave and that ultimately leads to the release of synaptic neurotransmitters. Membrane FA composition affects the ability of photons to transform rhodopsin to the activated state (56–58). The rhodopsin activation in response to light involves a transformation of metarhodopsin I (M I) to metarhodopsin II (M II).

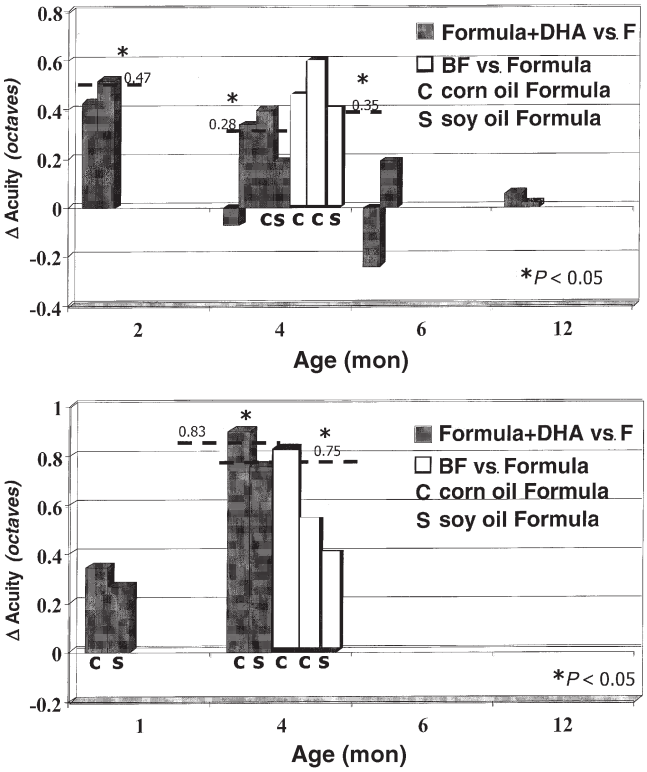


**FIG. 1.** Meta-analysis of visual acuity differences in preterm infants; solid bars represent randomized comparisons [group fed formula with long-chain polyunsaturated fatty acids (LC-PUFA) compared with group fed formula without LC-PUFA]. Open bars represent nonrandomized comparisons (group fed human milk compared with formula without LC-PUFA). Dashed line represents mean of estimate from combined data for various studies for given month. C and S refer to comparison formulas containing corn and soy oil, respectively. (A) Acuity differences measured by behavioral tests (acuity cards); (B) acuity differences measured by visual-evoked potentials; all values expressed in octaves (one-octave difference is a 50% reduction in width of stimulus element). Data obtained from Reference 41. DHA, docosahexaenoic acid; F, formula; BF, breast feeding.

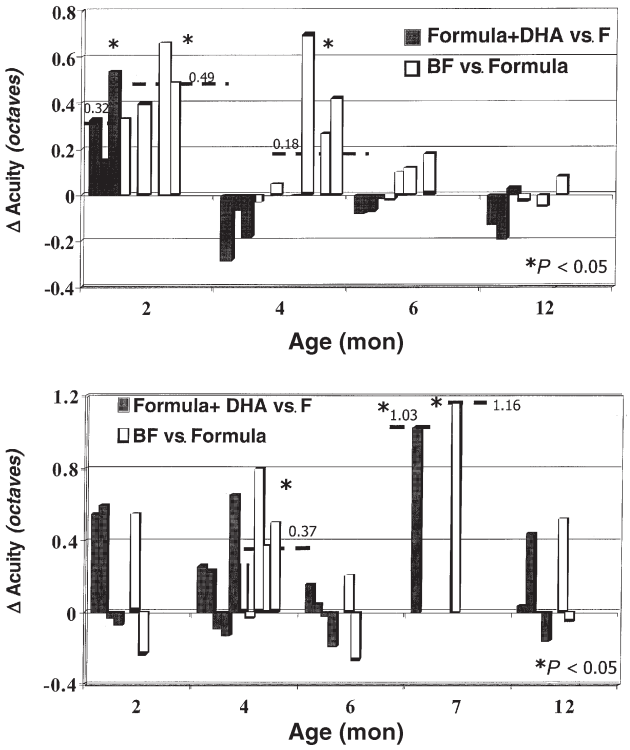


**FIG. 2.** Meta-analysis of visual acuity differences in term infants; solid bars represent randomized comparisons (group fed formula with LC-PUFA compared with group fed formula without LC-PUFA). Open bars represent nonrandomized comparisons (group fed human milk compared with formula without LC-PUFA). Dashed line represents mean of estimate from combined data for various studies for given month. (A) Acuity differences measured by behavioral tests (acuity cards); (B) acuity differences measured by visual evoked potentials; all values expressed in octaves (one-octave difference is a 50% reduction in width of stimulus element). Data obtained from Reference 42. See Figure 1 for abbreviations.

The MI → MII equilibrium constant is six times higher with di-DHA acylated PC than with di-myristoyl (14:0) PC. The di-DHA PC has an equilibrium constant that is almost identical to that of phospholipids extracted from native rod disks. The effect is explained mainly by the increase in membrane free volume. This greater mobility of rhodopsin within the lipid microenvironment most likely explains the change in G protein activation and the corresponding enhanced signal transduction to photon stimuli (58). The corresponding physiologic phenomenon is an increase in retinal sensitivity to light, such as we observe by providing DHA in the infant diet.

*EFA effects on electrophysiologic responses*. The role of membrane lipid composition in determining the electrical properties of cultured neuronal cells exposed to exogenous FA has also been investigated (59). Both n-3 and n-6 FA reduce the rate of rise and lower the amplitude of $Na^+$ action potentials. The opposite effects were observed when saturated or *trans* monoenoic FA were added to the culture (60). These effects are likely mediated by a change in the number of active $Na^+$ channels. A change in membrane composition or altered FA availability to the cells may explain this effect. Free LC-PUFA have been shown to modulate the inactivation of calcium and sodium channels in rat neural cells (61). There are also changes in cation currents in hippocampal neurons (60) and a higher seizure threshold in rat cortex (62). These effects appear to depend on free extracellular LC-PUFA concentrations and not on membrane phospholipid composition (61). The responsiveness of free LC-PUFA to dietary interventions, which alter tissue composition, remains unclear. The release of free LC-PUFA from membranes could have widespread effects on neurosensory function.

At the CNS level, interest in the effect of EFA on the maturation of visual function is based on their role as key structural components of cell membranes and their accumulation in visual and neural structures. Data from breast-fed infants who received DHA indicate that a higher content of this FA is present in brain cortex relative to infants fed formula (63). Neuringer and colleagues (64) established the need for n-3 FA in the diet, utilizing infant rhesus monkeys as a model system for n-3 FA deficiency. Following prenatal (maternal) and

**TABLE 3**
**Critical Aspects in Experimental Design of Studies of Long-Chain Polyunsaturated Fatty Acid (LC-PUFA) Supplementation on Visual and Neurodevelopment in Infants[a]**

| Design | Diet |
|---|---|
| Prospective randomized, controlled, double blind study | Content and balance of EFA: LA and ALA |
| Nonrandomized, formula compared with breast-fed reference group | Content and balance of each LC-PUFA |
| Descriptive, nonrandomized trial | Source of LC-PUFA: egg, single cell, marine |
| Preplanned or *post-hoc* analysis | Energy balance and content of critical nutrients (I, Fe, Zn, vitamin A, folate) |
| Sample size to detect effect | Digestibility and utilization |
| | Duration of the dietary intervention |
| **Subjects** | **Outcome** |
| Term or preterm infants | Sensitivity of selected outcome |
| Birth weight and gestational age | Variability in measurement of outcome |
| Special groups or representative population | Age of evaluation, time of day of evaluation |
| Socioeconomic and ethnic group | Time of effect relative to critical period |
| Maternal education | Biological and clinical significance |
| Home environment | Duration and reversibility of effects |
| Pre- and postnatal growth | Safety of intervention and side effects |

[a]Abbreviation: EFA, essential fatty acids. For other abbreviations see Table 1.

postnatal diets deficient in n-3 FA, the DHA concentrations in both the occipital cortex and the retina were reduced to 20% of that in control monkeys. The n-3 FA deficiency also impaired visual acuity as measured by preferential looking techniques. By 12 wk of age, the deficient monkeys presented Snellen acuities of ~20/125 vs. 20/50 in controls (20/20 is the average adult acuity; 20/50 is the average for 12 wk of age in monkeys). In addition, the b-wave amplitudes of ERG were reduced by 30% in the n-3 FA–deficient rhesus monkeys (64).

Several potential mechanisms by which early dietary EFA supply may affect visual and brain maturation and long-term function can be outlined on the basis of the available experimental data. The potential role of DHA as a modulator of membrane properties is supported by the *in vitro* studies of membrane fluidity and transport in neural cells modified in their membrane FA. The putative role of DHA in amplifying the phototransduction cascade is supported by the electrophysiologic findings in animals and humans. Dietary n-3 supplementation in these studies resulted in decreased retinal rod threshold and higher maximum amplitude, meaning that less light is required to trigger a retinal response and that more signal is being transmitted to the visual pathway, respectively. Moreover, the discovery of biochemical differences in phosphorylated microtubular-associated proteins in neurons from the visual cortex of light-deprived kittens during early development provides a mechanism for the classical observations by Hubel and Wiesel (65,66). The expression of microtubular proteins in the visual cortex induced by light plays a key role in establishing the dendritic arborization and interconnections necessary for visual perception; darkness inhibits the expression of this gene product (67,68). Thus the role of light-mediated stimuli in triggering cortical differentiation offers a plausible explanation for the phenomenon of a critical period for ocular dominance that has a biochemical basis as well as structural and functional correlates (65–70). We speculate that the effect of DHA on light transduction in the retina early in life may have lasting effects (i.e., imprint) on the organization and function of the visual cortex. The alternative possibility is that DHA has an effect on photoreceptors as well as an independent effect on cortical maturation itself. No definitive answer can be established from the available data. As previously indicated, the fact that human milk-fed infants exhibited more mature stereo acuity at 3 yr relative to formula-fed infants suggests that this phenomenon may be relevant to humans (16). Additional supportive evidence on the possible long-term effect of early DHA supply on maturation of the visual cortex comes from a large population-based prospective study of determinants of stereo acuity development conducted in Bristol, United Kingdom in collaboration with one of us (E.B.). The results indicate that a maternal antenatal diet rich in DHA is associated with enhanced stereopsis of infants at age 3.5 yr after adjusting for other environmental as well as maternal and infant factors (71).

In conclusion, evidence for a beneficial effect of AA + DHA supplementation on CNS development is strong. The followup of supplemented infants beyond infancy should help to address the question concerning the persistence of effects beyond early life. Studies summarized in this review provide evidence supporting the view that dietary EFA supply affects visual development of preterm and term infants. The preliminary information on cognitive development is insufficient to fully establish a claim for an LC-PUFA effect on mental development. Human infants require dietary AA and DHA to maintain normal FA composition of plasma and RBC membrane lipids, and presumably of brain and retina. Differential gene expression induced by EFA and changes in membrane biophysical properties are likely responsible for the observed functional effects. Further work should lead to a better understanding of the mechanisms by which EFA affect visual and brain development and allow for a better definition of optimal nutrition in early life. Optimal in this context implies the right amount and balance of nutrients to obtain a desirable outcome (i.e., enhanced cognitive development or decrease in burden of disease in adult life) considering that there are inherent risks from excesses as well as deficits.

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# Perinatal Characteristics May Influence the Outcome of Visual Acuity

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**ABSTRACT:** Visual-evoked potential (VEP) acuity has been used to assess the effects of dietary fats on the integrity of the visual pathway of infants. We investigated prognostic determinants of VEP acuity at 16 wk of age. The results of two randomized dietary intervention trials designed to assess the effect of dietary fatty acids on the visual development of term infants were combined. At entry to both trials (~day 5 of life), a blood sample to assess polyunsaturated fatty acid (PUFA) status was collected along with sociodemographic and perinatal characteristics. At 16 ± 0.9 wk of age, infants underwent VEP testing to measure acuity. There was no effect of dietary treatment on these outcomes within or between trials. Multiple linear regression models were constructed to investigate the effect of perinatal and nutritional variables at study entry on VEP acuity of 185 infants. Higher birth weight was associated with an ability to resolve smaller checkerboard patterns [$r^2$ = 0.05; 95% confidence interval (CI), –0.10, –0.04 log units]. Male gender ($r^2$ = 0.03; 95% CI, 0.01, 0.07 log units), day 5 plasma 22:5n-6 ($r^2$ = 0.04; 95% CI, 0.02, 0.20 log units), day 5 red cell membrane 20:3n-9 ($r^2$ = 0.03; 95% CI, 0.03, 0.13 log units), and the number of smokers in the household ($r^2$ = 0.02; 95% CI, 0.00, 0.04 log units) were all associated with poorer VEP acuity scores. It is possible that a combination of perinatal factors could accumulate to either mask or enhance effects of diet on VEP acuity, given the relatively modest effects of long-chain PUFA on visual outcome.

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Intervention trials with dietary long-chain polyunsaturated fatty acids (LC-PUFA), such as docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6), have generally used visual acuity as a primary outcome measure. Studies of dietary n-3 fatty acid deficiency in rhesus monkeys documented that reduced n-3 LC-PUFA status was linked with impaired visual acuity and altered retinal function associated with reduced brain and retinal LC-PUFA composition (2,3). Randomized trials involving preterm infants have also consistently demonstrated that infants allocated to LC-PUFA–supplemented diets have improved visual acuity and retinal sensitivity compared with infants fed unsupplemented diets (4–6). However, trials involving term infants have demonstrated equivocal results, with some trials reporting modest positive effects of LC-PUFA supplementation on visual acuity and others reporting no effect (1).

Various factors have been put forward as possible explanations for these disparate results, including dose of DHA, the background level of α-linolenic acid (ALA, 18:3n-3) (7), sample size, and different methodologies. However, none adequately explains the differences in the results of the six current published trials of LC-PUFA intervention and visual outcome. We hypothesized that the inconsistent results of LC-PUFA randomized trials in term infants may be related to confounding effects from baseline perinatal factors, sociodemographic characteristics, and/or a relatively small LC-PUFA effect size (1).

We recently reported two randomized controlled trials that were designed to test the effect of dietary fatty acids on visual acuity as assessed by visual-evoked potential (VEP). One trial assessed the effect of an LC-PUFA intervention (8), whereas the other evaluated the effect of altering the ratio of essential fatty acids (9). Neither trial demonstrated an effect of dietary intervention on VEP acuity when infants were 16 wk of age (8,9). This is in contrast to a previous trial we conducted, where LC-PUFA supplementation of term infants resulted in improved VEP acuity compared with unsupplemented infants (8,9). Because of these conflicting results, we undertook a retrospective analysis of our two trials to identify prognostic variables of VEP acuity in healthy, term infants at 16 wk of age.

## METHODS

The data from two randomized controlled trials of formula feeding as well as their breast-feeding reference groups were combined (8,9). The trials had the same inclusion and exclusion criteria as well as the same data collection protocol. The only difference between the two trials was the dietary intervention. In one trial, infants were randomly allocated to either an infant formula with a linoleic acid (LA, 18:2n-6) to ALA ratio of 17:1.5 and no LC-PUFA or an identical formula but with 0.35% DHA from tuna oil or 0.34% DHA and 0.34% AA from egg phospholipid (8). In the other trial, infants were allocated to either a standard formula (LA/ALA ratio of 16.9:1.7) or one with high ALA (LA/ALA ratio 16.6:3.3) (9). In both trials, we also recruited a parallel group of breast-fed infants (8,9). Information on sociodemographic and perinatal

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; LA, linoleic acid; LC-PUFA, long-chain polyunsaturated fatty acid; MAR, minimum angle of resolution; VEP, visual-evoked potential.

characteristics was collected at study entry (approximately day 5 of life) as was a baseline blood sample by heel prick to assess plasma and erythrocyte membrane fatty acids (8,9). Infants had an ophthalmic examination at 10–12 wk of age and were excluded if their refraction was outside the range of –3 to +5 diopters or they had severe astigmatism (≥1.75 diopters) or strabismus. Infant VEP to different checkerboard patterns (7, 10, 14, 20, 28, 42, and 55 min arc) was tested at 16 wk of age (8,9). The peak-to-peak amplitude of the VEP (N1–P1) response was measured and plotted against the log of the angle subtended by each check size. The linear portion of the plot was extrapolated to 0 μV to give the theoretical value that would just elicit a response [log of the minimum angle of resolution (log MAR)]. Hence, lower log MAR values represent better visual acuity, as they indicate detectable responses to smaller checkerboard patterns. Points were excluded from the regression if they were not on the linear portion of the stimulus–response function or represented amplitudes of ≤2 μV (8,9). VEP acuity extrapolations were accepted as valid only if there were at least three points and the regression line was significant ($P < 0.05$). There were no differences in VEP acuity values between dietary groups within either trial or between the two trials.

*Statistics.* Multiple linear regression models were constructed to evaluate the possible effects of baseline sociodemographic and perinatal variables on VEP acuity at 16 wk of age. The independent variables considered for the regression model were gender, gestational age, birth size, birth order, parental smoking, education and social scores, number of siblings, and infant plasma and erythrocyte membrane polyunsaturated fatty acids measured on day 5 of life. Infants with incomplete data sets were excluded. The models were constructed using the independent variables that were associated ($P < 0.2$) with the dependent variable. Independent variables were removed if the independent variable's presence or absence did not influence the model. The regression-standardized residuals of the dependent variable, VEP acuity, were normally distributed. All analyses were performed using SPSS for Windows 7.5 (SPSS Inc., Chicago, IL).

## RESULTS

In the original trials, a total of 261 infants underwent VEP testing at 16 ± 0.9 wk of age [95% confidence interval (CI), 15.2–17.7 wk]. The age bracket of testing was narrow, and, as a result, there was no association between age at testing (postnatal age) and VEP acuity.

Among the 261 infants tested, 194 had successful VEP acuity extrapolations (Table 1). These 194 infants had significantly lower mean birth weight and lower mean birth length compared with infants with unsuccessful VEP acuity extrapolations, even following adjustment for gender (Table 1). There were no other significant differences in sociodemographic or perinatal characteristics between infants with successful and unsuccessful VEP acuity determinations.

**TABLE 1**
**Characteristics of Infants with Successful and Unsuccessful Visual-Evoked Potential (VEP) Acuity Extrapolations at 16 wk of Age**

| | Infants with successful VEP acuity extrapolations | Infants with unsuccessful VEP acuity extrapolations[a] |
|---|---|---|
| *n* | 194 | 67 |
| Gender ratio (M/F) | 99:95 | 41:26 |
| Gestation (wk ± SD) | 39.3 ± 1.3 | 39.7 ± 1.2 |
| Birth weight (g ± SD) | 3408 ± 475 | 3563 ± 425* |
| Birth length (cm ± SD) | 50.6 ± 2.0 | 51.6 ± 1.9** |
| Birth head circumference (cm ± SD) | 34.8 ± 1.4 | 35.2 ± 1.5 |
| 5-min Apgar score (±SD) | 9.2 ± 0.6 | 9.1 ± 1.0 |
| Maternal smoking (% yes) | 24 | 24 |
| Maternal education[b] (score ± SD) | 3.0 ± 1.1 | 2.0 ± 1.1 |
| Maternal social score[c] (±SD) | 5.0 ± 1.1 | 4.0 ± 1.1 |
| Partner smoking (% yes) | 35 (*n* = 184) | 36 (*n* = 64) |
| Partner education[b] (score ± SD) | 3.0 ± 1.2 (*n* = 182) | 3.0 ± 1.2 (*n* = 64) |
| Partner social score[c] (±SD) | 4.0 ± 1.2 (*n* = 180) | 4.0 ± 1.2 (*n* = 63) |
| Day 5 plasma phospholipid fatty acids (expressed as % total phospholipid fatty acids ± SD) | | |
| 20:3n-9 | 0.27 ± 0.18 | 0.28 ± 0.16 |
| 18:2n-6 | 14.7 ± 3.5 | 14.7 ± 4.4 |
| 20:4n-6 | 14.6 ± 3.2 | 14.5 ± 2.6 |
| 22:4n-6 | 0.53 ± 0.14 | 0.54 ± 0.10 |
| 22:5n-6 | 0.53 ± 0.16 | 0.54 ± 0.14 |
| 20:5n-3 | 0.37 ± 0.12 | 0.40 ± 0.16 |
| 22:5n-3 | 0.39 ± 0.18 | 0.42 ± 0.31 |
| 22:6n-3 | 4.64 ± 1.05 | 4.46 ± 0.86 |

[a]Single asterisk denotes $P < 0.05$ after adjustment for gender, **$P < 0.005$ after adjustment for gender.
[b]Median score; education was ranked using a six-point scale: 1 = primary school level, 2 = mid secondary school level, 3 = completion of secondary school, 4 = completion of a certificate or diploma, 5 = tertiary degree, 6 = higher degree.
[c]Median score; the highest rank (1) was assigned to professionals and academic occupations and the lowest rank (6) was assigned to unskilled occupations.

Of the 194 infants with successful extrapolations, 185 were included in the regression model to predict VEP acuity. Eight infants were excluded because they had incomplete data sets, and the ninth one excluded was an outlier (>3 SD from the mean acuity). A combination of perinatal factors explained 17% of the variance in VEP acuity at 16 wk of age (Table 2). Birth weight explained 5% of the variance in VEP acuity, and a 1-kg increase in birth weight was associated with an ability to resolve smaller checkerboard patterns by 0.07 log units (Table 2). Male gender, day 5 plasma 22:5n-6, day 5 red cell 20:3n-9, and the number of smokers in the household were all associated with poorer VEP acuity scores (Table 2). Day 5 DHA status was not associated with VEP acuity at 16 wk.

## DISCUSSION

The use of VEP acuity as a test of visual/neural development in infants has been extensive in the area of LC-PUFA research although the long-term relevance of this test is yet to be fully assessed. The modest and inconsistent differences reported to date between infants receiving LC-PUFA–supplemented formula and those fed unsupplemented formula make it important to ensure that the data are free from potential confounders or effect modifiers so that the full effect of dietary LC-PUFA can be determined.

It is interesting to note the similarity of reported VEP acuity values across study centers, despite the diversity of VEP acuity testing procedures. The actual VEP acuity values of breast-fed and formula-fed infants at 16 wk of age observed in the combined data from our two trials were similar to values observed in the placebo formula-fed infants in our original study measured using different hardware (10). Other trials have demonstrated VEP acuity values in breast-fed and formula-fed infants similar to the data reported here (11). The VEP acuity values recently reported by Birch *et al.* (12) at 17 wk of age are approximately 1 SD lower than our data (8,9) and those of Auestad *et al.* (11). However, it should be noted that all trials report acuity values within the expected normal range.

**TABLE 2**
**Multiple Linear Regression Results for Independent Factors Predicting VEP Acuity at 16 wk of Age[a] ($n$ = 185, mean VEP Acuity 0.747 ± 0.105)**

| Variable | *B* (95% CI) | β | Adj.cr | *P* |
|---|---|---|---|---|
| Birth weight (kg) | –0.07 (–0.10, –0.04) | –0.309 | 0.05 | <0.001 |
| Day 5 plasma 22:5n-6 (% total phospholipid fatty acids) | 0.109 (0.02, 0.20) | 0.170 | 0.09 | 0.015 |
| Male gender | 0.042 (0.01, 0.07) | 0.203 | 0.12 | 0.004 |
| Day 5 red cell 20:3n-9 (% total phospholipid fatty acids) | 0.076 (0.03, 0.13) | 0.206 | 0.15 | 0.003 |
| Number of smokers in the house | 0.020 (0.00, 0.04) | 0.148 | 0.17 | 0.031 |

[a]*B*, understandardized regression coefficient; CI, confidence interval; β, standardized regression coefficient; Adj.cr, adjusted cumulative $r^2$. See Table 1 for other abbreviation.

Importantly, we could find no direct effect of DHA status near birth on VEP acuity. It has been suggested that the n-3 LC-PUFA status of infants at birth may be prognostic (antecedent variable) of later visual and neurological development (13,14). This postulate has been put forward as a possible explanation for inconsistent findings of randomized trials of formula feeding of term infants with and without LC-PUFA supplementation. It is proposed that n-3 LC-PUFA status near birth may confound any subsequent effect of dietary LC-PUFA on visual outcomes (13). In other words, those infants born with the highest DHA status may be less responsive to dietary supply. In our data, DHA was not a predictor of VEP acuity and only 22:5n-6 (which may be an indirect index of n-3 LC-PUFA status) predicted VEP acuity at 16 wk of age. This may be an insignificant association, as the regression model predicts that a 1% change in plasma 22:5n-6 level would be required for a 0.109 log unit change in acuity, which is unlikely even in infants fed n-3-deficient diets (15). Therefore, our data do not provide support for the hypothesis that n-3 LC-PUFA status at birth is a significant prognostic variable of visual outcome.

In our regression model to predict VEP acuity, higher birth weight emerged as a small but significant influence on improved acuity. This finding is similar to that reported by Jorgensen *et al.* (16). It is interesting to note that infants without VEP acuity extrapolations had higher birth weights compared with infants included in the regression model. This may indicate that the maximum of the spatial tuning curve of these infants occurred at the smaller checkerboard patterns, hence leaving too few points on the linear portion of the curve for extrapolation to the threshold (acuity) value (9). It is therefore likely that the infants excluded from the regression model had acuity values beyond the sensitivity of the test and that the effect of birth weight on VEP acuity is thus underestimated in our regression model. In any event, it may be important to adjust for birth weight in any analysis of VEP data.

Male gender also emerged in the regression model and was associated with poorer outcome. These findings are consistent with the negative association between male gender and global measures of neurodevelopment (17,18) and highlight the need for adequate gender stratification in randomized trials. Similarly, parental smoking was associated with poorer VEP acuity, independently of birth weight.

It is possible that the factors that have emerged as being prognostic of acuity in our data set may have confounded the outcome of previous trials. A combination of the independent variables birth weight, gender, and the number of smokers in the household can predict VEP acuity, and the magnitude of this association is similar to the postulated difference in VEP acuity between LC-PUFA–supplemented and unsupplemented infants at about 4 mon of age (1). It is therefore possible that a number of perinatal factors could be additive and

thereby mask or enhance dietary effects on VEP acuity, particularly since the effects of LC-PUFA on visual outcome are likely to be modest.

It should be noted that mothers and infants in our trials might have had different social and demographic characteristics from study samples in other trials. The infants in our trials were from families where, on average, both parents completed secondary education and infants had birth characteristics indicative of well-nourished and uncomplicated pregnancies. Thus, it is reasonable to suppose that the perinatal factors that are predictive of outcomes in other study populations may be different from those reported here.

So what do our results mean to researchers in this area? The effects of gender and birth weight may be balanced in any trial but only if adequate randomization and stratification procedures are in place. The other effects we have seen may be too small to be clinically important or even strong enough to influence an effect of diet. However, it is important for researchers to conduct their own tests of their data, *post hoc*, in order to assess actual power and whether or not they may have confounding influences in their data sets.

In summary, using the largest sample of VEP reported to date on healthy term infants and tested at the same postnatal age, we have demonstrated that a variety of perinatal characteristics including birth weight, gender, and number of smokers in the household need to be considered in relation to VEP acuity.

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# Polyunsaturated Fatty Acids and Infant Growth

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**ABSTRACT:** Because of the rapid rate of growth during infancy, and the potentially deleterious effect of differences in the availability of dietary essential nutrients, growth is an important outcome variable in any study assessing a diet designed for infants. Nearly 10 yr after the first demonstration of reduced growth in preterm infants fed a fish oil-enriched formula, there is very little additional information to confirm or refute the finding that long-chain n-3 polyunsaturated fatty acid (LC-PUFA) intake can modulate growth in infants. To evaluate the issue of a possible relationship between PUFA intake and growth of infants, we reviewed a total of 32 randomized studies, 13 in preterm infants and 19 in term infants. From the data published to date, it seems clear that long-chain n-3 fatty acids can reduce growth achievement in preterm and term infants under some experimental conditions. However, the effect of n-3 PUFA supplementation on the growth of preterm and term infants appears to be minimal and of questionable clinical and/or physiologic relevance. Nonetheless, n-3 fatty acids have an effect on gene transcription, at least in some species, and this finding may provide important clues to the mechanism by which n-3 and n-6 fatty acids regulate growth.

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Despite the common use of growth assessments, the interpretation of these assessments is not necessarily straightforward. In retrospect, this could be said to be the case for several randomized studies in prematurely born human infants who were fed very long chain n-3 fatty acids [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] and showed lower growth in supplemented vs. control groups (1–3). Lower growth in preterm infants was perceived as an adverse or potentially adverse outcome. Nearly 10 yr after the first such study was reported, however, there is very little additional information to confirm or refute the finding that long-chain n-3 polyunsaturated fatty acid (LC-PUFA) intake can modulate the growth of infants. Surprisingly, few data are available to help evaluate the clinical significance of the growth effects that were observed (4).

Studies evaluating LC-PUFA supplementation in infants were designed primarily to assess the effects of supplementation on PUFA status and visual, cognitive, and/or behavioral development. It has become clear that supplementation of formulas with n-3 and n-6 LC-PUFA is desirable for visual and/or neural development. However, only a few studies were designed specifically to evaluate growth, even if virtually all have included reports of anthropometric measures. These studies utilized formulas with a variety of linoleic acid (LA)/α-linolenic acid (ALA) ratios, as well as various amounts and/or sources of LC-PUFA. Among the studies, the duration of supplementation and/or followup, the outcome variables evaluated, and the number and birth weights of infants included in each group were also quite variable. Furthermore, the effect of gender has seldom been studied, and the growth of infants not always normalized to reference growth curves. Without these considerations, and in the absence of normalization to an established standard of growth, it is difficult to conclude that supplemented infants who grew less well than their own controls were at any real risk for growth impairment.

To address the issue of a possible relationship between PUFA intake and infant growth, we reviewed 13 randomized studies in preterm infants and 19 randomized studies in term infants. We included those studies that examined growth data of at least two groups of infants fed formulas with different fatty acid patterns, i.e., either different LA/ALA ratios or different amounts of LC-PUFA. Few of the studies were designed as growth trials, *per se*. Not all had enough power to detect an effect of PUFA supplementation on growth. If we assume that a difference of 1 SD in weight and/or length is of physiologic relevance, then the sample size required to observe such a difference in growth between two groups ($\alpha$ 0.05, power 0.80) is, theoretically, 16–18 infants/group. If the expected effect on growth is smaller, 0.7 SD or 0.5 SD, for example, then ~34 or ~66 infants, respectively, must be included to detect an effect on growth with the same power and $\alpha$. Because effects of n-3 fatty acid supplementation on growth of preterm infants have not been observed before ~10 wk of feeding (1), we may postulate that studies of <10 wk duration and with 16 infants cannot be used to conclude that there were not effects of n-3 supplementation on growth.

Because of the need to present a complete perspective on this subject and to evaluate all potential indicators, we provide here a summary of the evidence from the relevant studies. The data for preterm and term infants are delineated separately and results obtained in the larger studies are emphasized. The final section reviews molecular effects of n-3 fatty acids that may hold clues to the mechanism by which n-3 and/or n-6 fatty acids regulate growth.

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LC-PUFA, long-chain n-3 polyunsaturated fatty acids; NCHS, National Center for Health Statistics; PG, prostaglandin; PMA, postmenstrual age; PPAR, peroxisome proliferator-activated receptor.

## PUFA INTAKE AND GROWTH OF PRETERM INFANTS

Three studies in human preterm infants have reported effects of n-3 fatty acid intake on growth (1–3). In each of these studies, DHA was fed without a source of arachidonic acid (AA, 20:4n-6) although the formulas contained large amounts of the essential fatty acid linoleic acid (LA), the precursor of AA.

In the human infant study that found the most pronounced effect on growth of feeding an n-3 LC-PUFA–enriched formula (1), all anthropometric assessments from 0 to 12 mon corrected age were lower in the supplemented vs. the control group. Infants had an average birth weight of ~1.1 kg. There were a number of factors in the design that likely contributed to the size of the effect. First, infants were fed a large total amount of long-chain n-3 fatty acids because the fish oil supplement contained 0.3% EPA as well as 0.2% DHA, the intended additive, and the formula already contained a high amount of ALA. Second, the supplemented formula was fed for 11 mon. Third, the formula did not contain AA to balance the long-chain n-3 fatty acids, although it did contain a large amount of LA. Plasma concentrations of phosphatidylcholine AA (the average at 2, 4, and 6.5 mon) were used as the measure of infant AA status. AA status explained 7–23% of weight and length attainment during the time interval from 0 to 12 mon adjusted age (5). It was speculated that the greater decline in normalized growth in infants randomized to receive high-EPA fish oil supplementation compared with control formula was related to the effects of fish oil n-3 fatty acids on AA status (1).

The two other studies that involved the finding of an adverse effect of fish oil–supplemented formula on the growth of preterm infants had close to 30 infants/group (2,3). In both of these studies, however, the design should have mitigated the effects of fish oil on infant AA status. In one of these (2), the n-3 fatty acid–supplemented formula was fed for ~19 wk to ~1 kg birth weight infants (until 2 mon adjusted age). The source of DHA was low-EPA fish oil, which effectively reduced the intake of long-chain n-3 fatty acids by half compared with the first study (1) but provided the same amount of DHA. Normalized weight, length, and head circumference of the infants showed no overall effect of diet. However, n-3 LC-PUFA–supplemented infants had a lower normalized weight at 6 and 9 mon, lower weight-for-length at 2, 6, 9, and 12 mon, and lower head circumference at 9 mon. In particular, the lower weight-for-length of the infants fed the n-3 fatty acids suggested they might be somewhat leaner than the infants fed the commercially available formulation.

Ryan *et al.* (3) reported data of infants fed a low-EPA fish oil for 24 wk. Mean birth weight of infants was ~2 kg, considerably higher than that in all but one (6) of the studies involving the feeding of experimental formula supplemented with fish oil without AA (1,2,7,8). The data were normalized for age, but males and females were studied independently so that the actual comparison groups included ~15 infants of the same gender. Compared with males who were given the control formula, males fed the experimental formula had consistently lower Z-scores for weight, length, and head circumference throughout the study to 4.5 mon corrected age, but the differences were not significant. Absolute weight and length of the male infants, but not the female infants, were statistically lower in the experimental group than in the control group at 4.5 mon. In addition, n-3 fatty acid–supplemented male infants had a lower head circumference at 2.5 and 4.5 mon. Estimates of body composition, determined by total body electrical conductivity, indicated that males fed the supplemented formula had significantly lower fat-free mass at 2.5 and 4.5 mon. A lower fat mass also was observed for those in the fish oil-fed group, but the difference was not significant.

Three other studies reported growth of preterm infants fed fish oil without additional AA (Table 1). One was conducted at the same time as the first study discussed above. In this study, supplemented infants received slightly greater amounts of LC-PUFA, and the experimental formula was fed for only 25 wk (7) compared with 48 wk (1). There was no effect of the fish oil-supplemented formula on normalized weight, length, or head circumference at 0, 1, 2, 3, or 4 mon corrected age in infants with an average birth weight of 1.3 kg. The study had adequate power to detect a difference in growth of ~1 SD.

Growth data were also reported in a study in which low-EPA fish oil was fed to one group of 29 infants for only 4.5 wk (6), as well as in a study in which low-EPA fish oil was fed for 23 wk to a group of 11 infants (8). Neither study was intended as a growth study, and the studies lacked either a sufficient number per group, or a long enough duration of feeding to reach conclusions about growth.

Other studies in preterm infants provided both DHA and AA in the experimental formula (Table 2). The sources of LC-PUFA have included egg lipids (9), egg phospholipids (10), egg triglycerides (11), single-cell oils (11–16), and fish oil (11). Two of these studies (11,16) found lower growth rates in infants supplemented with DHA and AA compared with those in a control group but no significant effects on actual weight, length, or head circumference. In one study (16), a lower mean increase in length from 0 to 12 mon corrected age was observed in the group fed the single-cell–enriched formula. In the second study (11), a lower length gain between study entry and 4 mon corrected age and a lower head circumference gain (females only) were observed in the group fed the enriched formula with egg triglycerides and fish oil. In contrast to these studies, however, another study (14) found higher growth during the period from enrollment (30 wk postmenstrual age, or PMA) until 56 wk PMA (4 mon corrected age) with DHA and AA supplementation. None of these three studies (11,14,15), which included large groups of infants ($n$ = 58–144) that should have been adequate to detect differences in growth of <0.5 SD, demonstrated a significant effect of DHA + AA supplementation on the actual weight, length, or head circumference in the supplemented groups. The remaining four studies that provided DHA and AA to preterm

**TABLE 1**
**Effects of Formula Supplementation with Long-Chain n-3 Polyunsaturated Fatty Acids on Growth in Preterm Infants[a]**

| Reference | Diet | Duration of diet | Assessment ages/results[b] |
|---|---|---|---|
| 1 | High-EPA FO ($n$ = 31) vs. C ($n$ = 34) | 48 wk | Lower normalized W, L, HC in the high-EPA FO group at 0, 2, 4, 6, 9, and 12 mon. VLBW infants. |
| 7 | High LA/ALA ($n$ = 18), low LA/ALA ($n$ = 20), and high EPA FO ($n$ = 22) | 25 wk | No difference in normalized W, L, HC, TST, or SSF at 0, 1, 2, 3, and 4 mon. VLBW infants. |
| 2 | Low-EPA FO ($n$ = 26) vs. C ($n$ = 33) | 19 wk | No overall difference in W, L, HC at 0, 2, 4, 6, 9, and 12 mon. Lower W at 6 mon, W and HC at 9 mon, W-for-L at 2, 6, 9, and 12 mon in the low-EPA FO group. VLBW infants. |
| 6 | Low-EPA FO (2 groups combined, $n$ = 29) vs. C (3 groups combined, $n$ = 81) | 4.5 wk | No difference in W, L, and HC gain between start (10 d of age) and end of study (42 d of age). LBW infants. |
| 3 | Low-EPA FO ($n$ = 32) vs. C ($n$ = 31) | 24 wk | W, L, and HC assessed at –0.25, 0.75, 1.75, 2.75 and 4.5 mon. No effect of diet in females. Males in the low-EPA FO group had lower normalized L at 4.5 mon and W, L, and HC at termination of the study. LBW and VLBW infants. |
| 8 | Low-EPA FO ($n$ = 11) vs. C ($n$ = 12) | 23 wk | No difference in W, L, and HC at 0, 3, and 6 mon. VLBW infants. |

[a]C, control; LBW, low birth weight (<2500 g); VLBW, very low birth weight (<1500 g); L, length; W, weight; HC, head circumference; TST, triceps skinfold thickness; SST, subscapular skinfold thickness; FO, fish oil; LA, linoleic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid.
[b]Age expressed from expected term delivery.

infants were not designed to measure growth as an outcome. One (10) had sufficient power to detect a difference of 1 SD in growth, but the three other studies (9,12, 13), were too small, or of too limited duration, to permit detection of anything other than extremely large effects of diet on growth.

In summary, three of six reports of preterm infants supplemented with n-3, but not n-6, LC-PUFA showed lower growth. The other three studies in this group may have had too few subjects to detect an effect on growth (7,8) or have provided an experimental formula for only a short period of time (6). It seems clear that long-chain n-3 fatty acids can reduce growth achievement in preterm infants under some experimental conditions. One may speculate from the combined results of these studies that growth is more likely to be affected under conditions that would have the largest effect on the already marginal AA status of preterm infants (17). The most recent trials (11,14,15) have provided substantial data on the effect of balanced addition of DHA and AA to preterm formulas. Excepting some inconsistent effects on rates of growth, they have not demonstrated significantly different absolute growth in the experimental groups compared with the control group (Table 2).

**TABLE 2**
**Effects of Formula Supplementation with Long-Chain n-3 and n-6 Polyunsaturated Fatty Acids on Growth in Preterm Infants[a]**

| Reference | Diet | Duration of diet | Assessment ages/results[b] |
|---|---|---|---|
| 9 | Egg lipids ($n$ = 12) vs. C ($n$ = 11) | 10 wk | No differences in W and L gain. VLBW infants. |
| 12 | SCO DHA + AA ($n$ = 15), vs. C ($n$ = 16) | 21 wk | No difference in W gain. VLBW infants. |
| 10 | Egg PL ($n$ = 21) vs. C ($n$ = 16) | 19 wk | No difference in W, L, and HC gain from enrollment to 3 mon LBW and VLBW infants. |
| 13 | SCO DHA + AA (3 levels $n$ = 12–18/group) vs. C ($n$ = 16) | 4 wk | No difference in W and L. LBW and VLBW infants. |
| 14 | SCO DHA, SCO DHA + AA and C ($n$ ~ 58/group) | 20 wk | W, L, and HC at 0, 2, 4 mon. L of SCO DHA + AA > L of SCO DHA at 0 and 2 mon. SCO DHA + AA = fed reference group at 0, 2, 4 mon. Mostly VLBW infants. |
| 15,16 | SCO DHA + AA ($n$ = 77) vs. C ($n$ = 78) | 17 wk | No difference in W, L, and HC at 0, 2, or 12 mon. Lower mean increase in L from 0 to 12 mon in SCO DHA + AA compared with HM or C. LBW and VLBW infants. |
| 11 | Egg TG + low-EPA FO ($n$ = 143), SCO AA + low-EPA FO ($n$ = 140) and C ($n$ = 144) | 60 wk | No difference in W, L, HC at 0, 2, 4, 6, 9, and 12 mon. Lower L gain from study to 4 mon and lower HC gain in females from study entry and term in egg TG + low-EPA FO vs. C. Mostly VLBW infants. |

[a]PL, phospholipids; SCO, single-cell oil; TG, triglycerides; DHA, docosahexaenoic acid; AA, arachidonic acid; for other abbreviations see Table 1.
[b]Age expressed from expected term delivery.

## PUFA INTAKE AND GROWTH IN TERM INFANTS

Because of concern about the effect of dietary manipulations of n-3 LC-PUFA on growth in animals as well as in preterm infants, the Expert Panel of the Life Sciences Research Office (LSRO) of the American Society for Nutritional Sciences (18) did not recommend the addition of AA or DHA to term infant formula.

Because of the variable LC-PUFA content of human milk, one might suggest that differences in the PUFA composition of human milk could affect the postnatal growth of term infants. No study to date has addressed this issue, but several studies have provided postnatal growth data on infants of mothers randomly assigned to a DHA supplement from microalgae, fish oil, or high-DHA eggs (19–21). These supplements significantly modified the fatty acid composition of breast milk and the plasma or erythrocytes of the infants, but growth of the various groups of breast-fed infants did not differ. These findings suggest that changes in the diet of the mother do not significantly alter the fatty acid status of their infants to an extent that would be sufficient to influence postnatal growth. However, because these studies included only a small number of infants, further studies are required to evaluate this issue.

To examine the possibility of a relationship between PUFA intake and growth of term infants, we reviewed 19 randomized studies that included growth data on at least two groups of term infants fed formulas with different fatty acid patterns, i.e., either different LA/ALA ratios or different amounts of LC-PUFA (Tables 3 and 4). Only one of the 19 studies suggests that the amount of n-3 fatty acids significantly affected the growth of term infants (22). In that study, infants were fed formulas containing ~16% of total fatty acids as LA, and 0.4, 0.95, 1.7, or 3.2% of total fatty acids as ALA (LA/ALA ratios from 5:1 to 44:1). The group of infants fed the highest ALA intake had the highest plasma phospholipid ALA, EPA, and DHA concentrations. Equally importantly, this group had the lowest plasma phospholipid AA and 22:5n-6 concentrations. At 120 d of age, and only at this study point, the mean weight of the group that received the formula with 3.2% of total fatty acid as ALA was significantly lower than that of the group that received the formula with 0.4% ALA. There was no significant difference among feeding groups in weight before or after 4 mon of age. There also was no significant difference among groups in length, head circumference, triceps, or subcapsular skinfold thickness, although all of these measurements tended to be lower in the infants who received the highest ALA intake. This study suggests that the differences in growth pattern could be related to differences in ALA intake. However, it does not differentiate between the effects of ALA intake *per se* vs. differences in the LA/ALA ratio. The absence of any significant effect of ALA intake or the LA/ALA ratio on growth in three other studies (23–25) is further evidence that large differences in n-3 PUFA intake are needed to affect growth.

In contrast to studies in preterm infants, no significant reductions in weight or length have been reported among groups of term infants fed formulas supplemented with egg lipid (26–29), egg phospholipid and/or triglyceride (30–33), various fish oils (31–38), or single-cell sources of DHA and/or AA (33,39,40). One study, however, reported a significantly lower head circumference at 4 mon of age in infants fed a formula enriched with low-EPA fish oil vs. the unsupplemented formula (35). However, the head circumference of the infants fed the enriched formula was within the normal range for age and also similar to that of a reference breast-fed group. In addition, Morris *et al.* (40) reported that the subscapular skinfold thickness of infants fed a formula enriched with single-cell oils (DHA + AA) was significantly lower than that of controls at both 6 wk and 3 mon of age. The triceps skinfold thickness and mid-arm circumference also were slightly lower than in controls, although the differences were not significant. These findings suggest a decreased fat deposition in infants fed an LC-PUFA–enriched formula.

In one of the largest studies published to date (33), an increase in weight gain for male infants was observed between enrollment and 4 mon in the group fed a fish oil + fungal oil–enriched formula compared to a control.

It should be noted, however, that with few exceptions (30–33,39,40), either small numbers of infants were included and/or the duration of supplementation was short, so it cannot

**TABLE 3**
**Effects of Formula Supplementation with n-3 Long-Chain Polyunsaturated Fatty Acids on Growth in Term Infants[a]**

| Reference | Diet | Duration of diet | Assessment ages/results |
|---|---|---|---|
| 38 | High-EPA FO + GLA ($n$ = 14) vs. C ($n$ = 12) | 30 wk | No difference in normalized W, L, and HC at 5, 15, and 30 wk. |
| 34 | Low-EPA FO (2 levels) vs. 2 LA/ALA ratios ($n$ = 16–21) | 16 wk | No difference in normalized W, L, and HC at 16 wk. |
| 37 | High-EPA FO + GLA ($n$ = 14), high-EPA FO ($n$ = 12) and C ($n$ = 11) | 4 mon | No difference in W, L, and HC at 4 mon. |
| 35 | High-EPA FO ($n$ = 23), low-EPA FO ($n$ = 24), and C ($n$ = 21) | 42 d | No difference in W, L, HC, and MAC at 42 d. |
| 36 | Low-EPA FO ($n$ = 12) vs. C ($n$ = 12) | 4 mon | No difference in W, L, and HC at 4 mon.<br>Lower HC at 4 mon in the supplemented formula-fed infants. |

[a]MAC, mid-arm circumference; GLA, γ-linoleic acid; for other abbreviations see Tables 1 and 2.

**TABLE 4**
**Effects of Formula Supplementation with n-3 and n-6 Long-Chain Polyunsaturated Fatty Acids on Growth in Term Infants[a]**

| Reference | Diet | Duration of diet | Assessment ages/results |
|---|---|---|---|
| 27 | Egg lipids vs. C (*n* = NA) | 3 mon | No difference in W and L at 7 d, 1 and 3 mon. |
| 29 | Egg lipids (*n* = 21) vs. C (*n* = 24) | 4 mon | No difference in W, L, and HC at 4 mon. |
| 26 | Egg lipids (*n* = 12) vs. C (*n* = 10) | 4 mon | No difference in W, L, and HC at 4 mon. |
| 32 | Egg PL (*n* = 46), low-EPA FO (*n* = 43), and C (*n* = 45) | 12 mon | No difference in normalized W, L, and HC at 1, 2, 4, 6, 9, and 12 mon. |
| 28 | Egg lipids (*n* = 20) vs. C (*n* = 20) | 4 mon | No difference in W, L, HC, SST, TST, and MAC at 3 mon. |
| 39 | SCO DHA (*n* = 26), SCO AA + DHA (*n* = 27) and and C (*n* = 26) | 17 wk | No difference in normalized W, L, HC, SST, and TST at 6, 17, 26, and 52 wk. |
| 30 | Egg PL + TG (*n* = 154) vs. C (*n* = 155) | 6 mon | No difference in normalized W, L, HC, MAC, and sum of ST at 6, 9, and 18 mon. |
| 31 | Egg PL (*n* = 28), low-EPA FO (*n* = 27), and C (*n* = 28) | 12 mon | No difference in W, L, and HC at 6, 16, and 34 wk and 1 and 2 yr. |
| 40 | SCO DHA + AA (*n* = 54) vs. C (*n* = 55) | 12 wk | No difference in W, L, HC, MAC, TST at 6 wk or 3, 6, and 12 mon. Lowest SST at 6 wk and 3 mon in the supplemented formula-fed infants. |
| 33 | Egg TG (*n* = 80), SCO AA + low-EPA fish oil (*n* = 82) vs. C (*n* = 77) | 12 mon | No difference in W, L, and HC during the 12-mon study. Higher weight gain for males between study start and 4 mon in SCO AA + low-EPA FO group vs. C. |

[a]ST, skinfold thickness; NA, not available; for other abbreviations see Tables 1–3.

be concluded that term infants might not show effects of n-3 LC-PUFA on growth like those that have occurred in some studies of preterm infants.

A relationship between n-3 PUFA intake and growth in formula-fed infants may exist in term infants, as indicated by the study of Jensen *et al.* (22). However, the data reported to date suggest that the effects of n-3 PUFA supplementation on the growth of term infants are observed only when comparisons are made between infants fed a formula supplemented with low vs. very high levels of n-3 PUFA. These effects were small and have questionable clinical relevance. Just as has been observed in preterm infants, the trials in term infants with balanced addition of DHA and AA did not result in findings of lower growth in the experimental group compared with the control group (Table 4). A difference in skinfold thickness in one of these studies suggests, however, a lower rate of fat deposition in infants fed an n-3 and n-6 LC-PUFA–enriched formula. In the absence of any direct data on body composition, and because of the small number of studies actually designed to validly assess the effect of LC-PUFA on growth, studies are needed to monitor the long-term growth and quality of growth (i.e., body composition) of term infants fed an LC-PUFA–enriched formula.

## DOES THE COMPARATIVELY REDUCED GROWTH RATE OF INFANTS FED n-3 PUFA–SUPPLEMENTED FORMULAS HAVE ANY CLINICAL RELEVANCE?

In general, any adverse effect on weight gain or achieved weight in infants, particularly preterm infants, is considered undesirable. However, it is not clear that this is the case with respect to the effect of n-3 PUFA on growth. The supplemented groups that grew more slowly than randomized controls appeared to experience excellent growth when held to an established standard of growth (4). Given these considerations, it is difficult to conclude that supplemented infants who grew less well than their own controls are at any real risk. For example, the observed effect of n-3 PUFA on growth was rather small in the study of Jensen *et al.* in term infants (22). The mean weight of infants who received the highest ALA intake was only 0.1 SD below the National Center for Health Statistics (NCHS) 50th percentile value for weight at 120 d of age, whereas the mean weights of the other groups were above the 50th percentile.

To evaluate the clinical relevance of the effect of high- or low-EPA fish oil supplementation on the growth of preterm infants, the data on the growth of preterm infants published by Carlson *et al.* (1,2) were compared with NCHS data (41) on growth of term infants to generate percentiles for norms. However, a possible limitation of the preterm studies that showed effects of feeding long-chain n-3 fatty acids alone on growth is that all were conducted with populations having a high proportion of infants of African descent (1–3). The growth of term infants from one of the study sites indicated that first-year growth of term infants of primarily African descent may have been at a slower rate than that of infants included in the NCHS (Z-score 0 means growth at the 50th percentile for the reference group, here the NCHS group) (42; Carlson, S., unpublished data). Therefore, growth data of term infants (*n* = 61) enrolled in the same hospital and followed over the first 12 mon of life were also used to create other normative data from the same population (called the reference group) and are reported with growth data of preterm infants fed control and high-EPA fish oil–containing formulas (Fig. 1) or control and low-EPA fish oil–containing formulas (Fig. 2).

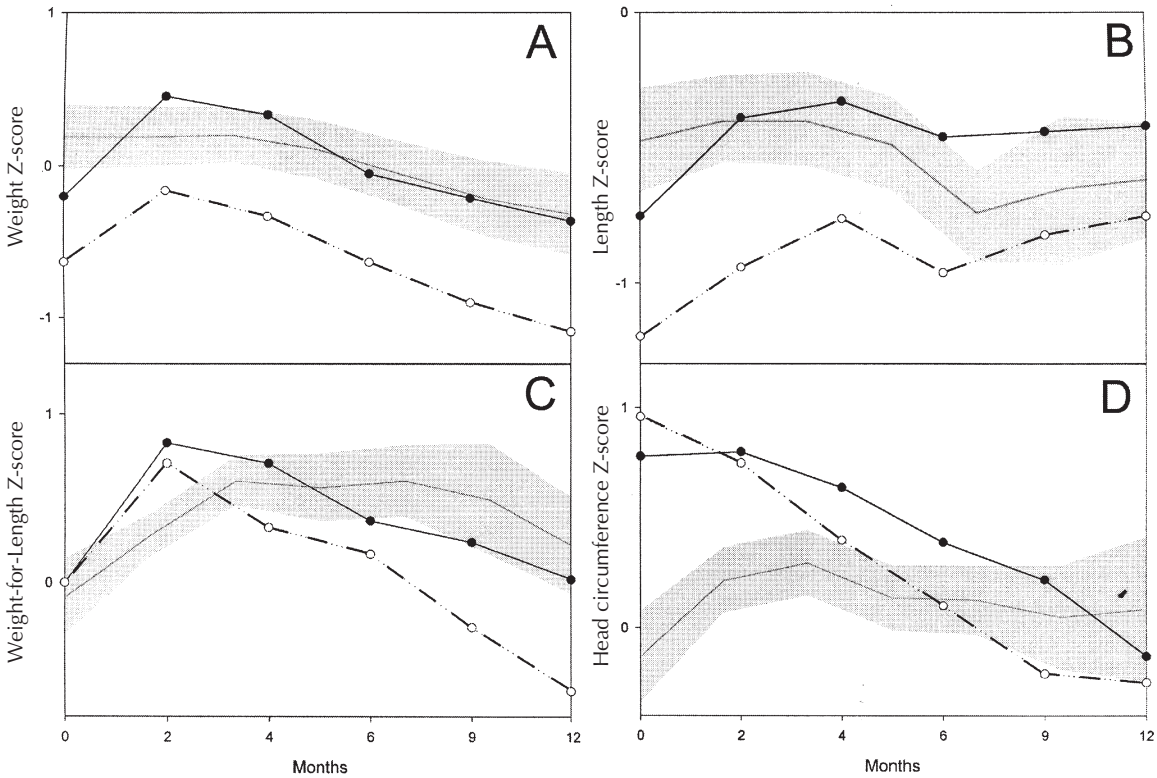


**FIG. 1.** The shaded areas are the normalized (±SEM) weight (A), length (B), weight-for-length (C), and head circumference (D) for 61 normal term infants enrolled from the same hospital and with similar demographics compared with the preterm infants fed control formula (●) and formula containing high-eicosapentaenoic acid (EPA) fish oil (○). See Reference 1 for population characteristics of preterm infants and other aspects of study design.

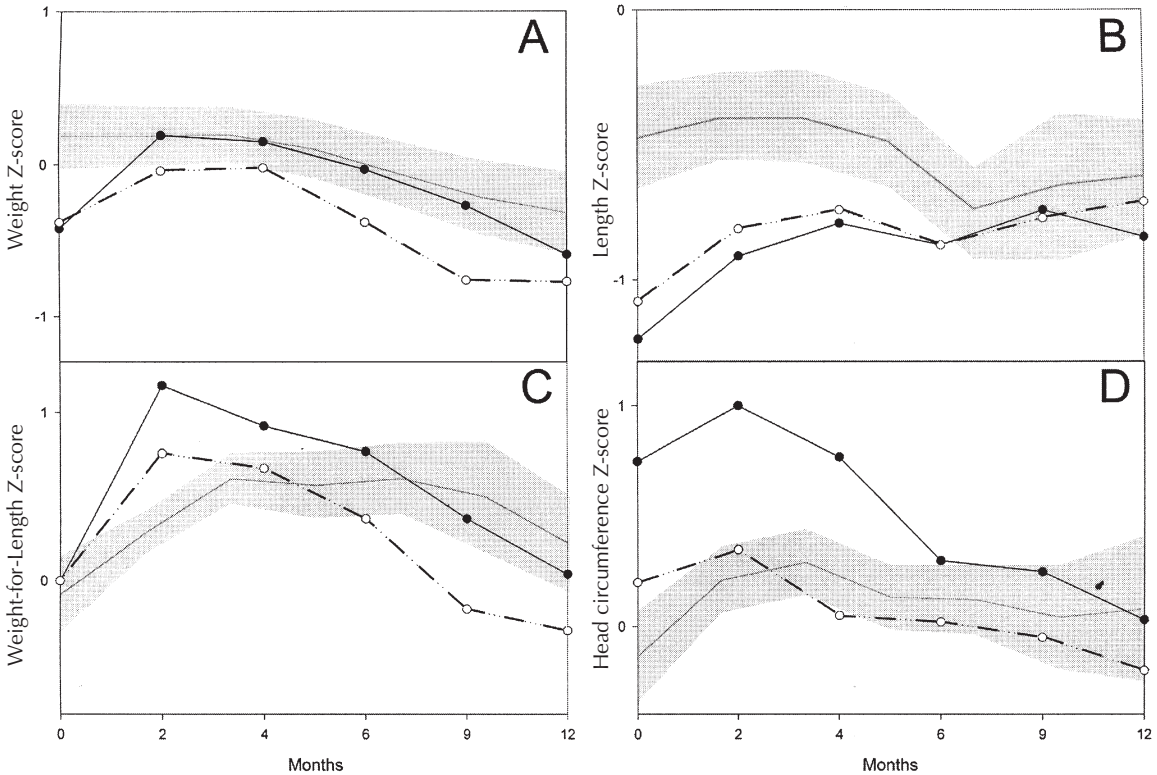


**FIG. 2.** The shaded areas are the normalized (±SEM) weight (A), length (B), weight-for-length (C), and head circumference (D) for 61 normal term infants enrolled from the same hospital and with similar demographics compared with the preterm infants fed control formula (●) and formula containing low-EPA fish oil (○). See Reference 2 for population characteristics of preterm infants and other aspects of study design, and Figure 1 for abbreviation.

Figures 1A and 1B illustrate the weight and length, respectively, of preterm infants fed the high-EPA fish oil–containing formula or the control formula. The randomized control group had caught up to and exceeded the 50th percentile for weight of the reference group of term infants by 2 and 4 mon. From 6 to 9 and from 9 to 12 mon, the mean normalized weight of the preterm control group declined progressively but was virtually identical to that of the reference group. Normalized length of the preterm control group exceeded that of the reference group by 4 mon corrected age and remained greater for year 1 of life. The normalized weight of infants fed the high-EPA fish oil–enriched formula showed the same pattern as the reference group and was significantly lower than the randomized control group of preterm infants at every age studied (0.4–0.8 SD). The linear growth of the experimental group was 0.2–0.3 SD below that of the reference group by the corrected age of 12 mon, but it was 0.5–0.6 SD below that of the randomized control group. Compared with the growth of the reference group from the same population, it is clear that the control randomized preterm group grew as well (weight) or better (length) than infants born at term. If catch-up growth in the supplemented group was slower than in the controls, it was also clear that the linear growth achieved by 12 mon corrected age in the experimental group was only slightly less than that of the reference group. In fact, growth of these ~1.1 kg birth weight infants was as good as or better than that reported for much larger infants studied in a multicenter trial during the same period (43).

The linear growth of preterm infants fed the low-EPA formula was unaffected by diet at any age (2; Fig. 2B). It can be seen from Figure 2A that a difference in weight began to emerge at the adjusted age of 2 mon, when the experimental formula was stopped. At 12 mon corrected age, the weight of preterm infants fed the experimental and control diet was the same but was 0.3–0.4 SD lower than the reference group. Linear growth of the two preterm groups was virtually identical from 0 to 12 mon of age. By 6 mon of age, both groups were 0.2 SD lower than the linear growth of the reference group, and at 9 and 12 mon, both groups were within the SEM for linear growth of the reference group (Fig. 2B). It can therefore be concluded that, compared with the growth of healthy term infants recruited from the same hospital and having the same demographic characteristics, the growth of preterm infants randomized to receive n-3 fatty acids from a low-EPA fish oil appeared to be only minimally compromised.

Head circumference data (Figs. 1D and 2D) indicate the rather remarkable variance of head circumference of preterm infants from both the NCHS reference and the term reference group from the same population. It is not clear at which point the variance in head shape due to prematurity might give way to the actual size of the brain as a predictor of head circumference in these preterm infants, if ever. It can be stated simply that head circumference of all four study groups fell within 1 SEM of the reference group by 12 mon corrected age.

Taking into account the observations described above, it can be argued that the apparent effects of ALA intake on weight gain of term infants, and of fish oil on the weight (Figs. 1A and 2A) and weight-for-length (Figs. 1C and 2C) of preterm infants are of questionable clinical and/or physiologic relevance (4). The effect of n-3 PUFA on growing infants may prove to be desirable in certain aspects of body composition, particularly if it reflects a lower rate of fat deposition, as the lower skinfold thickness and the reduced weight-for-length ratio suggest. Regardless of whether infants were fed high-EPA fish oil for 11 mon (Fig. 1C) or low-EPA fish oil for 5 mon (Fig. 2C), weight-for-length fell below 1 SEM of the reference group at 6, 9, and 12 mon corrected age. This was not the case for the randomized control group. With the increasing incidence of obesity in infants, older children, and adolescents, it seems wise to begin devising strategies for reducing fat deposition without reducing deposition of lean body mass, even during infancy. Modification of the quality of dietary fat, with or without a modest decrease of nonprotein energy as fat, conceivably could modify weight gain of infants without adversely affecting linear growth.

## PHYSIOPATHOLOGY

The mechanism whereby PUFA may affect the rate and/or composition of weight gain in infants is not clear. None of the studies performed in either term or preterm infants provide specific clues to address this issue. In general, differences in rates of growth among groups of similar infants reflect differences in nutrient intake, nutrient absorption, and/or nutrient utilization/deposition. Most of the studies cited included an assessment of intake, and differences between groups were minimal. None included an assessment of nutrient absorption, but there is little reason to expect differences in this area. How n-3 fatty acids affect nutrient utilization and/or deposition is not known, but data recently obtained, primarily in rodent models or cell cultures, offer potential clues.

Some studies have shown that growth is significantly correlated with the plasma and/or erythrocyte phospholipid content of AA and other n-6 fatty acids (1,2,22). Others have shown a negative association between DHA status and growth (31), but most have shown no significant correlation between any fatty acid and growth (24,38). Moreover, the correlations that have been reported, although significant, were usually quite weak. These discrepancies among studies raise the question whether the correlations between fatty acids and growth represent a cause-and-effect relationship. Because the mean AA content of erythrocyte phospholipid is usually lower in n-3 fatty acid–supplemented vs. unsupplemented groups, correlations between AA status and growth would be expected even if the effects of n-3 fatty acids on growth and on the AA content of plasma and/or erythrocyte phospholipid were independent. Therefore, it remains controversial whether the decrease in n-6 PUFA status (i.e., AA) or the increase in n-3 PUFA intake plays a major role in lowering weight gain and skinfold thickness in infants fed a formula supplemented with n-3 fatty acids, and the mechanism of the growth effects remains unclear. On the other hand, it should be noted that a decreased rate of growth is a prominent feature

of n-6 fatty acid deficiency (44,45). Indeed, infants who were fed a formula based on skim milk, or who were kept on lipid-free parenteral nutrition, developed growth retardation with dermatitis. The role of n-6 PUFA deficiency in growth was confirmed when growth failure and other symptoms could be prevented or cured if LA or AA were added.

A large body of literature suggests that the fatty acid composition of dietary lipid and, hence, tissue lipid membranes influence both hormonal signaling events and the molecular events governing gene expression (46,47). Numerous studies, performed primarily in rodents, have demonstrated that the ingestion of fats rich in n-3 PUFA (e.g., a large amount of fish oil) suppresses hepatic lipogenesis (47,48), reduces hepatic triglyceride output (49), enhances ketogenesis (50), and induces fatty acid oxidation in both liver and skeletal muscle (51,52). Further, these changes in fuel metabolism are accompanied by a decrease in body fat deposition (51,53). Although the repartitioning of fatty acids away from triglyceride synthesis and toward oxidation may explain the lipid-lowering effects of dietary n-3 PUFA, it cannot explain why fat deposition is less efficient in animals fed diets that are rich in n-3 LC-PUFA (46). Such an explanation requires the induction of biochemical processes that enhance heat production and are inducible by dietary PUFA. In this regard, peroxisomal fatty acid oxidation and the uncoupling of mitochondrial oxidative phosphorylation appear to fulfill this requirement. Indeed, there are data suggesting that PUFA induce the thermogenic pathways of peroxisomal fatty acid oxidation and uncoupled mitochondrial oxidation, and also reduce weight and fat deposition (46,47,54). The mechanisms by which PUFA are responsible for these metabolic outcomes appears to be exerted largely at the genomic level. Peroxisome proliferator-activated receptor (PPAR), particularly PPARα, which is expressed primarily in the liver, appears to be the major transcription factor that coordinately up-regulates genes encoding proteins involved in lipid oxidation and thermogenesis. Of major interest is the finding that several fatty acids bind to PPARα. In this respect, oils that are rich in n-3 PUFA (e.g., fish oils) appear to be even more efficacious than vegetable oils rich in n-6 PUFA. Thus, the activation of PPARα by n-3 PUFA, with up-regulation of the transcription of genes involved in lipid oxidation and thermogenesis, if it occurs *in vivo* in infants, could explain the lower rates of weight gain and/or skinfold thickness observed in infants fed a formula supplemented with n-3 PUFA.

In addition to their role in the expression of genes involved in lipogenesis and fat oxidation, PUFA appear to function in the control of adipogenesis. In this regard, adipogenesis is influenced more by n-6 PUFA than n-3 PUFA because PUFA themselves appear to have moderate effects on this regulation compared with various arachidonate metabolites. Mature adipocytes and cultured preadipocytes produce significant amounts of prostaglandins (PG) from AA ($PGE_2$, $PGD_2$, $PGF_{2\alpha}$, and $PGI_2$). PG have been shown to modulate preadipocyte differentiation by either promoting or inhibiting differentiation (55). This suggests that the balance between the two pathways is important and could determine the net effect of PG on the differentiation program. In this regard, it has been observed that 20:5n-3 inhibits fat cell differentiation, which suggests that 20:5n-3 may give rise to a biologically less potent activator of adipose differentiation (56). To date, there is more evidence to suggest that AA metabolites may promote rather than inhibit preadipose differentiation (55). Specifically, some of the PG were shown, at micromolar concentrations, to stimulate adipose differentiation *in vitro* through their interaction with PPARγ (56,57). Possibly in coordination with other transcription factors, PPARγ activates target genes, which promote differentiation of preadipocytes to adipocytes, and appears to play a key role during the last steps of differentiation (55). On the basis of these findings, we may postulate that a decrease in AA status in infants may reduce adipocyte status, whereas an increase in AA status may promote adipocyte differentiation, and hence, fat accumulation and deposition. Furthermore, the observation that plasma and/or erythrocyte content of 20:5n-3 is higher in all studies showing a negative effect of n-3 fatty acids on growth of infants supports the hypothesis that a biologically less potent activator of adipose differentiation was produced from 20:5n-3.

To our knowledge, the proposed mechanisms described above (i.e, up-regulation of genes encoding for fat oxidation, thermogenesis, and regulation of adipose differentiation) have not been shown to occur *in vivo* in humans, although they have been shown to occur in rodents and cell cultures. It is also possible that several other mechanisms may explain how n-3 and n-6 PUFA can modulate growth. Indeed, AA and its bioactive metabolites mediate the secretion of several hormones associated with growth and metabolic functions. These include luteinizing hormone, prolactin, adrenocorticotropic hormone, and corticotropin-releasing hormone (58). The hypotheses described above are consistent with the lower rates of weight gain and possibly fat deposition observed in infants fed formulas supplemented with n-3 PUFA. Discrepancies in the possible effects of n-3 PUFA on growth among the studies may be explained by the observation that effects of n-3 PUFA on growth were observed in studies utilizing relatively large amounts of total n-3 PUFA (ALA alone or ALA + fish oil). This raises the possibility of a dose response or threshold value for the n-3 fatty acids to reduce rates of weight gain and fat deposition in infants. In this regard, data in rodents support the possibility of a dose response of n-3 PUFA on fat deposition. On the basis of the difference in magnitude of the effects of n-3 PUFA supplementation on growth and/or fat deposition between rodents and humans, it may be postulated that the intensity of the cellular effects of PUFA in rodents and humans may differ, with a much higher response in rodents than in humans (59). Nonetheless, it seems reasonable to postulate that the mechanisms involved are similar in both species.

In conclusion, it is clear that there are significant effects of n-3 fatty acids on growth of both term and preterm infants from the above summary of published sets of data. A more relevant issue, however, is the biological significance of this effect. It appears that the mean rates of increase in both weight and length of all groups (i.e., control or supplemented) in some studies were close to, or actually greater than the mean rates of normal

control term infants. This raises the question whether the growth effects observed have any biological significance (4).

The primary effect of n-3 fatty acids on growth appears to be inhibition of the rate of weight gain rather than the rate of increase in either length or head circumference. It could be argued that this effect may be desirable if it reflects a lower rate of fat deposition. Some biological data from both animal models and cell cultures suggest that n-3 PUFA reduces fat deposition and n-6 PUFA induces adipogenesis. These mechanisms, theoretically, result in lower rates of fat deposition in infants receiving an n-3 PUFA–enriched formula. In this regard, the lower skinfold thickness or weight-for-length ratio in some studies may be biologically relevant. However, the lack of information concerning the effects of n-3 fatty acids on body composition and energy expenditure of human infants makes it very difficult to suggest a precise mechanism for the apparent inhibitory effect of n-3 fatty acids on growth of infants.

Although such an effect on fat deposition is clearly desirable for adults, it is not clear that it would be desirable for infants, especially preterm infants. One could argue that different rates of growth between two groups of preterm infants could impose some risk for the slower-growing group. To achieve the same subsequent size as the term infants, the preterm infants must experience more rapid rates of growth (4). In this situation, any dietary factor that inhibits growth for any period of time decreases the likelihood of the preterm infant's achieving the same size as the infant born at term. But even in these infants, there are, to date, few data to support this argument, and the consequences of the lower rates of growth of infants fed n-3 fatty acid–supplemented vs. –unsupplemented formulas are not known.

From this review, it is obvious that further studies are warranted not only to elucidate the mechanisms involved in the regulation of growth by LC-PUFA, but also to assess the quality (i.e., body composition) of early growth in infants fed LC-PUFA–supplemented formulas and to determine the long-term effects of various n-3 and/or n-6 LC-PUFA supplements on adiposity and growth.

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# Models and Methods for Studying Behavior in Polyunsaturated Fatty Acid Research

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**ABSTRACT:** This report examines a range of models and procedures applicable to polyunsaturated fatty acid (PUFA) research and considers their relative merits. Considerations pertaining to cost, efficiency, and scientific rigor are of particular interest. Parallel activities in other areas of behavioral neuroscience, such as behavioral pharmacology and toxicology, that have profitably exploited various behavior designs for the study of human and animal cognition are noted. Special attention is given to the utility of operant conditioning models and schedules of reinforcement, which are currently underrepresented in PUFA research. Investigations of analogs of complex human behavior as well as implications for generalizing laboratory results to clinical phenomena are addressed.

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The study of fatty acids (FA), particularly the essential fatty acids (EFA), has generated numerous investigations relative to behavioral correlates in sensory, cognitive, learning, and similar systems. There are constant reminders of the need to focus on establishing functional and/or behavioral implications of insufficient levels of EFA or their balance (ratio). From the perspective of the long historical developments in biochemistry and the neurosciences, behavioral studies in FA research are relatively new, and the participation of behavioral scientists is sorely underrepresented. Although diverse designs have been employed, it would seem useful to pause and reflect on the rationale that underlies the selection of a particular animal or human subject, or the specific assessment protocol that is chosen. It may also be useful for nonbehavior specialists to appreciate how the practitioners of experimental psychology are likely to evaluate a protocol of choice. Important works by leaders in the field of nutrition and FA research (e.g., 1–6) have already properly noted the conceptual issues that confront the execution of behavior studies, the caution required in interpreting the findings, and the frequent failure to establish evidence for behavior deficit in the face of known changes in membrane or neurochemical composition. This report will not attempt to provide a definitive summary of all of the published work nor to extricate conflicting findings. Rather, it will attempt to sketch the outlines of domains of concern, to reflect on the utility and failings of different animal and infant human analogs, and to clarify the advantages that might be realized by adopting alternative strategies. Elegant and sophisticated experimental investigations by physiologists, biochemists, biophysicists, and neurobiologists often appear to reflect an incomplete appreciation of some of these issues, and it is for them as well that some basic review of established experimental psychology methodologies is provided.

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Abbreviations: DRL, differential reinforcement of low rate; EFA, essential fatty acids; FA, fatty acids; PUFA, polyunsaturated fatty acid(s); VI, variable interval; VR, variable ratio.

## SELECTED CRITERIA FOR MODEL SELECTION

Initially, the dependent variables for assessing behavior changes are arrived at by creating a test condition analogous to an anecdotal report by a patient, or from observations in an otherwise uncontrolled environment. Behaviors of interest are also defined by reasonable expectations of functional change, when chemical or structural changes appear in an experimental or clinical preparation. It is quite reasonable therefore to expect some changes involving visual function when phospholipid changes in the retinal membranes are noted secondary to a FA-deficient diet. However, the varieties of measurements that can describe "visual function" are many, and not all visual behaviors may be sensitive to the physiological change. The design of a test to establish changes in more complex forms of functioning, such as "cognitive functions," becomes even more difficult, not least because of the difficulty to operationally define "cognitive function," a problem that has been recognized not only among behaviorists but also by other neuroscientists (7,8). When extrapolation to clinical conditions is undertaken and clinical efficacy is predicted from an animal study, there is the additional major problem and complication of a lack of resemblance between the models used and the clinical condition, as well as the diversity of the potential mechanisms of action of the test compounds (9). In an issue related to the EFA, when the subject studied is the fetus or infant, it was noted by an expert panel that "mechanisms appear to exist in a normal fetus and infant that can compensate for some nutritional and environmental diversity, which may obscure some of the effects of *trans* fatty acids" (10). Similarly, the attribution of behavior deficits or restorative effects to the presence or absence of EFA is thereby complicated as well.

Primarily problematic to the behavior researcher is the ambiguity of terms used to describe a function or ability for which the model was created. Deficits in "visual function," "memory," or "learning" are examples of performances of

interest; yet, depending on the conception of the investigator, each of these hypothetical states or functions can be assessed by a variety of procedures and the respective outcomes may not necessarily agree with one another. We have learned our lesson from years of "IQ" testing or "anxiety" research that the multivariate nature of the concept cannot be comfortably assessed by any single test result. Similarly, to evaluate the role and utility of FA in behavior, it is essential that the specific features of the concept or treatment in question be sufficiently operationalized to permit a translation for an experimental protocol. For example, before claiming any global advantages for nutritional "supplementation," it is necessary to distinguish among three conditions (at least) in which "supplementation" can be used: (i) as a prophylactic and protective agent, (ii) as a restorative agent to reverse or compensate for deficient diet or levels of FA or docosahexaenoic acid, for example, or (iii) as a facilitative agent to improve some aspect of behavior in otherwise nondeficient organisms. Especially problematic is the lack of precision with regard to discussions pertaining to cognitive function or chronic disorders, in which the implementation of an animal or human infant model that is able to integrate the biological, clinical, and behavioral aspects efficiently is a challenge of long standing (11,12).

It is also worth noting the obvious truism that significant findings in animal models may not translate to humans, and that an outcome in one animal species does not ensure similar outcomes for all species or even for members of the same order. Perhaps counterintuitive are the findings of different responses to an n-3 FA deficiency between rats and mice. For example, water maze learning was significantly different between mice fed n-3 FA-deficient diets and those fed sufficient diets (13), a result that was not reproduced for rats (14). Some inconsistencies in experimental reports may be attributable in part to diets in which n-3 alone is rendered deficient, compared with diets in which both n-3 and n-6 are deficient. In the latter case, several reports have shared the view that "we are afraid that these maze tests are too simple to evaluate behavioral changes induced by n-3 fatty acid deficiency in the presence of n-6 fatty acids" (13). In the case of the "dual deficiency" condition, the effectiveness of the introduction of the experimental intervention may depend upon the developmental stage of the animal, thereby adding yet another complication to understanding the deleterious effects of an n-3 deficiency, such that a deprivation of these fatty acids during the gestational phase led to irreversible impairment in Y-maze learning, whereas lactational deprivation led to impairment that could be remediated, and postweaning deficiency had no observable effect on learning performance (15). In the case of human studies, the selection of preterm vs. healthy term infants is a critical consideration in establishing the full EFA requirements and the ultimate influence on subsequent development and cognitive function (16,17). Above all, investigators are often challenged to ensure that any learning, memory, or cognitive changes attributable to the status of FA cannot readily be explained by alterations in maturational, visual, or motivational systems.

## EXTRAPOLATION TO COMPLEX BEHAVIOR

To the extent that biochemical pathways may implicate several behavior systems concurrently, it is quite reasonable to undertake a study whose outcome is expected to approximate behaviors that would be encountered in a clinical or "real world" setting. For example, given that FA deficiency has reliably been shown to affect certain visual performance measures, selected changes in the immune system, and modulation of serotonin, for example, one would be justified to expect observable changes in performances for tasks in which the separate biological systems are known to exert their influence. As such, studies of maze learning performance, memory, avoidance, visual discrimination, and a host of other comparable behavioral performances are essentially extrapolations from laboratory tasks or contrived experimental settings. The interpretations derived from those data are then employed as support for reflecting the patency of a biological/behavioral system, which is itself a hypothetical construct. Often elegant attempts are devised to simulate a "memory" task to be presented to an infra-human or infant human subject, and to interpret the outcome in a manner consistent with our appreciation of what is intended and understood by the concept of "memory." To be sure, "memory" is a requisite condition even for the simplest model of conditioning, far more complicated than what is popularly presented in the introductory psychology textbook. It is quite clear that an animal's conditioned response to a signal does not depend simply on the contiguity between the stimuli in the experiment, but does depend largely on the predictive value of that stimulus to alert the performing subject that something of consequence is about to happen. Without a doubt, the subject must be capable of remembering the critical features of earlier trials, and must be able to "process information" relevant to its success in the experiment. But conditioning models by themselves generally do not qualify as tests for memory or cognitive abilities, although there are options for conducting such tests. The literature dealing with the design and execution of different behavior models, species-specific characteristics, selection of dependent variables, and a theoretical framework for undertaking the behavioral experiment is vast. Publications abound with discussions and guidelines for properly understanding the experimental results, to ensure some measure of validity and to generalize to performance outside the laboratory setting. Separate discussions relevant to research in mice, rats, primates, and humans are available, and although a complete review or list of bibliographic citations is beyond the scope of this paper, a few publications can be offered as representative and as a valuable resource to provide an entry to the larger library of publications (18–23). Common to most of these scientific efforts is the utilization of operant conditioning protocols. Because operant conditioning dominates many of the activities surrounding animal research (in particular), and because it often serves as the basis for the study of many cognitive skills in animals and infants, it is useful to examine the essential nature of both the experimental proce-

dures and some of the underlying theory that define this methodology.

## SCHEDULES OF REINFORCEMENT, TIMING, AND IMPULSIVITY

The familiar Skinner box is, generically, a relatively restricted environment that contains provisions for the subject to respond (a manipulandum), for the response datum to be collected (counted, measured, recorded, depending on the nature of the data), for signals or cues to be presented, and for rewards (reinforcers) or noxious stimulations to be presented. It serves as the popular experimental setup for examining behavior under a wide variety of conditions. In addition to the study of basic principles of learning, the Skinner box allows for investigating behaviors that seem to meet the requirements for modeling more complicated constructs. Rather than discrete trials, the conventional operant design allows the experimenter to examine a stream of behavior as the subject engages in responding throughout the session. The strength of the correlation between the occurrence of a stimulus or environmental event and a subsequent response, together with the absence of the response in the absence of the stimulus or event, is described as "stimulus control." A powerful tool in the world of stimulus control derives from an observation made by Humphreys over 60 years ago, and for a while known as Humphreys' paradox, that learning trials orchestrated under conditions in which rewards are administered continuously whenever a correct response is made leads to extinction (or decrease) in the learned response at a faster rate than decreases in comparable learning when rewards are made available on some (but not every) occasion in which a correct response occurs. In other words, there is a greater resistance to extinction and forgetting following learning under conditions in which rewards are made available only some of the time. True for all organisms, this phenomenon, the Partial Reinforcement Effect, which was further developed by the work of B.F. Skinner, established formal paradigms of reward parameters for implementing partial reinforcement, i.e., Schedules of Reinforcement. Each of the schedules, or rules that govern the conditions when an experimenter will make reinforcers available, is an instance of Partial Reinforcement. Interestingly, however, the nature of the reinforcement schedule generates a pattern of responding, i.e., a "style of learning," that is characteristic for the respective schedule. Rate of responding, frequency and duration of interresponse pausing, and local changes in response rate are but some of the molecular aspects of an operant performance that may be observed in the visual record of a cumulative response graph or in the digitized information that can be presented as a table. In addition, by imposing restrictions, linkages, or conditional criteria, each schedule offers possibilities for examining analogs of complex human and animal behaviors, including but not limited to extrapolations to functions such as choice, counting, memory, and timing. Other cognate disciplines in the neurosciences such as pharmacology and toxicology have profitably investigated the effects of various compounds, stimulations, or extirpations on the separate properties that are derived from schedule performances.

A particularly sensitive index of operant performance concerns the role of time. The study of time in animals has been examined aggressively by experimental psychologists (24,25). To be sure, there are studies designed to examine time estimation (which itself can take a variety of procedural forms), whereas others purport to enable the study of elapsed time, and so forth. In particular, when demands are placed on the performing subject to learn to increase or decrease the response rate (i.e., frequency of responses per unit of time), one commonly observes a degradation of behavior in the presence of a chemical or surgical intervention. Although deserving of more examples in the FA areas, this variable has previously been shown to yield significant results for nutritional variables (26). The fact that time can play such an important role in modulating behavioral performance is indeed a testament to the "cognitive" nature of the task under study (27). The potential for meaningful assessments of FA effects on the separate aspects of operant responding and temporal stimuli would appear most promising and worthy of future research.

In addition to providing opportunities for evaluating molecular aspects of the learning task, schedules of reinforcement often set the occasion for behaviors that analogously reflect complex human activities. For example, if we consider a simple variable interval (VI) or variable ratio (VR) schedule of reinforcement, the subject will learn to respond at a fairly steady rate with rewards presented irregularly as programmed by the schedule. If we then add to this schedule a conditional restriction such as a differential reinforcement of low rate (DRL), the subject will learn to respond at a steady rate, but slowly enough to satisfy the conditions of the DRL requirement, i.e., the interresponse times will be increased. Response rates that exceed the DRL requirement are "not counted" toward the criterion needed to produce a reward. For many values of VI, VR, or DRL, subjects will usually be able to optimize performance on this amended schedule, as will be confirmed by distributions of the interresponse times and by noting that the responses produced the maximal amount of reinforcers that were programmed for the session under the conditions of the VI-DRL (or VR-DRL). The interpretation of successful DRL performance has often been offered in evidence to suggest an intact memory function or, of greater interest with poor DRL performance, to present evidence for impaired memory function. Such attributions have been offered for the effects of aging (28,29) as well as for the administrations of toxic compounds or diet and nutritional deficiencies (30). A more interesting, if somewhat anthropomorphic, interpretation of DRL performance has been offered to suggest that the subject is exercising "self control," i.e., refraining from responding at too fast a rate. When the subject is unable to meet the demands of the schedule, the interpretation takes the form of suggesting that the behavior gives evidence of "impulsivity." The experimental study of impulsivity has been of interest to the students of psychology, psychiatry, and

pharmacology (31,32), and the ability to provide an animal model constitutes a significant achievement. Although the DRL schedule focuses attention on the issues of environmental control with the attending power of reinforcement, considerable theoretical interest has been concerned with the role of the underlying neurobiology of this phenomenon (33). Perhaps most interestingly, the implications of impulsivity as a clinical disorder and its relationship to FA have not gone unnoticed (34). Other examples of using schedules (with variations) to represent complex and cognitive behaviors can be found.

## EPILOGUE

The armamentarium of behavior technologies for assessing the role of FA in sensory learning and higher functioning ("cognitive") in both humans and animals is far richer than the limited number of designs that have appeared in the published literature. Simple, one-trial or single-session tests for visual discrimination, maze, attention, or avoidance and escape performance may suffice for many conditions. However, if the concern is genuinely with relatively permanent changes that take place with exposure to rehearsal and practice (i.e., learning), there is much to be gained by adding other experimental protocols to the use of the T-maze or spatial maze, specifically to consider operant schedule performance. If the research question is directed at "cognitive" abilities, it is probably wise not to rely on maze performance for an answer. Schedules of reinforcement together with other laboratory procedures already exist for more directly assessing the effects of experimental variables on such behaviors as choice, fear, probability estimation, or the cost and utility of a reward. Such paradigms are more likely to approximate the investigator's intention of describing "cognitive" function and, in the context of the underlying FA biochemistry, are more likely to demonstrate the profound effects of lipid chemistry on behaviors that are essential for quality living

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# Recent Advances in Infant Cognition: Implications for Long-Chain Polyunsaturated Fatty Acid Supplementation Studies

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**ABSTRACT:** The assessment of cognitive function in early life has recently become an issue for consideration in long-chain polyunsaturated fatty acid (LC-PUFA) supplementation studies. This article reviews the various means by which such assessment has been done in past LC-PUFA supplementation studies and provides some background on recent advances in the measurement of infant cognition that may need to be considered when planning or designing future supplementation studies. These include (i) consideration of the specificity of LC-PUFA effects on cognition, (ii) inclusion of multiple tasks or levels of measurement as outcome measures, and (iii) a stronger emphasis on developmental processes in the design of such studies.

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Given the putative importance of some long-chain polyunsaturated fatty acids (LC-PUFA) to central nervous system (CNS) integrity and function, it has long been suspected that LC-PUFA play some role in mammalian behavior and behavioral development. The contribution of LC-PUFA to learning and cognition has been investigated in both animals (1–3) and humans (4,5). In addition, LC-PUFA deficiencies have been theoretically linked with a range of behavioral disorders from sudden infant death (6), to dyslexia (7), to schizophrenia (8).

For some time now, an understanding of the science of infant behavior and development has been relevant to scientists investigating the effects of LC-PUFA on behavioral development. There is an intriguing and plausible theoretical case to be made for the importance of LC-PUFA for the development of the CNS and retina during the pre- and postnatal periods (9–16). Thus, it has been hypothesized that early manipulations (either supplemented or deprived diets) of LC-PUFA should affect visual and cognitive development. Rather than wait until subjects reach maturity, many of these investigators have chosen to assess the effects of such manipulations with measures of visual and cognitive function in infant participants. As such, the period of infancy has served as a preliminary "proving ground" for such early nutritional manipulations.

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Abbreviations: AA, arachidonic acid; CNS, central nervous system; DHA, docosahexaenoic acid; ERP, evoked response potentials; HR, heart rate; LC-PUFA, long-chain polyunsaturated fatty acids; MDI, Mental Development Index.

## INFANT COGNITION AND LONG-CHAIN POLYUNSATURATED FATTY ACIDS (LC-PUFA)

A critical point in studies of this sort is the choice of the dependent measure. Studies of infant visual function have been widely conducted in this area for some time (e.g., Ref. 17), showing both positive (18–26) and null (27–31) effects for LC-PUFA supplementation. The choice of dependent variable within this realm is perhaps facilitated by the fact that there is a finite set of such measures for visual function (e.g., acuity or contrast sensitivity), and that the standardized measures that do exist (e.g., Ref. 32) possess good validity and reliability. However, other investigators have chosen to examine whether manipulations of LC-PUFA early in life affect broader cognitive functions. In choosing dependent measures designed to reflect early manifestations of cognition in infancy and toddlerhood, these investigators face a number of difficult issues (see Ref. 33), many of which remain unresolved among those in the field of early cognitive development. The purpose of this article is to try to address some of these fundamental issues on this topic for the audience that may be interested in the effect of LC-PUFA on cognition and behavioral development in early infancy. Although some data on standardized tests will be reviewed briefly, the primary focus of the paper is on recent advances in laboratory tasks of infant cognition. The aim is to provide a current description of this field and to explicate the implications of recent advances in this area for researchers interested in applying these tasks as outcomes in studies of early nutrition. Although the article will make reference to the extant data from clinical studies of LC-PUFA and behavior in infancy, methodological differences among those studies that may have given rise to differential outcomes on such measures will not be addressed here.

## MEASURES OF INFANT COGNITIVE/INTELLECTUAL FUNCTION

The first issue facing investigators interested in this topic of research is that there is no one widely accepted measure for assessing cognition or cognitive development in the infant/toddler age range. There are two broad classes of assessment tools. One class is comprised of normative, standardized tests of general behavioral or developmental function. The second class is comprised of laboratory tasks that have been designed to tap a specific cognitive process. In the sections that follow,

these two classes of assessment are generally described, along with a brief review of studies of LC-PUFA manipulations that have employed them.

## Standardized Normative Measures of Infant Development

There are a number of traditional standardized measures of infant cognitive or "mental" development. These include tests such as the Bayley Scales of Infant Development (34), the Kaufman Assessment Battery for Children (e.g., Ref. 35), the Battelle Developmental Inventory (e.g., Ref. 36), and the Denver Developmental Screening Test (e.g., Ref. 37). Many of these tests are derived from infant scales constructed during the 1920s or 1930s; their construction is based on the assumption that developmental outcome can be characterized as some aggregate of the infant's attainment of normative developmental milestones across a number of domains (motor, imitation, language). The summing of items across domains to achieve a single score is perhaps attributable to an implicit assumption that a construct of "general intelligence" exists in infancy (38–40).

Other more recent normative tests for the infant/toddler age ranges are constrained to a particular domain. Most prominent among these are tests of language or communicative development. These include the Sequenced Inventory of Communicative Development (41), the Reynell Developmental Language Scales (42), and the MacArthur Communicative Development Inventory (43). These tests avoid the pitfalls associated with the assumption of a general intelligence factor, but still calculate the optimality of outcome based on the comparison of the infant's performance against some standardized norms.

*LC-PUFA effects on laboratory-based tasks.* Standardized tests have been used in a number of follow-up studies of infant LC-PUFA supplementation. These include both positive and null findings. Carlson *et al.* (44) reported an advantage on the Bayley Mental Development Index (MDI) for preterm infants who were fed a LC-PUFA-supplemented formula. In a series of follow-up reports, Agostoni *et al.* (45,46) reported a strong correlation between LC-PUFA composition of the red cell membrane and improved neurodevelopmental performance on a standardized psychomotor developmental test (Brunet-Lexine Scale) at 4 mon of age. In a large-scale clinical trial, Scott *et al.* (47) reported no effects of LC-PUFA supplementation on Bayley MDI scores at 12 mon of age in a clinical trial, and in fact reported negative effects for LC-PUFA supplementation on some standardized language measures. Birch *et al.* (48) report an advantage of nearly half a standard deviation on the new Bayley scale at 18 mon for infants supplemented with docosahexaeonic acid (DHA) and DHA plus arachidonic acid (AA) until 17 wk of age. In contrast, Makrides *et al.* (49) found no differences among infants assigned to placebo, DHA supplementation, and DHA + AA supplementation groups at 12 and 24 mon on the Bayley Scales. In the last-mentioned study, however, it may be worth noting that breast-fed infants outperformed all three of the formula-fed groups on the Bayley at 24 mon of age. Clearly, the results of these studies are mixed; although positive outcomes were observed in a majority of the studies, these are tempered by null findings in larger-scale clinical trials.

## Laboratory Measures of Infant Cognition

Along with the standardized tests, some researchers have adapted a number of specific laboratory tasks as cognitive outcome variables in experiments and clinical trials of LC-PUFA. In general, such tasks were developed initially for purposes of conducting basic research on early cognitive development, and were designed explicitly to assess specific components of information processing or CNS function in infancy. As such, they are advantageous relative to the standardized tests because they presumably tap more specific cognitive domains with greater depth and accuracy, and their interpretation does not rest on assumptions about the structure of the intellect in infancy. Their disadvantage, however, is that they are not standardized in either their administration or interpretation (39,40). As a result, the outcomes of different studies may vary as a function of how the tasks are conducted. Furthermore, some ambiguity may exist in the interpretation of results. Let us first consider those tasks and/or measures that have been used in prior research with LC-PUFA research.

*Visual habituation.* In this task, a visual stimulus is repetitively presented to the infant, and the duration of the infant's looking is measured over the course of these presentations. The presentations may proceed on the basis of parameters chosen by the investigator ("fixed trial" habituation procedures), or may be provisional upon the infant's looking ("infant-controlled" procedures; see Ref. 38 for a procedural review). The duration of infants' looking declines across such repeated presentations, and this decline (or "habituation curve") is generally taken to reflect the infant's visual learning. The procedure is appropriate for infants from birth through ~10 mon of age. Many variables have been culled from this habituation curve that are thought to represent some index of the efficiency or rapidity of this learning. These variables include the slope or magnitude of the decline, trials to some criterion of decrement, or some measure of the infant's duration of looking (50).

*Paired-comparison tasks.* In novelty preference tasks, the infant is presented ("familiarized") with a visual stimulus to study for some amount of time, and then tested for whether s/he is able to recognize the stimulus by simultaneously pairing the familiarized stimulus with a novel one and allowing the infant to compare between the two. Recognition is generally indicated by some systematic preference for looking at one of the stimuli during this "paired-comparison" phase. Most typically, this is a preference for the novel stimulus, but under conditions of insufficient familiarization or increased cognitive demands, this preference may be expressed in terms of a preference for the familiarized stimulus (see Ref. 38). The initial familiarization period may be conducted using a habituation procedure. More commonly, however, the familiarization is

conducted by allowing the infant to study the stimulus for some fixed amount of time. A set of paired-comparison tasks (in which stimuli, familiarization times, and tasks protocols were standardized) comprised the Fagan Test of Infant Intelligence (FTII; 51). The FTII was a popular instrument through most of the 1990s in followup studies of various sorts, including some studies manipulating dietary LC-PUFA. This procedure is appropriate for infants from 2 to ~12 mon of age.

It is worth noting that some of the variables that can be culled from the habituation curve (most notably measures of look duration) are also available from an analysis of infant looking during the familiarization phase (52).

*Problem-solving tasks.* A number of tasks that tap higher-order cognitive functions have also been used in evaluating the effects of LC-PUFA supplementation. One is the "A-not-B" task (e.g., Ref. 53). Here, the infant is shown an object (e.g., a toy) being hidden under a cup or in a well at some location ("A"), and is then allowed to search and retrieve the object from the location. A brief delay (e.g., 5–15 s) may be imposed between the hiding and search. After several successful retrievals, the object is then hidden at a second location ("B"). Between 8 and 12 mon of age, infants will generally search at the first location rather than the second, that is, the infant searches at location A, not at B, thus giving rise to the name of the task. This perseverative error is generally attributed to the infant's inability to inhibit the previously successful response of searching at location A, and it has been taken to reflect immaturity of the dorsolateral frontal cortex.

Other problem-solving tasks have been used as well. These typically involve having the infant pull a string to retrieve an object or move one object out of the way to get to another. Thus, the infant must engage in some goal-directed behavior ("means") to attain a satisfactory outcome ("ends").

*LC-PUFA effects on laboratory-based tasks.* Several studies have documented significant differences between infants whose diets were supplemented with LC-PUFA (particularly DHA) in performance of such tasks. In general, the most consistent finding has been demonstrated for infant look duration culled from paired-comparison tasks. This finding has been generally interpreted as reflecting more rapid or more efficient visual learning. Werkman and Carlson (54) first observed this effect in preterm infants fed a DHA-supplemented diet through 9 mo of age. Assessment points were at 6.5, 9, and 12 mo of age. Carlson and Werkman (55) subsequently reported the same effect in 12-mon-old infants whose supplementation ended at 2 mon of age. Reisbick *et al.* (56) experimentally deprived infant monkeys of DHA and observed the opposite effect; the DHA-deprived infants looked for significantly longer durations. It is worth noting that, in each case, dietary supplementation affected look duration, but did not affect subjects' novelty preference performance.

Willatts *et al.* (57) also observed briefer duration looks during habituation in DHA-supplemented infants, but only in infants whose attentional patterns were characterized as more "disorganized" (i.e., with nonlinear/nonmonotonic declines). Willatts *et al.* (58) subsequently reported that DHA-supplemented infants performed better on means-ends tasks at 10 mon of age (see also Ref. 59).

## RECENT ADVANCES IN THE MEASUREMENT OF INFANT COGNITION

These findings are provocative and intriguing, and are generally in line with other results that suggest that cognitive function is affected positively by dietary LC-PUFA. These results have been obtained with procedures that might have been considered advanced or experimental in the early 1990s. However, infants' performance in each of the procedures outlined above may be interpreted in various ways (38,39). Indeed, there have been significant advances in the field of infant cognition since the mid-1990s to which researchers interested in using these tasks may profitably attend.

### Tasks for More Specific Processes and Underlying Substrates

The first advance is attributable to the growing influence of cognitive neuroscience in the field of developmental psychology. The scope of cognitive neuroscience is to identify and understand the neural substrates to which cognitive/behavioral processes may be attributed. The elucidation of such substrates is complicated by developmental processes (60), but substantial progress has been made in many areas, such as the development of the ability to plan and execute motor responses in infancy (53) and in the development of visual attention in infancy (61).

A by-product of this progress is a proliferation of tasks that tap specific processes and that presumably represent the function of specific underlying neural substrates. For example, in the area of visual attention, research has identified two separate neural substrates (see Fig. 1). One of these mediates the broadband detection of visual objects, the direction of attention to (and disengagement from) the location of such objects. The other mediates analysis of visual features and object recognition. These are sometimes referred to, respectively, as the "where" and "what" systems of visual attention. There is some crosstalk between the systems, but a primary pathway through which the systems interact is located in the frontal lobe. This frontal input may be thought of as providing "top-down" control over the two systems. For example, frontal areas probably provide the voluntary "holding" or maintenance of attention that is colloquially referred to as "attention span." A number of different functions have been identified for each of these systems, and for many of these functions, tasks have been adapted for use with human infants (see Table 1).

This point has several implications for follow-up research, such as LC-PUFA supplementation studies, that seeks to employ measures of infant cognition. First, some of the results of previous studies may be open to reinterpretation. For example, look duration in infants (which has been linked to DHA manipulations) has been interpreted as an index of rapidity or efficiency of learning. Recent work (62,63),

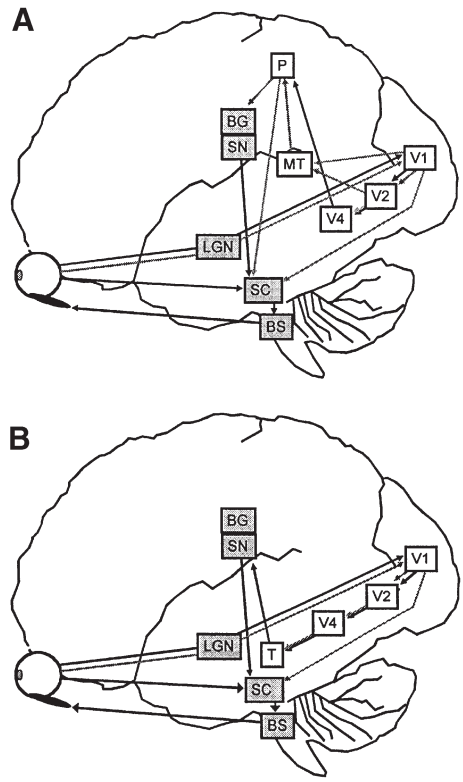


**FIG. 1.** Attention systems in the brain: (A) the dorsal or "where" pathway; (B) the ventral or "what" pathway. The figures represent schematics redrawn from various sources (78–81). Unshaded structures are cortical structures; shaded structures are subcortical. Legend: BG, basal ganglia; BS, brain stem; LGN, lateral geniculate nucleus; MT, medial temporal lobe; P, parietal lobe; SC, superior colliculus; SN, substantia nigra; T, temporal lobe; V1, V2, V4, areas of visual cortex.

however, suggests that look duration (at least during the middle portions of the first year) may reflect the infant's ability to disengage attention from visuospatial loci (a function of the dorsal, or "where" system). Interestingly, it has been argued that the paired-comparison task is mediated by the ventral attentional system (60). The finding that LC-PUFA affect look duration but do not improve paired-comparison performance (e.g., Ref. 56) supports the dissociability of the tasks, and also the possible specificity of the LC-PUFA effect.

**TABLE 1**
**List of Visual Attention Components Tested in Infancy[a]**

| |
|---|
| Spatial orienting |
| Smooth pursuit |
| Fast (involuntary/express) saccades |
| Disengagement of attention |
| Inhibition of return |
| Attention to visual object features |
| Sensitivity to color/form |
| Attention to color/form compounds |
| Intrastimulus shifting |
| Object cue dominance |
| Endogenous (voluntary) control of attention |
| Interstimulus shifting |
| Inhibition of saccades |
| Sustained attention |

[a]Adapted from Ref. 61.

A side implication of such a high degree of "modularity" of cognitive measures in infancy is that it is entirely possible to obtain effects that are not readily reconcilable. For example, how could Carlson and colleagues (54,55) and Reisbick *et al.* (56) observe improvements in look duration as a function of DHA status, but Scott *et al.* (47) observe significantly lower language outcome at 14 mon of age in DHA-supplemented infants? If one holds to a unitary approach to intellectual function, these findings make no sense at all. If, however, one considers the possibilities that the substrates reflected in attention are different from the one reflected in language, and may thus be differentially affected, then the findings appear far less anomalous.

A final implication to be discussed here arises from the fact that the number of dependent variables now available to researchers interested in conducting follow-up studies is indeed quite large. Unlike the standardized normative tests, whose construction and scoring rest on the assumption that the infant's performance can be reduced to a single score that reflects general intelligence or overall cognitive function, research suggests that these different functions are dissociable in terms of their underlying neural substrates and developmental course (61). As such, the choices that followup researchers make in evaluating the effects of LC-PUFA with such new tasks should be based on a strong theoretical position as to what function LC-PUFA might be expected to affect, and at what age point this effect might be expected to be manifest (39).

## Multilevel Measurement

A second major change in the field of measuring infant cognition that has occurred at the cutting edge of the field is that researchers no longer rely solely on behavioral measures, i.e., 5–10 yr ago, it would have sufficed to measure infant looking in the habituation or paired-comparison paradigms. However, it has become more common to see such behavioral paradigms bolstered with convergent measures (64). Such convergent measures can be psychophysiologic (e.g., heart rate; HR) or electrophysiologic (e.g., evoked response potentials; ERP) in nature. The increasing availability of high-density ERP systems (with measurement from as many as 128 electrodes on the scalp) nearly approximates neuroimaging (65,66), although the choice of paradigms is constrained to some degree with such increasingly sophisticated measurement systems.

The addition of such multilevel measurement allows for two things. First, it is possible to observe internal reactions to events within paradigms that heretofore have not yielded data. For example, in a recent study (67), 4-mon-olds were observed in a fixed-trial habituation procedure; measurement of looking was augmented by simultaneous recording of HR. It was observed that infants routinely showed brief but significant accelerations to stimulus presentations; that is, upon illumination of the stimulus, infants' HR increased. One striking finding was that infants who looked for long durations had

significantly greater HR accelerations than infants who looked for brief durations. Thus, the simultaneous measurement of HR provided a finding that would not have been available with only behavioral measures of attention.

Second, the addition of measures allows for a much finer and more precise analysis of cognitive components during attention. In particular, Richards and Casey (e.g., Refs. 64,68,69) have taken the characteristic HR deceleration that occurs during infant looking and have parsed the looking into three distinct phases, each of which putatively reflects different types of information processing (see Fig. 1). "Orienting," which is the initial portion of the deceleration, reflects a simple reaction to the detection of the stimulus. "Sustained Attention," which is the asymptotic portion of the decelerative response, is presumed to reflect the voluntary maintenance of attention to the stimulus. Finally, "Attention Termination" is a period during which HR begins to increase from its decelerative asymptote, and reflects the end of processing. The hypothesized characteristics of the latter two phases have been supported by research over the 1990s (64,68,69). Such a framework has been useful in studying individual differences in infant cognition; indeed, one of the defining characteristics of infants exhibiting prolonged durations during an infant-controlled habituation, relative to their shorter-looking counterparts, was increased amounts of attention termination (70). That is, longer-looking infants spent more time looking when their HR patterns suggested that they were probably not processing very much information.

It goes without saying that access to new measures and the ability to conduct finer-grained analyses in familiar paradigms has great potential for followup work of the type that LC-PUFA supplementation studies might take.

### An Emphasis on Developmental Process

One last advance that is relevant to the use of measures of infant cognition in short-term outcome research is not the direct result of improvements in technology or the influence of other sciences. It is, rather, an advance that may be attributed to a growing theoretical shift in the field of developmental psychology.

The study of development is, by definition, the study of change. Over the past 20 yr, there has been a growing emphasis on the consideration of developmental processes, rather than a simple focus on behavioral products at particular points in time (71–73). In the 1990s, researchers working with LC-PUFA dietary manipulations were attracted to measures of infant cognition largely because those measures had been observed to be correlated to some degree with performance on standard tests of intelligence (i.e., IQ) and language during childhood and adolescence (38). Thus, habituation and novelty preference measures were used primarily as substitutes for, or facsimiles of, a cognitive product (i.e., IQ); the attraction, however, was that these measures could be taken during infancy.

This mind-set reflects a focus on static cognitive "products" that might simply be "tapped" in some emergent or precursor form early in the lifespan. In this way of thinking, it should be possible to implement an intervention (here, some dietary manipulation of LC-PUFA), choose a measure of early cognition, and then take a "snapshot" at one point during infancy to see whether the intervention makes a difference. This approach is inspired by a "psychometric approach" to infancy that was encouraged in some earlier publications (e.g., 38,50). The psychometric approach is attractive in its parsimony and simplicity, in that it leans heavily on the assumption that there will be a putatively direct causal relationship between early and mature cognitive function. A major drawback to this approach, however, is that it essentially ignores the processes by which cognitive functions develop and evolve over the lifespan. Indeed, one particular study of the paths through which the continuity of cognitive function travels from infancy to childhood has revealed that the significant relationships between early and later measures are not direct, but are rather mediated through a series of complex variables across age (74).

The consideration of such complex and cascading causal effects in development is generally within the realm of developmental systems theory (e.g., Ref. 75). The growing importance of this approach in understanding behavioral development has several implications for research in the area of LC-PUFA supplementation.

First, there will be an emphasis on measuring the developmental course of cognitive function in infancy, rather than simply taking such measures in a "snapshot" or "slice" at one particular age point. Indeed, researchers will have to conduct more extensive longitudinal measurements and use measures of the developmental course of variables in assessing whether nutritional supplementation affects cognitive outcome. Our own recent work on the development of look duration (76) suggests that the developmental course of look duration is complex, and that different phases of the course may reflect the functional onset of different neural substrates. As such, an LC-PUFA supplementation follow-up study that assesses look duration at one (or perhaps even two or three) particular age points will not provide an adequate means to evaluate the effects of the supplementation over all of these phases. It should be noted, however, that relatively intensive longitudinal measurement is more the norm than the exception in the literature on early manipulation of dietary LC-PUFA. Most of the studies on infant visual function and infant visual attention have collected data at three or more age points.

A second methodological implication of adopting a broader, developmental systems approach is that researchers should consider whether variables other than LC-PUFA manipulations interact with the manipulations themselves. For example, it is possible that LC-PUFA supplementation has larger effects in populations in which environmental quality (e.g., socioeconomic status or caregiver responsiveness) is poor, compared with populations in which environmental conditions are more optimal. Many other examples of such variables can be proposed (e.g., individual differences in metabolism or reactivity), all of which might interact with nutritional supplementation.

A third and final implication is that small or transient effects observed at one age point may not be unimportant. For example, in a recent meta-analysis, SanGiovanni *et al.* (77) concluded that DHA supplementation produces an early but transient improvement in visual acuity. Such transience has caused some to dismiss the finding. However, the fact that a control group will show the same visual acuity as a supplemented groups at 10 mon of age ignores the possibility that the acceleration of the visual acuity curve experienced by the supplemented group may have a longer-term effect. For example, the improvement in visual acuity in DHA-supplemented infants appears during a time period in which it has been shown that the visual substrates of the CNS can be greatly affected by environmental input. The maintained focus upon the acuity variable misses the possibility that even a small early advantage in lower-order function may serve to affect higher-order functions at some later point.

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# Effects of Docosahexaenoic Acid on Retinal Development: Cellular and Molecular Aspects

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**ABSTRACT:** We have recently shown that docosahexaenoic acid (DHA) is necessary for survival and differentiation of rat retinal photoreceptors during development *in vitro*. In cultures lacking DHA, retinal neurons developed normally for 4 d; then photoreceptors selectively started an apoptotic pathway leading to extensive degeneration of these cells by day 11. DHA protected photoreceptors by delaying the onset of apoptosis; in addition, it advanced photoreceptor differentiation, promoting opsin expression and inducing apical differentiation in these neurons. DHA was the only fatty acid having these effects. Mitochondrial damage accompanied photoreceptor apoptosis and was markedly reduced upon DHA supplementation. This suggests that a possible mechanism of DHA-mediated photoreceptor protection might be the preservation of mitochondrial activity; a critical amount of DHA in mitochondrial phospholipids might be required for proper functioning of these organelles, which in turn might be essential to avoid cell death. Müller cells in culture appeared to be involved in DHA processing: they took up DHA, incorporated it into glial phospholipids, and channeled it to photoreceptors in coculture. Both Müller cells, when cocultured with neuronal cells, and the glial-derived neurotrophic factor (GDNF) protected photoreceptors from cell death. These results suggest that glial cells may play a central role in regulating photoreceptor survival during development through the provision of trophic factors. The multiple effects of DHA on photoreceptors suggest that, in addition to its structural role, DHA might be one of the trophic factors required by these cells.

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The enrichment in docosahexaenoic acid (DHA) of membrane lipids in the nervous system has stimulated the search for the possible functions of this fatty acid in brain and retinal neurons. In the retina, DHA is the major fatty acid of photoreceptor phospholipids (1), and several pieces of evidence suggest that a close correlation exists between this high content of DHA and correct photoreceptor function. A decrease in DHA content in retinal lipids is associated with alterations in the electroretinogram (2,3) and impairment of visual acuity (4,5). DHA plays an important role during the development of the vertebrate retina. A deficiency of this fatty acid or its precursors in infant formulas affects human retinal development (6–8), and supplementation of infant diets with long-chain polyunsaturated fatty acids improves visual development (9). The puzzling question regarding the roles of DHA in the development, structure, and functionality of the retina, and particularly photoreceptor cells, has been the subject of considerable research and has mainly been studied from a structural point of view. Several studies suggest that DHA-enriched membranes can favor rhodopsin conformational changes and function (10–12). However, the precise functions of DHA in the retina are far from being established.

Recent reports from our laboratory have shown a novel role for DHA as a survival factor for photoreceptor cells. This fatty acid is able to rescue photoreceptors from apoptosis, the most frequent pathway of programmed cell death, during the early stages of their development *in vitro* (13–15). Elucidation of the molecular and cellular mechanisms involved in the regulation of photoreceptor apoptosis is one of the major aims in this area of investigation, particularly with the recognition that apoptosis is the main mode of photoreceptor death in mouse models of retinitis pigmentosa (16,17). However, until now the identification of specific trophic molecules able to effectively prevent photoreceptor degeneration has been elusive. Three trophic factors, namely, epidermal growth factor, basic fibroblast growth factor, and ciliary neurotrophic factor, along with other molecules such as laminin, taurine, and retinoic acid have been shown to have either protecting or differentiating effects on photoreceptor cells (18). More recently, the glial-derived neurotrophic factor (GDNF), produced and released by glial cells, has been added to the list of survival-promoting molecules (19).

The mechanism of action involved in DHA protection remains to be established. It is also unclear how retinal cells, and particularly photoreceptors, get the DHA required for building their membrane phospholipids. It has been proposed that DHA is transported from the liver, bound to plasma lipoproteins to reach photoreceptor cells in the retina (20). Non-neuronal cells in the retina may also synthesize and/or process DHA and deliver it to photoreceptors. Glial cells appear to have multiple functions in the retina and have been shown to be a source of trophic factors, including GDNF, required for the survival and maintenance of neuronal cells (21,22).

In the present paper, we review DHA survival- and differentiation-promoting effects; show that GDNF and glial cells,

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Abbreviations: BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; DHA, docosahexaenoic acid; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GDNF, glial-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; PI, propidium iodide.

when cocultured with retinal neurons, can also rescue photoreceptors from cell death; and present evidence suggesting that glia may modulate photoreceptor survival through the provision of DHA and GDNF.

## MATERIALS AND METHODS

*Materials.* Albino Wistar rats bred in our own colony were used in all the experiments. Plastic 35-mm culture dishes and multichambered slides (Nunc) were purchased from Inter Med. Fetal bovine serum (FBS) was from Centro de Virología Animal (Cevan). Dulbecco's modified Eagle's medium (DME) (Gibco) was purchased from Life Technologies. Bovine serum albumin (fraction V; fatty acid-free; low endotoxin, tissue culture tested) (BSA), poly-DL-ornithine, trypsin, trypsin inhibitor, transferrin, hydrocortisone, putrescine, insulin, polyornithine, selenium, gentamicin, 4,6-diamidino-2-phenylindole (DAPI), fluorescein-conjugated secondary antibodies, propidium iodide (PI), palmitic, oleic, and arachidonic acids, anti-glial fibrillary acidic protein (GFAP) IgG fraction antiserum antibody, and paraformaldehyde were from Sigma Chemical Co. (St. Louis, MO). Secondary antibody, *Alexa 488*-conjugated goat anti-mouse, *Alexa 546*-conjugated goat anti-rabbit IgG, and Mitotracker were from Molecular Probes, Inc. Monoclonal antibody against rhodopsin, Rho-4D2, was generously supplied by Dr. R. Molday (University of British Columbia). Monoclonal antibody HPC-1 was a generous gift from Dr. C. Barnstable (Yale University). DHA was isolated from bovine retinas, by a combination of chromatographic procedures (13). [$^{14}$C]22:6 (specific radioactivity 160 mCi/mmol) was from NEN (Boston, MA). Solvents were high-performance liquid chromatography grade, and all other reagents were analytical grade.

*Retinal neuron cultures.* Purified cultures of rat retinal neurons were prepared by methods previously described (13,23), with slight modifications. In brief, 1–2-d-old rat neuroretinas were dissociated for 12 min with trypsin (0.1%) in $Ca^{2+}$–$Mg^{2+}$-free Hanks' balanced salt solution, and the resulting cell pellet was incubated with trypsin inhibitor. The cells were sequentially rinsed, resuspended in a serum-free, chemically defined neuronal medium, and then subjected to a gentle dissociation with a glass pipette. About $0.8 \times 10^5$ cells/cm$^2$ were seeded on 35- or 100-mm diameter dishes or on coverslips placed in these dishes. Culture dishes and coverslips had previously been sequentially treated with polyornithine and schwannoma-conditioned medium (24).

*Pure glial cell cultures*. Pure glial cell cultures were prepared from 1–2-d-old rat retinas dissociated as described above; retinal cells were then resuspended in DME with 10% FBS and seeded at a density of $2.5 \times 10^5$–$3 \times 10^5$ cells/cm$^2$ on a 35-mm diameter plastic dish, with no pretreatment. The culture medium was routinely replaced every 2–3 d to eliminate neuronal cells.

*Coculture of retinal neurons with glial cells.* To obtain neuron–glia cocultures, the dissociated cells were resuspended in neuronal medium and seeded at a density of $2.5 \times 10^6$–$3 \times 10^6$ cells per 35-mm diameter plastic dish, with no pretreatment, as described above. After 2 d, the culture medium was replaced by DME with 10% FBS, to allow the growth of glial cells.

*Fatty acids and GDNF supplementation.* DHA, palmitic, oleic, and arachidonic acids, complexed with BSA, were added to 35- or 100-mm diameter dishes at day 1 in culture, 4–6.7 mM final concentration (13). The same volume of a BSA solution of the same concentration was added to control cultures. After different incubation times, the cells were thoroughly washed and scraped from the culture dishes, and lipids were extracted as described below.

GDNF was added to the cultures immediately after seeding the cells at a final concentration of 10 ng/mL in DME. A similar volume of DME was added to control samples.

*Lipid analysis.* Rat retinas were excised using a dissecting stereomicroscope and immediately homogenized for lipid extraction (25). To prepare lipid extracts from neurons in culture, the incubation media were removed, and, after the cultures were rinsed, the cells were transferred to glass tubes and centrifuged for 10 min at 1000 rpm (13). Neuronal lipids were then extracted (25). All samples were kept under an $N_2$ atmosphere.

To analyze neuronal fatty acid composition, free fatty acids were separated by thin-layer chromatography (13); the methyl ester derivatives of phospholipid and triacylglycerol fatty acids were then prepared (26) and analyzed by gas–liquid chromatography (13). Distribution of radioactivity among lipids was determined (15). Unlabeled lipids prepared from bovine retina were added to the samples as carriers.

*Labeling of glial and neuronal cells with [$^{14}$C]DHA.* To investigate the possible metabolic coupling between glial cells and retinal neurons, pure glial cell cultures were incubated for 6–10 d until they reached confluence. The serum-containing media were then replaced by chemically defined medium supplemented with [$^{14}$C]22:6 (0.1 μCi, 6.7 μM). Unlabeled 22:6n-3 was also added, in the amount required to reach the desired concentration. After 1 d, this medium was removed, and glial cells were thoroughly washed to eliminate nonincorporated label. Fresh serum-free, chemically defined culture medium was added (1 mL), and pure neuronal cultures, grown on coverslips, were then placed on top of and facing glial cells and cocultured for 3 d. The coverslips were then removed, and both glial cells and neurons were fixed for 1 h with 2% glutaraldehyde in phosphate-buffered saline (0.9% NaCl in 0.01 M $NaH_2PO_4$, pH 7.4) and processed for autoradiographic analysis (23).

*Cytochemical methods.* The cultures were fixed as described above and permeated with Triton X-100 (0.2%). Neuronal cell types were identified by immunocytochemistry with the monoclonal antibodies HPC-1, for amacrine cells (27,28), and Rho-4D2, for photoreceptor cells (29), as previously described (13), using *Alexa 488*-conjugated goat anti-mouse as the secondary antibody. Glial cells were identified by their flat morphology and by their immunoreactivity to GFAP, which selectively recognizes Müller cells in the retina.

Controls for immunocytochemistry were done by omitting either the primary or the secondary antibody. Dead cells were identified by fluorescence microscopy, incubating the cultures with PI (0.5 μg/mL final concentration in culture) for 30 min just before fixation (30). Nuclei integrity was determined with DAPI (14).

Mitochondrial activity was determined by incubating the cultures for 30 min before fixation with the fluorescent probe Mitotracker (0.1 μg/mL), which selectively stains active mitochondria in culture. Active mitochondria, displaying a bright red fluorescence, and damaged mitochondria, showing a pale red fluorescence, were identified by microscopy.

*Statistical analysis.* For cytochemical studies, 10 fields per sample were analyzed in each case. Each value reported from cytochemical, compositional, and lipid labeling studies represents the average of at least three experiments ± SD. Statistical significance was determined by Student's two-tailed *t*-test.

## RESULTS

*Neuronal and glial cultures.* When retinal cells were incubated in chemically defined medium and in the absence of specific trophic factors, they developed and differentiated mainly as photoreceptors and amacrine neurons (13,14). Photoreceptors have a small, round cell body with a single neurite at one end and sometimes display a connecting cilium at the opposite end, but they failed to develop their characteristic outer segments (Fig. 1A). Opsin was diffusely distributed over the cell body, as observed with the Rho4-D2 monoclonal antibody. Amacrine neurons are bigger than photoreceptors and have multiple neurites (Fig. 1B). Both cell types developed normally for 3–4 d. Photoreceptors then started an apoptotic pathway leading to the death of most of these cells by day 14 (14). In contrast, amacrine neurons continued their survival and differentiation for up to 17 d *in vitro*.

Dissociated retinal cells resuspended in 10% FBS in DME and seeded at high density on low adhesive substrata developed mainly as flat cells, with big oval nuclei, that survived in culture for several days. Using the GFAP antibody, these cells were identified as Müller cells (Fig. 1C).

*DHA had multiple effects on photoreceptor survival and differentiation.* Analysis of the fatty acid composition of neuronal lipids showed that DHA amounted to only 4–6% of the total esterified fatty acids; this proportion was the same as found in the 1–2-d-old retinas used for the cultures. While the percentage of DHA in retinal lipids increased steadily during development *in vivo*, it remained constant in neurons *in vitro* (Fig. 2A). However, addition of DHA to the culture medium showed that neurons had active mechanisms for taking up and esterifying this fatty acid, because the proportion of DHA in neuronal lipids increased to over 20%, reaching similar values to those found *in vivo* (Fig. 2A,B).

Unexpectedly, this increase in the proportions of DHA in neuronal lipids was paralleled by an increase in photoreceptor survival (13). Addition of DHA had a protective effect on photoreceptors, rescuing them from the degeneration pathway observed in control conditions. At day 10, about 80% of photoreceptors were apoptotic in control cultures, whereas in DHA-treated cultures this percentage was reduced to nearly 60% (Fig. 3A). This effect was specific for photoreceptors; amacrine cells showed no signs of apoptosis and were unaffected by DHA addition.

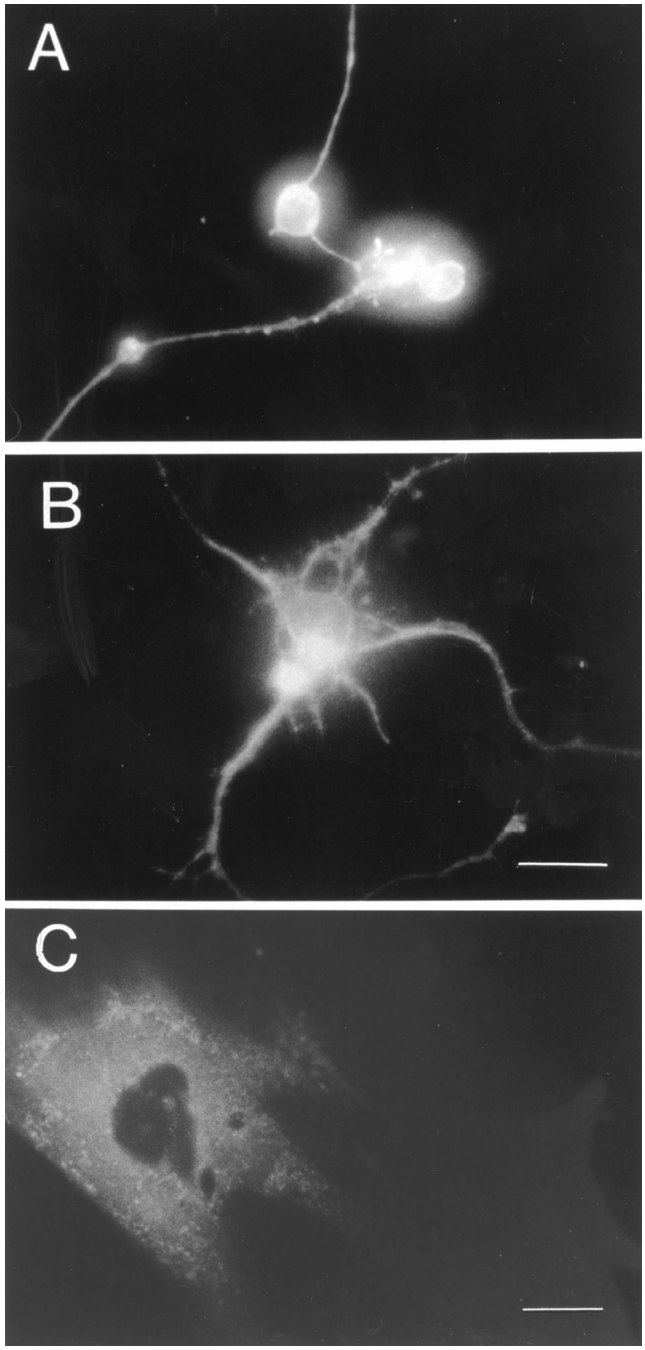


**FIG. 1.** Neuronal and glial cell identification. Fluorescence photomicrographs of (A) photoreceptors labeled with the Rho4-D2 monoclonal antibody, (B) amacrine cells visualized with the HPC-1 monoclonal antibody, and (C) glial cells, identified with antibodies to glial fibrillary acidic protein (GFAP). Rat retinal neurons were cultured for 7 d in a chemically defined medium; glial cells were cultured until they were confluent in 10% fetal bovine serum in Dulbecco's modified Eagle's medium. The bars represent 10 μm (A and B) and 20 μm (C).

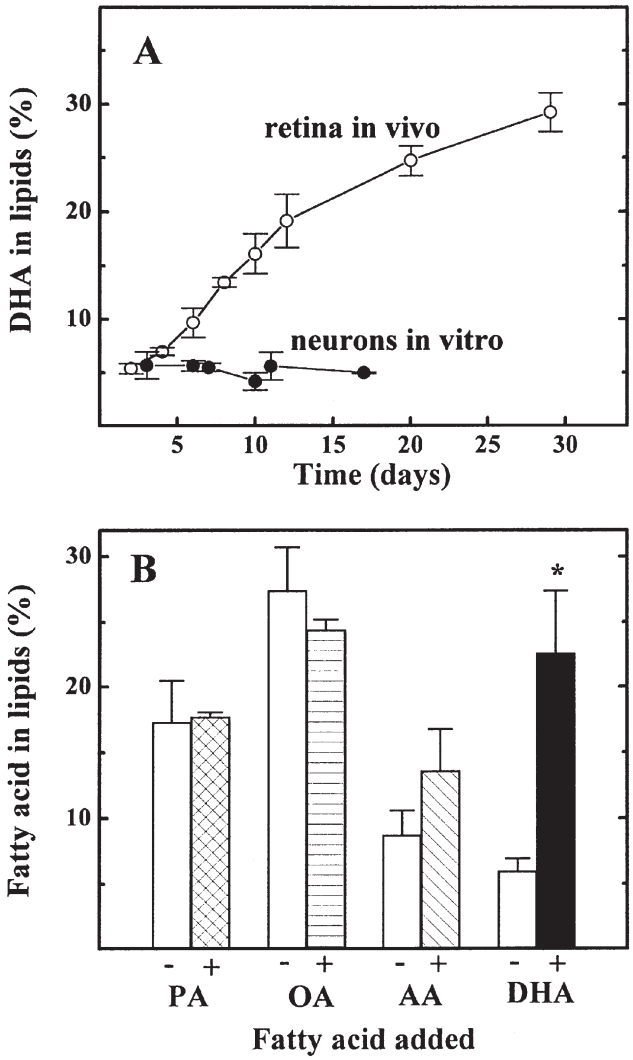


**FIG. 2.** Fatty acid composition of the retina *in vivo* and of retinal neurons in culture. Whole rat retinas were excised at different postnatal days *in vivo*, and retinal neurons were collected at different times of development *in vitro*. In fatty acid-supplemented cultures, the fatty acid (4 μM) complexed with bovine serum albumin (BSA) was added at day 7 *in vitro*; the same volume of a BSA solution was added to control cultures. At day 11, lipids were extracted, and the combined fatty acid composition of phospholipids and triacylglycerols was analyzed. (A) Variations in the percentage of docosahexaenoic acid (DHA) in lipids in the retina *in vivo* and retinal neurons *in vitro* during development. Values represent the means ± SD of three to seven samples. (B) Comparison of fatty acid composition of neuronal membranes in control cultures and cultures supplemented with palmitic acid (PA), oleic acid (OA), arachidonic acid (AA), or DHA. Bars represent the percentage of each fatty acid in lipids of retinal neurons in control cultures (–) and in cultures supplemented (+) with same fatty acid. Mean values ± SD of at least three samples are given. *Statistically significant difference with respect to control cultures ($P < 0.05$).

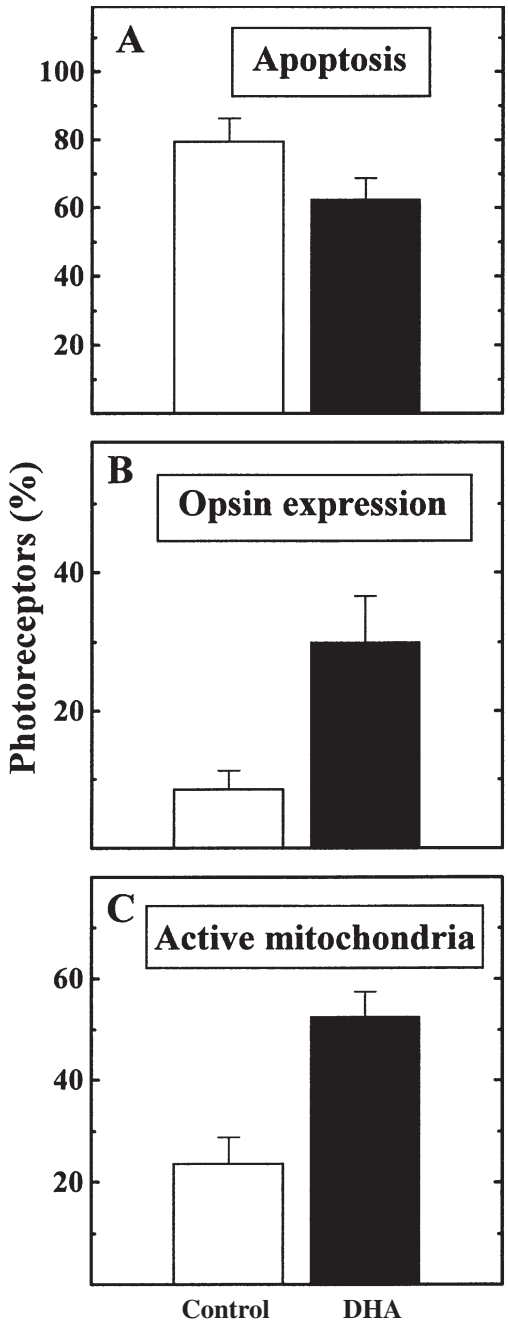


**FIG. 3.** Effects of DHA on photoreceptor survival, differentiation, and mitochondrial activity. Pure neuronal cultures were supplemented at day 1 with either 6.7 μM DHA or with a BSA solution (control), as described in Figure 2. Cells were fixed at day 10, and (A) photoreceptor apoptosis was determined by counting the number of these cells with fragmented nuclei, labeled with 4,6-diamido-2-phenylindole (DAPI); (B) opsin expression was evaluated by counting the number of Rho-4D2-positive photoreceptors; and (C) mitochondrial activity was analyzed with the fluorescent probe Mitotracker. Mean values ± SD of at least three samples are shown. See Figure 2 for abbreviations.

Even when photoreceptors displayed such characteristic apoptotic features as fragmented nuclei, they maintained some basic functions for several days. At early stages of apoptosis, most cells had a normal appearance and even expressed opsin. Depending on the severity of the damage, nonviable cells could be visualized by the pale to bright red fluorescence they exhibited after incubation with PI. At day 1, about 40,000 photoreceptors/dish were nonviable; by day 10, this number had sharply increased, and about 700,000 cells/dish were PI-positive (Fig. 4). Addition of DHA markedly reduced the number of nonviable cells, to about 470,000 photoreceptors/dish (Fig. 4).

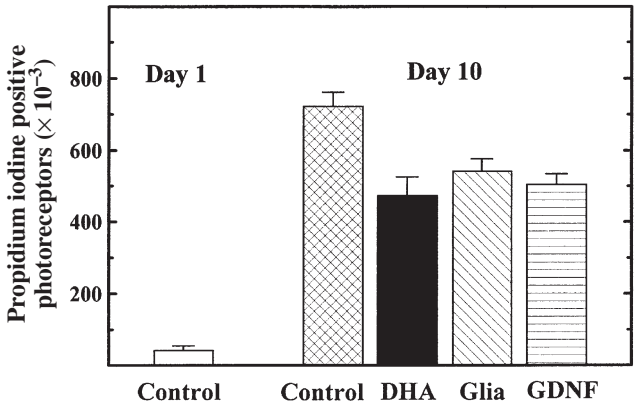


**FIG. 4.** Effect of DHA, glial cells, and glial-derived neurotrophic factor (GDNF) on photoreceptor survival. Pure neuronal cultures supplemented with either DHA, as described in Figure 2, or GDNF (10 ng/mL), added immediately after seeding the cells or neuron–glia cocultures, were incubated for 10 d. Dead photoreceptors were identified with propidium iodide (PI). The bars represent the number of dead, PI-positive photoreceptors in each culture condition, as means ± SD of at least three samples. See Figure 2 for abbreviations.

To find out if these effects were specific to DHA, neuronal cultures were supplemented with other fatty acids, such as palmitic, oleic, or arachidonic acid. The fatty acid composition of neuronal membranes remained the same upon addition of these fatty acids (Fig. 2), because none of them was able to accumulate in membrane lipids. Concomitantly, these fatty acids were unable to stop photoreceptor degeneration and had no effect on apoptosis (14). Hence, DHA was the only fatty acid having an anti-apoptotic effect and this effect, was consistent with its increased proportions in neuronal lipids.

Searching for clues to understand the anti-apoptotic activity of DHA, we analyzed characteristic parameters of photoreceptor differentiation and found that both the development and the differentiation of these cells were enhanced upon DHA addition. As described above, under control conditions, a small percentage of photoreceptors expressed opsin, and the photoceptors did not develop their characteristic outer segments (Fig. 1A). However, upon DHA supplementation, the proportion of photoreceptors displaying apical processes at the end of their cilium (13,15) and showing opsin expression was markedly increased (Fig. 3B). Thus, the percentage of photoreceptor cells expressing opsin at day 10, either in the cell bodies or in their apical processes, increased from 8.6% in control cultures to 30% in DHA-treated cultures (Fig. 3B). In addition, in DHA-treated cultures, opsin tended to lose its axon localization and to concentrate either in photoreceptor cell bodies or in their apical processes. All these changes were indicative of photoreceptor differentiation.

Mitochondria have been proposed to have a central role in triggering apoptotic death (31). Impairment in mitochondrial activity seems to be closely related to the onset of cell death. Looking for possible molecular pathways leading to the protective effect of DHA, we investigated mitochondrial functionality in control and DHA-treated cultures. Most amacrine cells had several active mitochondria at every time studied (not shown). In contrast, mitochondrial activity was severely reduced in photoreceptor cells under control conditions: by day 10, only about 25% of these cells were left with active mitochondria. DHA supplementation seemed to ameliorate mitochondrial damage: after the same time in culture, 53% of photoreceptors still displayed active mitochondria (Fig. 3C). Hence, the anti-apoptotic effect of DHA might be related to the maintenance of mitochondrial activity.

*Glial cells ameliorated photoreceptor cell death* in vitro. We investigated the effects of glial cells on neuronal survival by coculturing retinal glia with neuronal cells. The presence of glial cells led to a significant increase in photoreceptor survival: the amount of PI-positive photoreceptors at day 10 was reduced from 700,000 cells/dish in pure neuronal control cultures to about 550,000 cells/dish in neuron–glia cocultures (Fig. 4). Glial cells seemed to exert this effect by delaying the onset and retarding the progression of apoptosis, in a similar manner to DHA (Insua, M.F., Rotstein, N.P., and Politi, L.E., unpublished data). Moreover, as observed in DHA-supplemented cultures, glial cells increased the formation of apical processes in photoreceptors (Insua, M.F., Rotstein, N.P., and Politi, L.E., unpublished results).

We then analyzed glial cell fatty acid composition and the ability of glia to take up DHA from the culture medium. The major fatty acids present in glia total lipids were oleic and palmitic, with arachidonic as the major polyunsaturated fatty acid; almost 15% of the fatty acids were DHA (Table 1). The proportion of this acid was considerably higher than that found in neuronal lipids. When glial cells were supplemented with 6.7 μM DHA, the percentage of this fatty acid in lipids increased to about 27% with a concomitant decrease of oleic acid. Therefore, glial cells were able to take up DHA and accumulate it in their lipids.

**TABLE 1**
**Effect of Docosahexaenoic Acid (DHA) Supplementation on the Fatty Acid Composition of Glial Cells *in vitro*[a]**

| Fatty acid | –DHA (%) | +DHA (%) |
|---|---|---|
| 16:0 | 19.25 ± 1.33 | 17.26 ± 1.54 |
| 16:1 | 3.42 ± 1.48 | 1.80 ± 0.03 |
| 17:0 | 1.21 ± 0.27 | 0.97 ± 0.05 |
| 18:0 | 15.21 ± 2.66 | 20.10 ± 2.67 |
| 18:1 | 23.78 ± 7.66 | 12.33 ± 0.31 |
| 18:2n-6 | 1.37 ± 0.18 | 0.80 ± 0.06 |
| 20:4n-6 | 18.59 ± 4.80 | 15.50 ± 1.78 |
| 20:5n-3 | 0.59 ± 0.45 | 2.07 ± 0.22 |
| 22:4n-6 | 0.93 ± 0.44 | 0.69 ± 0.12 |
| 22:5n-6 | 0.81 ± 0.06 | 0.52 ± 0.10 |
| 22:6n-3 | 14.73 ± 3.18 | 27.13 ± 0.65 |
| 24:5n-3 | 0.06 ± 0.09 | 0.31 ± 0.08 |
| 24:6n-3 | 0.04 ± 0.04 | 0.51 ± 0.24 |

[a]Glial cells were cultured for 6 d in 10% fetal bovine serum in Dulbecco's modified Eagle's medium until they reached confluence and were then incubated in neuronal medium either with 6.7 μM DHA complexed with bovine serum albumin (BSA) (+DHA) or with the same volume of a BSA solution (–DHA). After 2 d, the cells were collected and centrifuged, their lipids were extracted, and the fatty acid composition was analyzed as described in Figure 2. The values are percentages of total fatty acids and are shown as means ± SD from three or four samples.

*Glial cells can transfer DHA to neurons in coculture*. The question remaining was whether glial cells could channel DHA to neurons. Glial cells previously labeled with [$^{14}$C]DHA were cocultured with neurons grown on coverslips, and then glial cells and neurons were again separated. Autoradiographic analysis showed a diffuse distribution of the silver grains in glial cells, indicating [$^{14}$C]DHA uptake and dispersion over the entire cells (Fig. 5). In contrast, silver grains were more densely concentrated in neuronal cells, preferentially in photoreceptors; moreover, in these cells, the most intense staining was observed in the regions corresponding to apical processes. Overall, these results suggest that glial cells incorporated the fatty acid and managed to convey [$^{14}$C]DHA to neurons in coculture and that photoreceptors avidly took it up and concentrated it in the newly formed apical processes.

*GDNF protects photoreceptors from cell death.* Glial cells might rescue photoreceptors from cell death by releasing trophic factors other than DHA, such as GDNF. The possibility that this molecule might protect photoreceptors from degeneration *in vitro* was investigated. When cell death was evaluated (Fig. 4), the number of PI-positive photoreceptors at day 10 was reduced from about 720,000 cells/dish in control cultures to 500,000 cells/dish in GDNF-supplemented cultures. This reduction was similar to that induced by glial cells and DHA (Fig. 4). Therefore, GDNF, DHA, and glial cells were able to rescue photoreceptors from cell death triggered by the absence of trophic factors.

## DISCUSSION

The mechanisms by which photoreceptor cells avoid natural programmed cell death during normal development in the retina are still unknown. The identification of the possible trophic factors involved in this process is of paramount interest, not only to increase our understanding of retinal functioning but also to prevent or treat human inherited degenerative

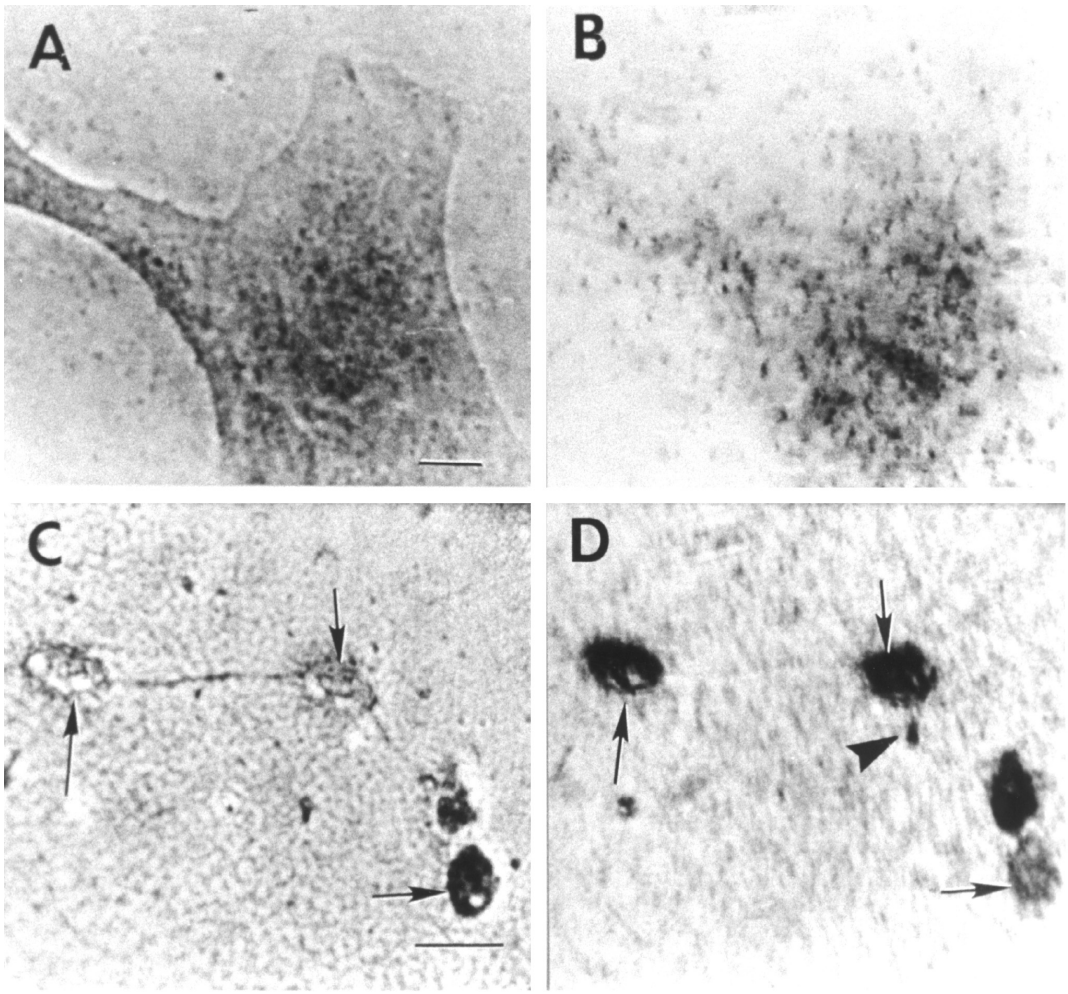


**FIG. 5.** Channeling of [$^{14}$C]DHA from glial to neuronal cells in coculture. Phase-contrast (left) and bright-field (right) pictures of autoradiographies of glial (A, B) and neuronal (C, D) cells. Glial cultures were incubated with [$^{14}$C]DHA for 1 d and, after thorough removal of the fatty acid, cocultured with pure neuronal cultures for 3 d. Both cell types were then separated, and [$^{14}$C]DHA labeling was analyzed by autoradiography. In glial cells, silver grains were diffusely distributed, whereas these grains were concentrated in photoreceptors in neuronal cultures. The bars indicate 10 μm. For abbreviation see Figure 2.

diseases affecting the retina, such as retinitis pigmentosa. The multiple effects of DHA on photoreceptor survival and differentiation shown in the present and previous reports (13–15) suggest a novel role as a neurotrophic factor for this lipid molecule. DHA rescues photoreceptors by delaying the onset and slowing down the progression of apoptosis. Moreover, our present results confirm previous reports that DHA, like other trophic factors, advances the differentiation of this single neuronal cell type. In the mature retina, photoreceptors must have highly differentiated characters at both the molecular and structural levels to accomplish their visual function: they must develop apical processes to fully differentiate the outer segments where opsin is to be concentrated for phototransduction to occur. Under control conditions *in vitro*, the diffuse distribution of opsin over the entire plasmalemma and the lack of apical differentiation mirrored immature stages of photoreceptor differentiation (14,15). DHA addition promoted the formation of apical processes, enhanced opsin expression, and favored its localization in these newly formed apical processes of photoreceptor cells.

The DHA-promoted survival and differentiation of photoreceptors contrast with the well-known harmful and proapoptotic effects of arachidonic acid in other nervous tissues (32). The protective effects of DHA paralleled an increase in its content in neuronal lipids upon DHA supplementation, similar to the accretion of this fatty acid in the retina *in vivo* during early stages of development (Fig. 2). Although the enzymes required for the esterification and turnover of several fatty acids are active in retinal neurons (15), DHA was the only fatty acid able to accumulate in neuronal lipids and modify their acyl chain composition when added to the culture media (Fig. 2). This was consistent with DHA being the only fatty acid having a neurotrophic activity (13–15). This suggests that the ability to form new DHA-containing phospholipids might be somehow related to the survival-promoting and, even more likely, the differentiating actions of DHA. The availability of such phospholipids, essential components of rod outer segments, might help redirect opsin to its correct localization (33,15), and the right combination of protein and phospholipids might allow the formation of apical processes. In patients with retinitis pigmentosa, and in animal models of this disease, a decrease in DHA content in plasma (34) and in both retina and photoreceptors (35) has been described. This decrease might contribute to the failure of photoreceptors to develop their outer segments and the ultimate death of photoreceptors in retinitis pigmentosa.

However, roles played by DHA are probably not only structural. Addition of DHA also has a protective action on monocytes, reducing tumor necrosis factor-induced apoptosis in these cells (36). These protective effects suggest that DHA could induce the release of a survival signal or, alternatively, it could prevent the release of a death signal during apoptosis development. Death signals, such as cytochrome c, are known to be released from damaged mitochondria during apoptotic processes (reviewed in Ref. 31). Our results show that an increase in the number of apoptotic photoreceptors was consistent with increased mitochondrial impairment in these cells. Mitochondrial damage has also been shown in other apoptotic processes affecting photoreceptors (37). Addition of DHA to the cultures partially prevented the loss of mitochondrial activity, suggesting a possible role for this acid in sustaining mitochondrial function. Mitochondrial failure is closely related to alterations in membrane properties, such as transmembrane potential and $H^+$ gradient, mainly due to the opening of a large-conductance channel (31). Polyunsaturated fatty acids have been shown to be involved in the regulation of ion channels, thus regulating neuronal survival in other systems (38,39). Accumulation of DHA in mitochondrial lipids might similarly participate in the modulation of the aperture of mitochondrial channels, partially preventing or postponing the changes in membrane properties and thus slowing down the apoptotic death of photoreceptors. Further research to obtain a better understanding of the mechanisms involved in DHA protection of mitochondrial function by DHA is warranted.

The essentiality of DHA for photoreceptor survival makes it essential for retinal cells to accumulate this fatty acid. DHA is tenaciously retained in the retina, even upon prolonged dietary deprivation (2,40), and efficient mechanisms have been developed to avoid loss of DHA during the daily recycling of photoreceptor discs (41). The liver has been proposed to be in charge of DHA provision to the retina (20). This fatty acid can also be synthesized by both the pigment epithelium (42) and the retina itself (43–45), although experiments conducted with the whole retina have not determined whether this synthesis was performed by neurons or glial cells. In the brain, astrocytes take charge of DHA synthesis and delivery to neurons (46). Our results showed that glial cells had a protective effect on photoreceptors, delaying cell death in a similar fashion to DHA. In addition, the proportion of DHA was higher in glial than in neuronal lipids. This prompted us to investigate whether glia could provide DHA to neurons in coculture, and we found that not only did glial cells transfer [$^{14}$C]DHA to neurons but these cells avidly incorporated it and esterified it in their lipids as well. Radioactive DHA was particularly concentrated in photoreceptors, suggesting that either glial cells preferentially channeled it to these neurons or that photoreceptors had the most efficient mechanisms for the uptake of DHA. Glial cells have been shown to modulate several neuronal functions, including photoreceptor survival through the supply of trophic factors (21,22). Provision of DHA might be at least one of the mechanisms involved in the protective role played by glial cells in the regulation of photoreceptor survival in the retina.

There is growing consensus that a combination of trophic factors is probably required for sustaining photoreceptor survival (47,48). DHA is undoubtedly required at a precise developmental period to postpone the triggering of photoreceptor apoptosis; however, this process eventually starts, with only a small population of photoreceptors being spared from apoptotic death (14). This suggests that other survival factors are required, acting in a sequential or synergistic manner to allow these cells to survive for longer time periods and

acquire fully differentiated characters. Our results show that GDNF was able to protect photoreceptors from cell death in the absence of other trophic factors *in vitro*, having a survival-promoting effect similar to those of DHA and glial cells. We have also shown that GDNF diminished apoptosis, acting coordinately with DHA (49). Since glial cells are known to release GDNF, this trophic factor may provide glia with another way of controlling photoreceptor apoptosis. Hence, glial cells might play a central role in the regulation of photoreceptor survival, by modulating the release of GDNF and DHA during development.

In conclusion, mounting evidence supports the hypothesis that a lipid molecule like DHA, acting in a coordinated, developmentally regulated fashion with other trophic molecules, may behave as a trophic factor essential for sustaining photoreceptor survival and differentiation.

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# Polyunsaturated Fatty Acids and Cerebral Function: Focus on Monoaminergic Neurotransmission

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**ABSTRACT:** More and more reports in recent years have shown that the intake of polyunsaturated fatty acids (PUFA) constitutes an environmental factor able to act on the central nervous system (CNS) function. We recently demonstrated that the effects of PUFA on behavior can be mediated through effects on the monoaminergic neurotransmission processes. Supporting this proposal, we showed that chronic dietary deficiency in α-linolenic acid in rats induces abnormalities in several parameters of the mesocortical and mesolimbic dopaminergic systems. In both systems, the pool of dopamine stored in presynaptic vesicles is strongly decreased. This may be due to a decrease in the number of vesicles. In addition, several other factors of dopaminergic neurotransmission are modified according to the system affected. The mesocortical system seems to be hypofunctional overall [e.g., decreased basal release of dopamine (DA) and reduced levels of dopamine $D_2$ ($DAD_2$) receptors]. In contrast, the mesolimbic system seems to be hyperfunctional overall (e.g., increased basal release of DA and increased levels of $DAD_2$ receptors). These neurochemical changes are in agreement with modifications of behavior already described with this deficiency. The precise mechanisms explaining the effects of PUFA on neurotransmission remain to be clarified. For example, modifications of physical properties of the neuronal membrane, effects on proteins (receptors, transporters) enclosed in the membrane, and effects on gene expression and/or transcription might occur. Whatever the mechanism, it is therefore assumed that interactions exist among PUFA, neurotransmission, and behavior. This might be related to clinical findings. Indeed, deficits in the peripheral amounts of PUFA have been described in subjects suffering from neurological and psychiatric disorders. Involvement of the monoaminergic neurotransmission function has been demonstrated or hypothesized in several of these diseases. It can therefore be proposed that functional links exist among PUFA status, neurotransmission processes, and behavioral disorders in humans. Animal models are tools of choice for the understanding of such links. Improved prevention and complementary treatment of neurological and psychiatric diseases can be expected from these studies.

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The presence in the brain of large amounts of polyunsaturated fatty acids (PUFA) from the n-3 and n-6 families is in agreement with their major role in the structure and function of this organ (1). These essential fatty acids (EFA) are exclusively provided by the diet in the form of precursors (18:3n-3 or α-linolenic acid, and 18:2n-6 or linoleic acid) and long-chain derivatives (LC-PUFA, mainly docosahexaenoic acid, 22:6n-3 or DHA; and arachidonic acid, 20:4n-6 or AA).

During the last decade, it has become evident that intake of PUFA constitutes an environmental factor able to act on the central nervous system (CNS) function. This is based on experimental studies that show behavioral abnormalities in animals consuming diets unbalanced in PUFA, and on clinical observations that describe abnormal levels of PUFA in the plasma and/or erythrocytes of subjects suffering from several diseases of the CNS. In agreement with these findings, we recently reported evidence that such behavioral dysfunctions might be related to neurochemical changes, especially in the monoaminergic neurotransmission processes.

This new field of research on the effects of nutrition on the neurotransmission processes opens up perspectives regarding the following: (i) the knowledge of mechanisms involved in the effects of PUFA on the CNS and (ii) the potential preventive and therapeutic use of PUFA in several neurological and psychiatric diseases.

## PUFA AND MONOAMINERGIC NEUROTRANSMISSION: EXPERIMENTAL STUDIES IN ANIMALS

The involvement of PUFA in CNS function can be assessed using dietary manipulation in animal models. It has already been shown that chronic dietary deficiency in α-linolenic acid in rodents greatly affects the fatty acid (FA) composition of cerebral membrane phospholipids (1–5). The main changes comprise reduction in DHA levels and a compensatory rise in n-6 PUFA levels, especially docosapentaenoic acid (22:5n-6). It was shown more recently that the composition of PUFA in cerebral membranes is not homogeneous throughout the brain, and is not modified in a similar way in response to PUFA deficiency. Analysis of specific brain regions showed that in rats and mice consuming a diet balanced in n-6 and n-3 PUFA, the amount of DHA was significantly higher in the frontal cortex than in other regions such as the striatum, hippocampus, and cerebellum (6,7). Moreover, the frontal cortex

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Abbreviations: AA, arachidonic acid; ADHD, attention deficit/hyperactivity disorder; CNS, central nervous system; DA, dopamine; $DAD_2$, dopamine $D_2$; DAT, DA transporters; DHA, docosahexaenoic acid; $D_nR$, dopamine receptor; Dopac, dihydrophenylacetic acid; EFA, essential fatty acids; FA, fatty acid; GABA, γ-aminobutyric acid; HPLC, high-performance liquid chromatography; LC, long chain; PUFA, polyunsaturated fatty acids; $VMAT_2$, vesicular monoamine transporter.

seemed to be more affected by α-linolenic acid deficiency than other regions (6-8).

In addition to these biochemical changes, α-linolenic acid deficiency impairs performance in a variety of learning tasks (3–5,9,10). These impaired behavioral responses often involve both learning ability and sensory, motor, or motivational processes (11). In particular, increased responses to several reinforcement factors and slower extinction observed in n-3 PUFA–deficient rats can be interpreted as changes in motivation.

Although behavioral responses cannot be precisely related to specific neurochemical pathways, we proposed that the behavioral effects of n-3 PUFA deficiency might be mediated through dopaminergic systems (6). This hypothesis was based mainly on the known role of dopamine (DA) as a major factor modulating attention, motivation, and emotion (12). This role of DA in behavior modulation is exerted mainly through the mesocortical and mesolimbic systems, whereas the nigrostriatal pathway is essentially involved in locomotor activity. Mesocortical DA neurons are thus involved in cognitive functions such as working memory, and mesolimbic DA neurons play a strong role in motivational behavior and emotional functions (12–14).

We therefore studied the effects of α-linolenic acid deficiency on several parameters of monoaminergic neurotransmission, and more especially, dopaminergic neurotransmission, in cerebral regions under such neurochemical control. In most of our experiments, we compared 2- to 3-mon-old male rats consuming a diet deficient in α-linolenic acid for several generations (the lipid ratio was provided by African peanut oil providing 1200 mg of linolenic acid and <6 mg of α-linolenic acid/100 g of diet) to age-matched rats consuming a diet balanced in n-6 and n-3 PUFA (the lipid ratio was provided by a mixture of African peanut oil and rapeseed oil, providing 1200 mg of linolenic acid and 200 mg of α-linolenic acid/100 g of diet). The n-6/n-3 ratio in the deficient diet was >200, whereas in the balanced control diet it was 6, which is considered as optimal to obtain and maintain a physiological level of DHA in developing and adult rats (15).

In the initial series of experiments, we measured the overall amounts of three monoamines, DA, serotonin, and noradrenaline, in tissue homogenates of the frontal cortex, striatum, hippocampus, and cerebellum obtained from rats consuming the α-linolenic acid–deficient or the control diet. The main modification observed in rats fed the deficient diet was a 40–60% decrease in the amount of DA in the frontal cortex, whereas only a slight decrease was observed in the striatum (6); both abnormalities persisted throughout life, from 2 to 24 mon of age (16). To refine these results, we turned our work to a dynamic approach allowing the study of several parameters of DA neurotransmission in live animals. For this, we used the intracerebral microdialysis technique. This method consists of implanting a probe, terminating with a semipermeable membrane, and perfused with a buffer medium, into a specific region of the brain. The pores of the membrane allow the passage of solutes of suitable size from the extracellular compartment along a concentration gradient (17). Neurotransmitters are thereafter measured in the dialysate fractions collected with an appropriate method, e.g., high-performance liquid chromatography (HPLC) associated with electrochemical detection. This measurement reflects the concentration of neurotransmitters in the fluid surrounding the dialysis probe, which represents a large population of nerve terminals. It must be emphasized that the experimental conditions of microdialysis are of major importance for the correct interpretation of the results (18).

We used microdialysis to study the release of DA and its main metabolites [dihydrophenylacetic acid (DOPAC) and homovanillic acid] in basal conditions and under pharmacological stimulation (drugs being administered through the probe or by systemic injection). This demonstrated that the decrease in DA in the homogenates of the frontal cortex from α-linolenic acid–deficient rats was probably due to abnormalities in the DA storage compartment, rather than to the cytoplasm compartment (19,20). Several findings thus suggest a deficit in the storage of DA in the presynaptic vesicles, which can be due to a decrease in the number of dopaminergic vesicles. Indeed, the vesicular monoamine transporter ($VMAT_2$), which is localized on the vesicle membrane and allows DA entry into vesicles, is decreased in the frontal cortex (21,22) and nucleus accumbens (20) of deficient rats. Although this decrease was found to occur in both regions, the dopaminergic function response to n-3 PUFA deficiency differed between cerebral regions. The results of pharmacologically stimulated release of DA were in accordance with hypofunction in the frontal cortex and hyperfunction in the nucleus accumbens (Fig. 1). The strong functional links existing between the frontal cortex and the nucleus accumbens (14,23,24) could explain in part this last finding. For instance, the enhanced basal level of extracellular DA measured in the nucleus accumbens of awake n-3 PUFA–deficient rats (20) might be related to the reduction in the level of DA in the frontal cortex, thus removing the inhibitory effect exerted by the frontal cortex efferent on the DA level in the nucleus accumbens. This last cerebral area is very involved in reinforcement processes, mainly through DA release soon after a reward (25,26). On this basis, Reisbick and Neuringer (27) recently proposed that the poorer performance in several cognitive tasks observed in n-3 PUFA–deficient rats might be attributed to increased reactivity to reward related to the dopaminergic function of the nucleus accumbens.

In addition to these effects on DA metabolism, it seems that α-linolenic acid deficiency is also able to induce changes in specific molecular targets of DA. The main targets are receptors localized on postsynaptic neurons, and autoreceptors and membrane DA transporters (DAT), both localized at presynaptic levels. Five subtypes of dopamine receptors have been distinguished to date on pharmacological, genetic, and molecular grounds. They belong to two families, i.e., the $D_1$-like family ($D_1$ and $D_5$ receptors), and the $D_2$-like family ($D_2$, $D_3$, $D_4$ receptors). The $D_1$ receptors ($D_1R$) are exclusively postsynaptic (28), whereas $D_2$ receptors ($D_2R$) are both post-

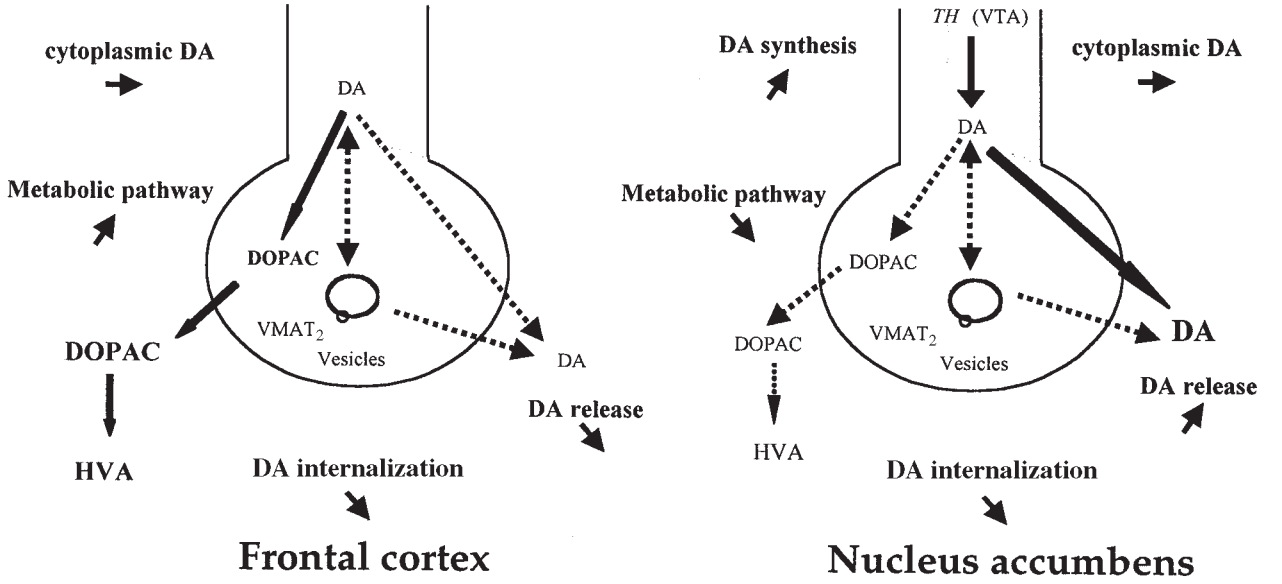


**FIG. 1.** Dopamine metabolism in the frontal cortex and nucleus accumbens of n-3 PUFA–deficient rats: hypothesis. In the frontal cortex as well as in the nucleus accumbens, the cytoplasmic compartment of DA is unmodified and the vesicle storage compartment (DA internalization) is reduced in α-linolenic acid–deficient rats. However, the metabolic pathway is increased in the frontal cortex (decreased DA release and increased DOPAC and HVA release), and increased in the nucleus accumbens (increased DA synthesis, increased DA release, decreased DOPAC and HVA release). This leads to a hypofunction of dopaminergic transmission in the frontal cortex, and an hyperfunction in the nucleus accumbens. Abbreviations: DA, dopamine; DOPAC, dihydroxyphenyl acetic acid; HVA, homovanillic acid, PUFA, polyunsaturated fatty acid; $VMAT_2$, vesicular monoamine transporter; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

synaptic and presynaptic (see Ref. 29 for recent review). The DAT has major physiological roles in regulating neurotransmission processes through rapid removal of DA from the synaptic cleft back into the presynaptic nerve endings. It also mediates the pharmacological effects of drugs such as cocaine and amphetamine (30), and is very involved in a variety of disease processes such as Parkinson's disease (31). It was therefore of great interest to study the potential effects of n-3 PUFA deficiency on these presynaptic and postsynaptic binding sites, which are involved in the physiological function of DA.

We found that neither $D_1R$ nor DAT seemed to be affected by α-linolenic acid deficiency (6,16), whereas $D_2R$ were slightly decreased in the frontal cortex (6,16) and strongly increased in the nucleus accumbens (20). These changes were observed at the following two levels: (i) protein expression measured by quantitative autoradiography using binding experiments with specific ligands, and (ii) mRNA expression measured by *in situ* hybridization. These modifications occurring in deficient rats could result in part from regulatory responses to the neurotransmission abnormalities already described. For example, the increase in $D_2R$ in the nucleus accumbens could be due to hypersensitivity of presynaptic autoreceptors in response to the increased DA levels observed in this cerebral region (20) because this type of regulatory mechanism has been described (32).

Our overall neurochemical findings, which demonstrate that chronic n-3 PUFA deficiency acts on the mesocortical and mesolimbic systems, are in accordance with several studies reporting behavioral effects of such deficiency related to motivation, response to reward, and learning ability. However, the precise mechanisms linking PUFA, neurochemical events, and behavior remain to be clarified. One of these mechanisms might involve the effects of changes in the relative amounts of PUFA in the neuronal membranes on the function of these membranes. Several findings show that changes in dietary PUFA act on membrane fluidity (33–35); it can therefore be hypothesized that membrane changes induced by chronic n-3 PUFA deficiency could decrease the formation of vesicles, which we observed. In agreement with this, it has also been shown that dietary α-linolenic acid deficiency can affect vesicle density in the rat hippocampus (36). As already proposed, biochemical modifications in neuronal membranes might also be involved in abnormalities of the neurotransmitter receptors that are included in these membranes (37).

In addition, neurochemical changes also suggest that modification of the lipid content in the diet is able to act on the regulation of gene transcription. The direct involvement of lipids, particularly PUFA, in the regulation of gene expression, transcription, and mRNA stability in different biological tissues has become increasingly apparent in the last decade. Such phenomena have been studied and described in hepatic, lipogenic, and immune tissues (38,39). We can therefore hypothesize that such regulation takes place in the brain and that dietary modulation of n-3 PUFA content influences gene expression and transcription, thus explaining differences in protein and mRNA expression between control and deficient rats.

Animal experiments therefore suggest that interactions among PUFA, neurotransmission, and behavior exist, and these might have repercussions for the improvement of human health.

## PUFA AND CNS DISORDERS: CLINICAL DATA

Several teams have recently focused on the peripheral amounts (plasma and/or red blood cells) of PUFA in subjects suffering from neurological or psychiatric diseases. It was shown that various neurological disorders such as Huntington's disease, multiple sclerosis, Alzheimer's disease, and adrenoleucodystrophia can be associated with deficits in n-6 and/or n-3 PUFA (40,41). However, little information is available to date. In addition, it is difficult to obtain relevant comparisons between affected and control subjects because peripheral amounts of PUFA seem very heterogeneous among populations (40). Findings are more consistent in the field of psychiatric disorders, and we chose to study three of these diseases in which the involvement of monoaminergic neurotransmission processes are hypothesized, i.e., schizophrenia, depression, and attention deficit/hyperactivity disorders (ADHD).

Schizophrenia is a psychiatric disease that affects ~1% of the population. The predominant hypothesis regarding the pathophysiology of this disease is dysfunction of the dopaminergic systems (see Ref. 42 for review). These systems seem to be unbalanced, thus inducing dysfunction at different cerebral levels under dopaminergic control, such as the frontal cortex, limbic regions, and basal ganglia (43). In addition, other neurotransmitter systems such as the glutamatergic pathways, which have strong interactions with DA, could be involved (44).

Horrobin *et al*. (45,46) first proposed that relationships could exist between schizophrenia and changes in the status of EFA, showing a tendency toward lower amounts of plasma PUFA, especially linoleic acid. However, further findings concerning the levels of EFA in erythrocytes suggested that two schizophrenic populations could be distinguished, i.e., one with EFA levels similar to those of controls and another with reduced amounts of n-6 and n-3 PUFA, especially AA and DHA (47–49). Several mechanisms could explain these deficits, including increased activity of phospholipase $A_2$, thus inducing increased extraction of AA and DHA from cerebral membranes (50–52). Another argument in favor of a relationship between schizophrenia and EFA deficit is that dietary supplementation in PUFA is able to alleviate symptoms of the disease (53,54). It seems therefore that schizophrenia might be an example of disease in which PUFA supplementation associated with pharmacological treatment might be beneficial, but extended evaluation of such treatment is still required (see Ref. 55 for review).

Depression is a complex disorder that particularly involves serotoninergic neurotransmission processes, especially serotonin receptors and membrane transporters (56). Several studies have described deficits in plasma and/or erythrocytes of depressed subjects (57–61), e.g., a 45% reduction in the levels of α-linolenic and DHA, thus inducing an overall increase in the n-6/n-3 PUFA ratio (59). However, no clear hypothesis has yet been proposed to explain the relationships between these findings and depression.

Attention deficit/hyperactivity disorder (ADHD) affects mainly boys and is characterized by increased impulsivity and hyperactivity. Several recent findings are in agreement with abnormalities in parameters of dopaminergic neurotransmission, especially the dopamine transporter, associated with this disease (62–65). The first study linking ADHD and PUFA was performed on 48 drug-naïve children compared with age-matched control subjects, and showed plasma decreases in DHA, AA, and dihomo-γ-linolenic acids (66). More recent reports have confirmed these findings (67–69), but the reasons for these deficits remain unclear.

It appears therefore that several psychiatric diseases could be associated with peripheral deficit in n-6 and more often n-3 PUFA. However, several mechanisms might be involved in these abnormalities. They include the following: (i) deficit in the dietary intake or digestive absorption of LC-PUFA or their precursors; (ii), poorer ability to convert the precursors to LC-PUFA; and (iii) incorrect PUFA incorporation into membranes or increased membrane extraction related to enzyme dysfunction such as phospholipase $A_2$ (according to the hypothesis proposed by Horrobin). In addition, it remains to be assessed whether abnormalities in PUFA levels in the plasma and/or erythrocytes are related to changes in the composition of cerebral membranes. Little information is available on this cerebral composition in subjects suffering from neurological or psychiatric diseases. Reduction in the level of total phospholipids (70) as well as decrease in several FA, including AA and DHA (71), was described in various cerebral areas in Alzheimer's disease subjects. Lower levels of PUFA, particularly AA and its precursor linolenic acid, were found *post mortem* in the frontal cortex (72) and caudate (73) in brains of schizophrenic subjects. These findings are in agreement with the possible occurrence of abnormal composition of FA in cerebral membranes of subjects suffering from diseases of the CNS, but remain to be confirmed.

Such clinical findings, which are still sparse, might be put together with experimental studies in animals. Because we have now shown that induced deficiency in n-3 PUFA in animals can cause changes in several aspects of the monoaminergic neurotransmission processes, it can be proposed that a deficit in these PUFA might be able to aggravate human diseases involving these processes. This proposal is therefore in favor of the following: (i) the detection of patients suffering from PUFA deficits and (ii) PUFA supplementation associated with pharmacological treatment in such patients.

## PROSPECTS

There is currently a great need for investigations that would provide understanding of the mechanisms linking PUFA, neurochemical events, and behavioral processes. Such knowledge is necessary to achieve new potency in clinical applications. Animal models are very relevant tools for this aim because dietary manipulation, neurochemical studies, and behavioral tests can be performed and compared. Several specific points would be thus tackled in animal models.

*Dose-effect of n-3 PUFA on monoaminergic neurotransmission.* Most of the above studies involve animals totally deprived of α-linolenic acid. However, less information is available concerning the effects of high dietary intake of n-3 PUFA. It has been shown that this type of diet induces changes in brain PUFA composition, i.e., a rise in n-3 PUFA (DHA, ecosapentaenoic acid) compensated for by lower amounts of n-6 PUFA (74,75). In addition, high dietary fish oil (rich in n-3 LC-PUFA) seems to improve learning ability in specific experimental conditions (76–78). We found recently that a similar diet also induced neurochemical modifications, including slightly reduced (striatum) and slightly increased (frontal cortex) amounts of dopaminergic $D_2R$ and a rise in overall DA levels in frontal cortex tissue (79). These last results might suggest opposing effects of n-3 overload and deficiency on dopaminergic function, but they are still preliminary. It would therefore be of great value to study the simultaneous effects of increasing amounts of n-3 PUFA, especially DHA, from deficiency to overload, on FA membrane composition, dopaminergic parameters, and response to behavioral tests. Few experiments have been performed in this field (80). Such information would be very useful with a view to nutritional supplementation in specific clinical situations as discussed above.

*Reversibility of neurochemical changes induced by n-3 PUFA deficiency.* Another major question is to establish whether the neurochemical changes observed under α-linolenic acid deficiency could be reversed by n-3 PUFA supplementation. It has been shown that, in terms of FA composition, the speed of recovery after deficiency is very slow in rats (81). It has also been shown in mice deficient in α-linolenic acid that intake of n-3 PUFA for 2 mon from the age of 7 wk is effective in reversing the biochemical and behavioral changes induced by the deficiency (82). It would be of great interest now to associate neurochemical parameters with these first biochemical and behavioral findings. In addition, the occurrence and swiftness of reversibility might be dependent on the age at which the supplementation is provided, and thus to a stage of cerebral development.

*Effects of PUFA on other neurotransmission systems.* The results obtained on the effects of n-3 PUFA deficiency on dopaminergic neurotransmission raise the question of the potential effects of such deficiency on other neurotransmission systems. It is not clear whether PUFA act on neurotransmission through membrane, genetic, or other routes that might simultaneously disturb several systems, or specifically disturb particular systems. In the first hypothesis, these effects might be exerted directly by PUFA. A few reports have described the effects of PUFA on various neurotransmitters, such as the reduced effects of DHA on the γ-aminobutyric acid (GABA) response (83), a rise in acetylcholine levels induced by DHA (84), and various effects of n-6 and n-3 PUFA on cerebral peptides (85). In addition to these direct effects, the effect of PUFA on several neurotransmission systems might also be the consequence of dopaminergic changes. It is indeed known that dopaminergic systems have many functional interactions with other systems, such as the serotoninergic, glutamatergic, GABAergic, and cholinergic, all of which are involved in behavioral processes. This therefore opens up a wide range of neurochemical investigations in relation to PUFA dietary intake.

This report brings together extensive evidence showing that PUFA are environmental factors able to act on several aspects of CNS function, such as neurochemical events and behavior. It is proposed that strong links exist among PUFA status, neurotransmission processes, and behavioral disorders. More evidence must be found to reinforce this proposal; the use of animal models is therefore a tool of choice in which diet, neurochemistry, and behavior can be studied simultaneously. The expected repercussions of such experiments are improved prevention and treatment of neurological and psychiatric diseases.

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# Mechanisms of Action of Docosahexaenoic Acid in the Nervous System

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**ABSTRACT:** This review describes (from both the animal and human literature) the biological consequences of losses in nervous system docosahexaenoate (DHA). It then concentrates on biological mechanisms that may serve to explain changes in brain and retinal function. Brief consideration is given to actions of DHA as a nonesterified fatty acid and as a docosanoid or other bioactive molecule. The role of DHA-phospholipids in regulating G-protein signaling is presented in the context of studies with rhodopsin. It is clear that the visual pigment responds to the degree of unsaturation of the membrane lipids. At the cell biological level, DHA is shown to have a protective role in a cell culture model of apoptosis in relation to its effects in increasing cellular phosphatidylserine (PS); also, the loss of DHA leads to a loss in PS. Thus, through its effects on PS, DHA may play an important role in the regulation of cell signaling and in cell proliferation. Finally, progress has been made recently in nuclear magnetic resonance studies to delineate differences in molecular structure and order in biomembranes due to subtle changes in the degree of phospholipid unsaturation.

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## DHA COMPOSITION

In the 1960s, the very high level of docosahexaenoic acid (DHA, 22:6n3) in the mammalian brain was already appreciated (1,2) although the first description by Thudichum (3) was nearly a century earlier [see review by Salem *et al*. (4)]. Yabuuchi and O'Brien (5) described the positional distribution of brain phosphoglycerides in 1968, detailing both the high concentration of DHA in position *sn*-2 and its concentration in the aminophospholipids, phosphatidylserine (PS), and phosphatidylethanolamine (PE). By the early 1970s, the very high concentration in brain synaptosomal plasma membranes (6) and synaptic vesicles (7) was described by Breckenridge and co-workers. Table 1 presents the DHA composition in the aminophospholipids of brain and other selected mammalian tissues (1,6–18). It is apparent that the DHA content of the nervous system is very high. The retina not only contains a very high level of DHA in the rod outer segment (ROS) membranes, but also contains a very considerable amount of di-DHA species (19) as well as ones with DHA coupled to other highly unsaturated fatty acids (HUFA). The sperm is another compartment enriched in DHA. Every mammalian cell contains DHA, and phospholipids of internal organs and muscles have a significant content. Human milk contains a relatively low content of DHA with a higher percentage in phospholipids than triglycerides, the main lipid component of milk.

It has long been known that when an adult mammal consumes a diet low in DHA and its n-3 precursors, the nervous system content of DHA is much less altered than are other organs, i.e., DHA is said to be tenaciously retained once neural development has occurred (for reviews, see Refs. 4,20). However, animal studies have shown that when n-3 fat sources are inadequate during early neural development, then the levels of brain and retinal DHA decline (4,20–24). This has also been confirmed in autopsy studies of human infants that were fed a vegetable oil-based formula with low n-3 fat sources vs. breast-feeding in which preformed DHA was present (25–27). This has naturally led to an interrogation of the functional consequences of neural DHA loss.

## ANIMAL STUDIES

Representative studies in the animal literature (28–42) concerning the n-3 fatty acid deficiency syndrome are presented in Table 2. Only studies that focus on neural functions, notably brain and retinal functions, have been included here. Typically, these studies involve a two-generation diet regimen in which the mother is raised on an n-3-deficient diet and her offspring are then studied. Such treatment has generally been found to be necessary to induce a marked decline in brain and retinal DHA; a decline of 50–80% is typical of those associated with a change in neural function. A variety of different tasks show impairment, including those in both the visual and olfactory modalities (Table 2). In addition to decrements in performance in simple associative learning types of tasks, losses in spatial memory (39) and olfactory set learning have been reported recently (43). Thus, the loss in brain DHA may be said to affect cognition, at least to the extent that it can be ascertained in the rat.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPAn-6, docosapentaenoic acid; DROSS, dipolar recoupling on-axis with scaling and shape preservation; HUFA, highly unsaturated fatty acids; LCP, long-chain polyunsaturates; α-LNA, α-linolenate; LO, lipoxygenase; M, metarhodopsin; MAS, magic angle spinning; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser enhancement spectroscopy; PC, phosphatidylcholine; PDE, phosphodiesterase; PE, phosphatidylethanolamine; PS, phosphatidylserine; ROS, rod outer segment.

**TABLE 1**
**Docosahexaenoic Acid (DHA) Content of Aminophospholipids in Various Mammalian Tissues**

| | | | | Phospholipid class (% DHA) | |
|---|---|---|---|---|---|
| Ref | Species | Tissue | Fraction | Phosphatidylserines | Phosphatidylethanolamines |
| 1 | Human | Brain | Gray matter | 36.6 | 24.3 |
| 1 | Human | Brain | White matter | 5.6 | 3.4 |
| 8 | Bovine | Brain | Gray matter | 28.7 | — |
| 8 | Bovine | Brain | White matter | 7.6 | — |
| 6 | Rat | Brain | Synaptic plasma membrane | 34.1 | 32.4 |
| 7 | Rat | Brain | Synaptic vesicles | 37.0 | 30.6 |
| 9 | Human | Retina | — | 18.5 | 22.2 |
| 10 | Bovine | Retina | Rod outer segment | 37.7 | 38.7 |
| 11 | Ram | Sperm | — | — | 40.5 |
| 12 | Bovine | Sperm | — | — | 37.8 |
| 13 | Rat | Heart | — | — | 23.4 |
| 14 | Rat | Liver | Plasma membrane | 14.1 | 6.9 |
| 15 | Rat | Muscle | — | — | 36.1 |
| 16 | Human | Platelet | — | 2.1 | 4.1 |
| 17 | Human | Erythrocyte (infant) | — | — | 5.9 |
| 18 | Human | Milk | — | — | 0.08 |

## DHA RECOVERY

It has long been known that once depleted, the brain recovers its DHA rather slowly (44,45). A recent study in rats provided the time courses of DHA recovery and the reciprocal decline in docosapentaenoic acid (DPAn-6, 22:5n-6) in the retina, brain, liver, and serum when the rats were repleted with a diet containing both α-linolenate (LNA) and DHA (46). The half-times for brain and retinal recovery of DHA were 2.9 and 2.1 wk, respectively, even though the liver and plasma half-times were only 0.3 and 0.5 wk, respectively. This suggests a rather slow transport of DHA into the brain/retina even in the case of a DHA-deficient nervous system (47).

It could be hypothesized then that if the functional consequences of dietary n-3 fatty acid deficiency were due to the loss in DHA, at least some neural functions may be restored as the neuronal and retinal DHA level is restored. Others may not be reversible due to missed opportunities in sequential development or changes in structural features of the brain (48). The first such study of functional recovery by Connor and

**TABLE 2**
**Animal Studies of Effects of Low n-3 Fatty Acid Diets on Neural Functions**

| Task | Reference |
|---|---|
| Rodent studies | |
| Reduced amplitude of a- and b-waves | Wheeler and Benolken, 1975 (28) |
| Y-maze performance | Lamptey and Walker, 1976 (29) |
| Active avoidance task | Mills *et al.*, 1988 (30) |
| Brightness discrimination | Yamamoto *et al.*, 1991 (31) |
| Shock avoidance | Bourre *et al.*, 1989 (32) |
| Death after neurotoxin | Bourre *et al.*, 1989 (32) |
| Exploratory activity | Enslen *et al.*, 1991 (33) |
| Scopolamine-induced locomotion | Nakashima *et al.*, 1993 (34) |
| Age of eye opening (mice) | Wainwright *et al.*, 1991 (35) |
| Morris water maze (mice) | Nakashima *et al.*, 1993 (34) |
| Electroretinogram, a-wave, peak-to-peak | Weisinger *et al.*, 1996 (36,37) |
| Delayed acquisition of olfactory discrimination | Sheaff-Greiner, *et al.*, 1999 (38) |
| Spatial task acquisition and memory | Moriguchi *et al.*, 2000 (39) |
| Cat study | |
| Electroretinogram, a- and b-wave implicit time | Pawlosky *et al.*, 1997 (40) |
| Primate studies | |
| Reduced visual acuity, longer implicit time | Connor and Neuringer, 1984 (41) |
| Impaired recovery of dark-adaptation | Neuringer *et al.*, 1986 (42) |

Neuringer (49) indicated that electroretinographic changes associated with low retinal DHA persisted after DHA repletion. However, Moriguchi *et al*. (50) recently presented evidence that spatial task acquisition and memory are reversible and, to a first approximation, correlate well with the level of brain DHA. However, Weisinger *et al*. (51) reported that when n-3-deficient guinea pigs were subsequently given a diet containing LNA, changes in mean arterial blood pressure and electroretinographic changes in the a-wave were not reversed even when the DHA levels were indistinguishable from the control levels. Thus, it appears that there may be no general answer to the question of reversibility of losses in function due to DHA losses in early development; the answer will depend on the type of function involved.

## HUMAN STUDIES

As mentioned above, formula-feeding of infants has been associated with a loss in brain DHA with respect to the level in those breast-fed (25–27). The pre-existing animal literature would predict that formula-fed infants would have a functional deficit if the neural DHA loss were of a sufficient magnitude. Of course, there are many differences between breast-feeding and formula-feeding; these involve not only differences in nutrients, but also maternal contact and care, and the association of breast-feeding with socioeconomic factors. Nevertheless, it appears that the DHA variable can explain a good portion of the benefits associated with breast-feeding.

Studies of preterm infants have generally shown a benefit when DHA is added to the formula in controlled experiments (Table 3) (52–59). The studies included here are limited to controlled studies of formula-feeding with or without addition of DHA or DHA plus arachidonic acid (AA). Also, only those in which neural outcomes were included are listed; studies of growth and other anthropometric measures are not included. Studies of preterm infants (52–58) indicated, with one exception (59), that there was a benefit to adding long-chain polyunsaturates (LCP) to formulas that contain only the 18-carbon essential fatty acids found in vegetable oils. In addition, a meta-analysis of visual acuity differences in premature infants at 2 and 4 mon of age found a benefit of LCP of 0.47 and 0.28 octaves, respectively (60). These observations, in combination with studies indicating the safety of the ingredients used to supply these nutrients, lead us to conclude that preterm infant formulas must contain DHA/AA.

Studies of full-term infants are listed in Table 4 (61–72). In half of these studies, the LCP supplement supported an increased visual acuity (61,63,67), neurodevelopmental score

**TABLE 3**
**Effect of Formula Supplementation with Docosahexaenoic Acid (DHA) or DHA and Arachidonic Acid (AA) on Brain and Retinal Function in Preterm Infants[a]**

| Authors | Reference | Year | Outcome tested | Results | | Age |
|---|---|---|---|---|---|---|
| Uauy *et al.* | 52 | 1990 | ERG threshold, $V_{max}$ | LCP > F | LCP = BF | 36 wk PCA |
| Birch *et al.* | 53 | 1992 | VEP, FPL | LCP > F | LCP = BF | 36, 57 wk PCA |
| Carlson *et al.* | 54 | 1993 | FPL | LCP > F | | 2, 4 mon |
| Carlson *et al.* | 55 | 1996 | FPL | LCP > F | | 2 mon |
| Werkman and Carlson | 56 | 1996 | Fagan NPT | LCP > F | | 6.5, 9, 12 mon |
| Carlson and Werkman | 57 | 1996 | Fagan NPT | LCP > F | | 12 mon |
| Faldella *et al.* | 58 | 1996 | ERG latency | LCP > F | LCP = BF | 52 wk PCA |
| Bougle *et al.* | 59 | 1999 | Motor nerve conduction | LCP < F | LCP < BF | 30 d |

[a]ERG, electroretinogram; LCP, long-chain polyunsaturates, i.e., DHA or AA/DHA; F, formula-fed; BF, breast-fed; PCA, post-conceptional age; VEP, visual evoked potential (log MAR, where MAR = minimum angle of resolution); FPL, forced choice preferential looking (Teller cards); NPT, novel preference test or Fagan test of infant intelligence.

**TABLE 4**
**Studies of Formula Supplementation with DHA or DHA and AA on Retinal and Brain Function in Full-Term Infants[a]**

| Authors | Reference | Year | Outcome tested | Results | | Age |
|---|---|---|---|---|---|---|
| Makrides *et al.* | 61 | 1995 | VEP | LCP > F | LCP = BF | 16, 30 wk |
| Agostoni *et al.* | 62 | 1995 | Brunet-Lezine[b] | LCP > F | LCP = BF | 4 mon |
| Carlson *et al.* | 63 | 1996 | FPL | LCP > F | LCP = BF | 2 mon |
| Agostoni *et al.* | 64 | 1997 | Brunet-Lezine | LCP = F | LCP = BF | 24 mon |
| Auestad *et al.* | 65 | 1997 | FPL, VEP | LCP = F | LCP < BF | 2, 4, 6, 9, 12 mon |
| Hornby Jorgensen *et al.* | 66 | 1998 | VEP | LCP = F | LCP = BF | 4 mon |
| Birch *et al.* | 67 | 1998 | VEP | LCP > F | LCP = BF | 6, 17, 52 wk |
| Willatts *et al.* | 68 | 1998 | Means-end problem solving | LCP > F | — | 10 mon |
| Scott *et al.* | 69 | 1998 | MCDI | LCP < F | — | 14 mon |
| Lucas *et al.* | 70 | 1999 | Bayley MDI, PDI | LCP = F | LCP = BF | 18 mon |
| Birch *et al.* | 71 | 2000 | Bayley MDI | LCP > F | — | 18 mon |
| Makrides *et al.* | 72 | 2000 | VEP, Bayley MDI | LCP = F | LCP < BF | 34 wk, 2 yr |

[a]VEP, visual evoked potential (log MAR); MCDI, Minnesota Child Development Inventory; PDI, psychomotor development index; MDI, mental development index. For other abbreviations see Table 3.
[b]Brunet-Lezine derived from Gesell test for psychomotor development.

(62,71), or problem-solving ability (68). In five of the trials, no effect was observed for the LCP supplement (64–66,70,72). In one trial, infants with the LCP supplement appeared to perform more poorly in a vocabulary test administered to 14-mon-old children (69). However, a recent trial with a larger number of preterm infants reported a positive effect of LCP-supplemented formula on vocabulary scores (73). San Giovanni *et al*. (74) performed a meta-analysis of the trials involving visual acuity and concluded that there was a 0.32 octave difference in visual acuity when supplemented and unsupplemented formula groups were compared, with the DHA-fed groups having the higher acuity. A somewhat larger difference (0.49 octaves) was observed when breast-fed infants were compared with unsupplemented formula-fed infants.

In most of these trials, a rather low level (0.1–0.35% of total fatty acids) of DHA supplement was given, corresponding to a "Western" level of DHA in milk. In a recent review, Jensen (75) calculated the average for DHA content in mature milks in Western and non-Western women to be 0.45 and 0.88% of total fatty acids, respectively. Thus, it is likely that more of the trials would have observed a benefit of DHA if given at a higher level that is more consistent with the range of present-day worldwide human milk values. Viewed from this perspective, it is rather surprising that some trials can succeed in demonstrating a benefit of a fat component that is only 0.1–0.2 % of the total fatty acids. This suggests that these LCP are potent and essential nutrients for optimal development.

It was also of interest to note that Jensen (75) found the ratios of AA/DHA to be very close to 1 in his averages of human milk from both Western and non-Western women. The average levels found in non-Western women of ~0.9% each AA and DHA may be a good starting point for future research. This is believed by some to represent a better standard than that of Western women because the composition is strongly influenced by the diet and Westerners have in the last century or two shifted their consumption of fats toward n-6 fats and away from n-3 fats due to the availability of linoleic-rich vegetable oils. Estimates of the Paleolithic diet indicate a much greater intake of LCP and a ratio of n-3/n-6 fats close to 1 (76). Thus, human infants likely received a much higher intake of DHA and other LCP from their mother's milk during human evolution. In modern times, this ensured supply of DHA during neurodevelopment has been abrogated by formula-feeding and by a very low maternal intake of n-3 fats in many modern women. Given the present state of knowledge from human and animal studies of changes in neural function associated with a low DHA status, coupled with biochemical and nutritional studies indicating the loss of DHA in both peripheral tissues and the nervous system when preformed DHA is not fed and the safety of ingredients (70,77) used to supply DHA, it is clear that a prudent course of action would be to supply sources of preformed DHA in the infant diet.

## MECHANISMS OF ACTION OF DHA

From the above, it should be clear that many effects of DHA status have been observed relating to physiologic and behavioral functions of the nervous system. What has been unclear are the mechanisms underlying DHA function. Perhaps the most perplexing aspect of this question relates to the phenomenal degree of specificity that is apparent in this effect. It must be recalled that these studies did not involve essential fatty acid deficiency and that there were adequate and often excessive amounts of n-6 fats present, usually in the form of linoleic acid (LA). There is a well-known reciprocal replacement of DHA with DPAn-6, in this case in the brain (78) and retina (37,46,49). These two fatty acids differ only with respect to the absence of the Δ-19 double bond in the DPAn-6 molecule; both are 22-carbon HUFA with their first five double bonds in the same positions with respect to the carboxyl end of the molecule. There is little in the modern disciplines of biochemistry, biophysics, and neuroscience to offer a conceptual framework to understand this extraordinary specificity.

The first and most reasonable hypothesis was that a cyclooxygenase or lipoxygenase (LO) product of DHA but not DPAn-6 was produced that had an important function in the central nervous system. This hypothesis was explored extensively after the early reports that cyclooxygenase products of DHA were produced in the rainbow trout gill (79). Early investigations indicated that products made by rat brain were sensitive to LO inhibitors (80–83). A correction of the trout gill work indicated that the DHA products were not prostaglandins but rather LO products (84). Aveldano and Sprecher (85) observed that platelet LO produced a monohydroxylated form of DHA, and Bazan *et al.* (86) found a similar product after incubations with rat retinas. However, Kim and co-workers (80,81) demonstrated that the brain DHA products were a racemic mixture and thus unlikely to be enzymatic products. They also demonstrated that many of the products observed in the brain were a result of the failure to remove platelets and other blood cells by perfusion of the brain before *in vitro* experiments. Apparently, what was being measured *in vitro* may have corresponded to the low level of nonenzymatic fatty acid peroxidation that is known to occur. This is not to deny the existence of LO in the capillary beds in the brain, because Moore *et al*. (87) demonstrated 12-*S*-LO activity in a microvessel fraction. Also, Sawazaki *et al*. (82) found a 12-LO product of AA and DHA in the rat pineal gland and Zhang *et al*. (88) subsequently observed that the formation of these products is regulated by the light–dark cycle and melatonin through the modulation of both 12-LO (88) and cytosolic phopholipase $A_2$ expression (89). They also reported that n-3 fatty acid deficiency had profound effects not only on the pineal lipid profile but also on pineal biochemical activity, resulting in significantly fewer LO products (90).

It is still quite possible that a "magic bullet" type of molecule may be found for DHA, i.e., a function for the nonesterified fatty acid or a metabolite that is extremely potent. Physiologic experiments, for example, have demonstrated that DHA or an anandamide analog of DHA has a potent effect on the $K^+$ channel (91–93). Leaf and co-workers showed that the nonesterified form of DHA has a potent effect on $Na^+$ (94–96) and $Ca^{2+}$ channels (96,97). Also, synaptic transmission (98) and long-term potentiation (99,100) in the hippocampus as well as

*N*-methyl-D-aspartate responses in the cerebral cortex (101) are altered by DHA. However, what is not clear is whether these actions of DHA and its analogs are operative *in vivo*. Moreover, the substrate specificity required to explain the n-3 deficiency syndrome has generally not been found in these studies.

The failure of this initial hypothesis led to proposals that are based on the concept that the active form of DHA is in the form of a phospholipid (102–104). Little progress was made on this intractable problem until workers focused on this hypothesis. Several approaches have been used including cell biological, biochemical, and biophysical attacks. Examples of each of these will be summarized in turn below with reference to apoptosis, protein-lipid interactions, and the physical state and membrane properties of DHA-phospholipids.

The first topic that will be taken up is G-protein signaling with a focus on rhodopsin. This line of inquiry is central to an understanding of the function of DHA-lipids in the visual system; it also serves as a model of other G-protein-coupled signaling receptor systems that helps us to understand how DHA may function in the brain.

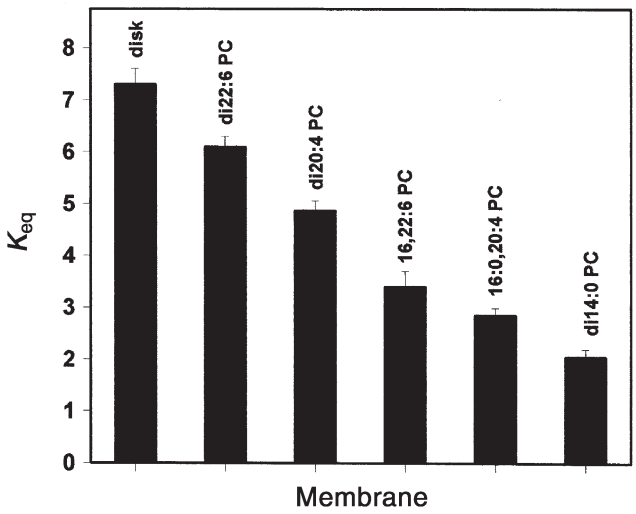


**FIG. 1.** The compositional dependence of the metarhodopsin MI↔MII equilibrium constant, $K_{eq}$, determined for rhodopsin in a series of compositionally defined bilayers varying in degree of unsaturation at 37°C. *Source*: Reference 107. M, metarhodopsin; PC, phosphatidylcholine.

## PROTEIN-LIPID INTERACTIONS: G-PROTEIN SIGNALING

Intercellular signaling is initiated through the activation of ligand-specific receptors imbedded in the lipid bilayers of cellular membranes. An understanding of the factors that govern the efficiency of signaling processes requires elucidating how the lipid composition of the membrane modulates the interactions of these receptors with the other membrane-bound protein components in the signaling pathway. To elucidate the role of n-3 fatty acids in the nervous system and visual process, the phospholipid acyl chain dependence of several steps in the visual transduction pathway was studied (105). This was accomplished by purifying several components of the visual transduction system and reconstituting them in phospholipid vesicles of defined lipid composition (106).

The visual transduction pathway is initiated by the absorption of a photon by rhodopsin, a prototypical member of the family of G-protein-coupled receptors that includes many neurotransmitter receptors such as those for serotonin and dopamine. Metarhodopsin (M)II is the conformation of photoactivated rhodopsin that binds and activates the visual G-protein, $G_t$, which in turn activates a cGMP-specific phosphodiesterase (PDE) (105). Hydrolysis of cGMP by the PDE results in the closing of cGMP-gated channels in the ROS plasma membrane, changing the transmembrane potential and initiating the neuronal response to light. In n-3-deficient animals, a reduced amplitude and delayed response are observed in the leading portion of the a-wave of electroretinograms (37,40,41). This portion of the a-wave is associated with the transduction pathway. To determine whether these observations can be linked to changes in membrane composition, we examined the bilayer dependence of MII formation, the kinetics and extent of MII-$G_t$ complex formation, and resulting PDE activity as a function of phospholipid acyl chain composition and cholesterol content of the bilayer. These topics will be treated in turn below.

$K_{eq}$ measures the extent of MII formation, which represents the formation of the activated ligand-bound receptor state, for the MI-MII equilibrium. This parameter depends critically on the level of acyl chain unsaturation (Fig. 1) (107). For both mixed-chain phosphatidylcholines (PC) and symmetrically substituted PC, the highest levels of MII formation were seen in DHA-containing bilayers. The addition of 30 mol/100 mol phospholipid to these systems lowered the level of MII formation. However, the lowest percentage reductions were obtained in the DHA-containing systems, suggesting that DHA-containing phospholipids are best able to buffer the inhibitory effects of cholesterol.

The first amplification step in the visual cascade is the activation of $G_t$. The initial step in this activation is the binding of $G_t$ to MII and is characterized by the association constant, $K_a$. This process is also affected by acyl chain composition. The value of $K_a$ in 18:0,22:6-PC is more than two times greater than that in 18:0,18:1-PC, indicating that twice as much MII-$G_t$ complex is formed in the DHA phospholipid than in the monounsaturated bilayer (Niu, S., Mitchell, D.S., and Litman, B.J. unpublished results). The number of $G_t$ molecules activated in the two bilayers should be proportional to the amount of complex formed, suggesting that at equivalent levels of MII and $G_t$, a higher signal amplitude will be observed in the DHA-containing bilayer.

Another important aspect of signaling is the response time. This aspect of signaling was addressed by measuring the kinetics of both MII and MII-$G_t$ formation in several bilayers. An important characterizing parameter in these measurements is the ratio of the rate of formation of MII-$G_t$ to that of MII. This parameter represents the lag time in appearance of the

complex after MII has formed and is a measure of the efficiency of the interaction of the receptor and $G_t$ protein. This ratio is 1.4 in the native ROS disk membrane, indicating a rapid complex formation after the appearance of MII (106). Complex formation in 18:0,22:6-PC and 18:0,18:1-PC is characterized by ratios of 3.5 and 4.9, respectively. Although the DHA PC phospholipid is not as efficient as the disk system, it does provide for more efficient MII-$G_t$ formation than the less unsaturated bilayer. Some of the enhanced efficiency of MII-$G_t$ formation in the disk membrane may be attributable to the more complex mixture of phospholipid classes that contribute a net negative surface charge to the membrane.

The overall measure of the signaling pathway is the dose-response curve, generated by determining the level of PDE activity brought about by increasing levels of rhodopsin activation. In these experiments, both $G_t$ and PDE were reassociated with rhodopsin-containing vesicles. At light exposure levels at which 1 in 1000 rhodopsin molecules was activated, ROS disks yielded 87% of their maximal PDE activity. Under similar light exposure conditions, 59 and 26% of maximal disk activity was obtained in 16:0,22:6-PC and 16:0,18:1-PC, respectively (106). Although not reaching the same activity as native disk membranes, the DHA-containing bilayer yields twice the activity of the monounsaturated bilayer. Thus, in the integrated function of the pathway, the DHA-containing bilayer yields higher activity levels than the monounsaturated bilayer.

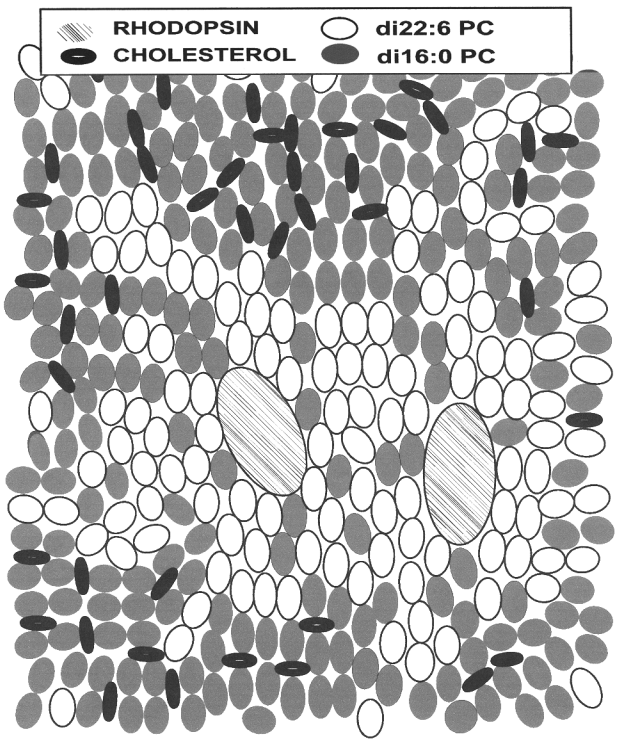


**FIG. 2**. Domain structure of rhodopsin-lipid model membranes. Fluorescence energy transfer studies of model membranes composed of a 3:7:3 mixture of di22:6-phosphatidylcholine (PC)/di16:0-PC/cholesterol and varying levels of rhodopsin show the presence of lateral domains. These domains are composed of a rhodopsin-containing region highly enriched in di22:6-PC and a second region highly enriched in di16:0-PC and cholesterol. The formation of these domains requires the presence of both rhodopsin and cholesterol, demonstrating the complex nature of the molecular interaction responsible for domain formation. These include a rhodopsin preference for docosahexaenoic acid (DHA) acyl chains and a preference of cholesterol for saturated acyl chains (108).

Recent studies suggest that lateral domain formation may play a critical role in the requirements for DHA-containing phospholipids (Fig. 2) (108). Fluorescence energy transfer experiments have provided evidence of the formation of lateral domains in reconstituted membranes consisting of di22:6-PC, di16:0-PC, cholesterol, and rhodopsin. In these domains, the lipid composition around rhodopsin is highly enriched in di22:6-PC, whereas the di16:0-PC is highly enriched in cholesterol. Domain formation requires the presence of both rhodopsin and cholesterol, indicating the complex nature of the interactions that drive lateral segregation of the constituents of this system. If the other components of the signaling pathway have the same preferable partitioning into a DHA-rich lipid domain as rhodopsin, then this would raise their local concentration and provide a greater efficiency for the signaling pathway.

The results presented here demonstrate that the visual signaling pathway is greatly dependent on the acyl chain composition of the bilayer. The steps in this signaling process involve unimolecular conformation changes required for rhodopsin activation and several protein–protein interactions for the activation of $G_t$ and PDE. Both of these types of processes were enhanced in bilayers containing DHA. The studies reported here were carried out in PC bilayers of varying acyl chain composition. The disk membrane contains ~42–45% of both PC and PE and ~10–12% PS. The differences in activity observed between the pure PC system and native disk membrane might be attributable to the lack of a surface potential supplied by the presence of PS or perhaps specific properties contributed by the PE. It should be noted that both PE and PS contain the highest levels of symmetrically substituted di-DHA species in the retina. Despite these differences, the results reported here suggest an explanation for the observations in the electroretinograms of n-3-deficient animals. The delay in the development of the leading edge of the a-wave is likely related to the increased lag time observed in the formation of the MII-$G_t$ complex, whereas the reduced amplitude may be explained on the basis of the reduced association constant observed for MII-$G_t$ complex formation.

## ANTIAPOPTOTIC EFFECT OF DOCOSAHEXAENOIC ACID

Next, we turn to a consideration of the effects of DHA at a cell biological level. Unlike AA, DHA is not easily released from neuronal membranes, but instead is retained by membrane phospholipids (109–111). In contrast, astroglia cells, which are known to support neuronal survival, release this fatty acid readily (110–113). This suggests that DHA fatty acid may act as a trophic factor, and enrichment of this fatty acid in neuronal

membranes may be an important aspect in neuronal survival. In neuronal membranes, DHA is highly enriched in aminophospholipids, especially PS (1,2,4,5–10,114). We and others have previously demonstrated that the enrichment of DHA in cell membranes increases PS synthesis and, conversely, that depletion of this fatty acid by an n-3-deficient diet or by chronic ethanol exposure decreases the accumulation of PS (115–118). Considering the fact that PS is the major negatively charged phospholipid class in many mammalian cell membranes and many of the signaling proteins such as protein kinases are influenced by PS (119–121), this alteration of PS content may have significant implications for cellular function.

In contrast to the well-documented apoptotic effect of DHA (122–127), only a few studies have indicated an antiapoptotic function for this fatty acid (128–131). In each case, an antiapoptotic effect was observed only after preincubation with DHA before the induction of apoptosis. Because DHA is prone to oxidation during the incubation period and much of the apoptotic effect is mediated through oxidative stress (126,127), it is difficult to successfully enrich cultured cells with DHA without accompanying lipid peroxidation. Therefore, to observe the antiapoptotic effect of DHA, it is crucial that an antioxidant such as vitamin E be added along with DHA to the culture medium (130,131).

In studies by Kim *et al*. (131) in which PC-12 or Neuro-2A cells were exposed to AA (1–25 μM) during serum deprivation, apoptosis determined by genomic DNA fragmentation decreased in a dose-dependent manner, but treatment with DHA or oleic acid had no effect. The insensitivity of the protective effect of AA to indomethacin or nordihydroguaiaretic acid indicated that the observed protective effect of AA was not mediated by either cyclooxygenase or LO derivatives, but rather through the direct action of AA (132). In contrast to the effect of AA, DHA became protective only after a prolonged period of incubation. In Neuro-2A cells, the protective effect required at least 24 h of enrichment, and a longer incubation led to an enhancement of the protective action of DHA. During this period, DHA was steadily incorporated into PS, and total cellular PS was increased. The protective effect is related to the extent of cellular PS accumulation. When cells were enriched with DHA in a serine-free medium, the PS content did not increase significantly and the antiapoptotic effect was diminished significantly.

Caspase-3 activity, which has been shown to mediate mammalian apoptosis, increased as the starvation proceeded, with the exception of cells enriched with DHA. Both AA- and DHA-treated cells initially showed less caspase-3 activity in comparison to nonenriched control or oleic acid-enriched cells. However, prolonged serum starvation abolished the protective effect of AA, and only DHA-treated cells maintained caspase-3 activity at a level similar to that of control cells kept in the 5% serum medium. The DNA fragmentation data and DNA ladder formation obtained after at least 48 h of serum starvation showed consistent results (Fig. 3). It was also observed that the 17-kDa active fragment of caspase-3 increased under serum-free conditions, and supplementation of Neuro-2A cells with DHA before serum starvation effectively prevented the increase of the 17-kDa fragment (131).

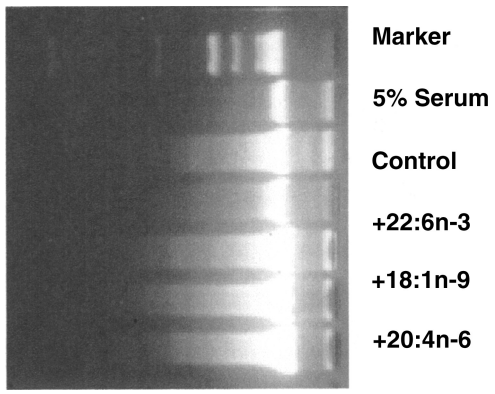


**FIG. 3**. Protection from DNA fragmentation after the enrichment of cells with docosahexaenoic acid for 48 h.

Enrichment of cells with DHA also altered expression of various proteins at the gene level because the levels of mRNA for caspase-3 decreased, whereas mRNA of Raf-1 increased (131,132). DHA has been shown to affect transcriptional activities through nuclear hormone receptors such as the peroxisome proliferator-activated receptor (133) or the retinoid X receptor (134). The antiapoptotic effect of DHA enrichment suggests that ensuring the survival of neuronal cells may be one of the reasons for the high level of DHA in brain. Alterations of the membrane PS content by DHA will influence not only the receptor activities but also the translocation of various signaling proteins as well as their activation (118–120,131). Although the release of DHA in neuronal cells may be minimal, it may be possible to reach a local concentration of intracellular DHA sufficient to activate nuclear receptors involved in transcriptional activity. It is likely that the antiapoptotic effect of DHA is the result of multiple regulations at various signaling stages, ranging from the plasma membrane to nuclear events, most of which have yet to be discovered.

## BIOPHYSICAL PROPERTIES OF POLYUNSATURATED LIPID MEMBRANES

As referred to above, there has been a lack of a conceptual basis in biophysics in which a meaningful difference in function could be predicted with respect to a DHA- vs. a DPAn-6-containing lipid. In fact, there have been few biophysical studies that discern appreciable differences in the properties of various polyunsaturated species. It has often been said that introduction of a double bond into a saturated lipid results in a large change in physical properties. Introduction of a second double bond also results in an additional effect; however, it is of a much smaller magnitude than that of the first double bond. Thereafter, introduction of additional double bonds (for a total of three or more) has little effect. However, the n-3 fatty acid deficiency syndrome described above indicates that there are significant biological effects measurable in the whole organism, e.g., by

behavioral or physiologic means, when DPAn-6 is substituted for DHA. Because these are pentaenoic and hexaenoic lipids, it would appear that a physical basis must exist for the differential biological function of various highly unsaturated fatty acids. Recent studies have begun to demonstrate that all highly unsaturated lipids indeed do not have the same physical properties. Some of the techniques and research approaches as well as data obtained from these investigations are presented below.

## MAGIC ANGLE SPINNING (MAS)

Solid-state nuclear magnetic resonance (NMR) spectroscopy on models of polyunsaturated membranes enables the measurement of dozens of parameters, probing every segment of the lipid bilayer with atomic resolution. This became possible after improving the performance of magic angle spinning (MAS) probes in combination with very high magnetic field strength. MAS NMR reduces the linewidth of lipid resonances to ~10 Hz (135). That is equivalent to or better than resolution of resonances for very small unilamellar liposomes that tumble rapidly enough to eliminate anisotropic interactions. However, in contrast to studies of liposomes, for MAS NMR, the membranes are not required to have small radii of curvature, and water content does not matter as long as the lipids remain in the liquid-crystalline state. Just a few milligrams of sample is sufficient to provide very high signal-to-noise ratios as evidenced by the spectra in Figure 4 (136).

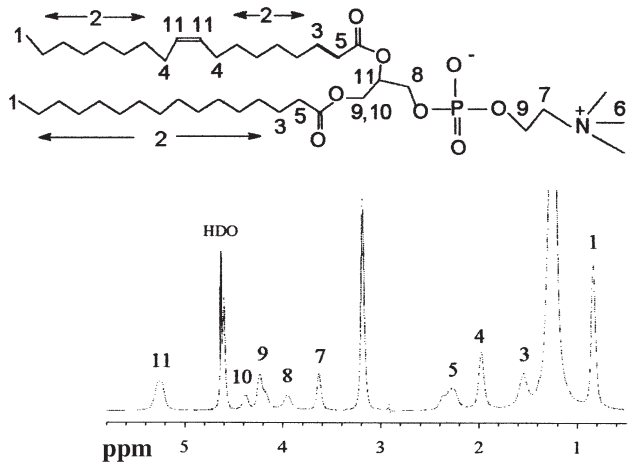


**FIG. 4.** $^1$H magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectrum of 16:0,18:1 PC in 50 wt% $D_2O$ recorded at ambient temperature and a spinning speed of 10 kHz. Signal assignment is provided by numbers in the spectrum and the formula. *Source*: Reference 137.

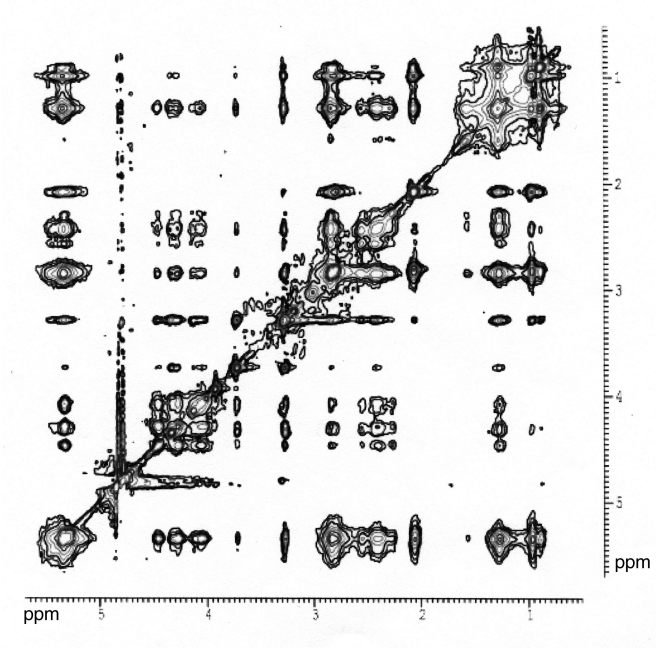


**FIG. 5**. Two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectrum of an 18:0-22:6 phosphatidylcholine (PC)/18:$0_{d35}$-22:6 phosphatidylethanolamine (PE)/18:$0_{d35}$-22:6 phosphatidylserine (PS)/cholesterol$_{d7}$ (4:4:1:3, by vol) mixture.

## NUCLEAR OVERHAUSER ENHANCEMENT SPECTROSCOPY (NOESY)

The excellent resolution of lipid resonances allows application of techniques that probe magnetization transfer between protons such as nuclear Overhauser enhancement spectroscopy (NOESY), well known for its important contribution to the structural determination of soluble proteins (137).

Magnetization transfer is the result of interactions between the magnetic dipoles of the protons in lipids. Rates of transfer become observable when the protons approach each other to within distances ≤5 Å (138). In the two-dimensional NOESY contour plot shown in Figure 5 (139), the peaks along the diagonal correspond to the one-dimensional resonances shown in Figure 4. The rate of magnetization transfer is reflected by the intensity of the off-diagonal crosspeaks. The surprising observation has been that magnetization is transferred between all lipid resonances, but at different rates. Even the most distant protons such as methyl groups of the choline headgroup and methyl groups at the end of lipid hydrocarbon chains exchange magnetization. For a long time, such surprising transfers were ascribed to a process called spin diffusion. Spin diffusion relays magnetization *via* coordinated flip-flops of magnetization along the proton network of a lipid molecule. In a series of experiments on protonated lipids in deuterated matrices, we demonstrated recently that this interpretation is incorrect (140). In the biologically relevant liquid-crystalline state, membranes are truly disordered to the point that lipid headgroups and ends of hydrocarbon chains meet once in a while, albeit with lower probability than membrane segments that are located closer to each other. The experiments also demonstrated that the unexpected long-range contacts are not taking place within one lipid molecule, but rep-

resent magnetization transfer to neighboring lipid molecules that surround the emitter (141).

Experiments on *sn*-1 chain perdeuterated 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (18:$0_{d35}$,22:6-PC) not only confirmed that polyunsaturated chains in membranes are similarly disordered, but also offered evidence that conformational disorder of the remaining degrees of freedom of a polyunsaturated chain is higher (135,142). Furthermore, the intensity of cross- and diagonal peaks as a function of mixing times allows judgment about motional correlation times in the polyunsaturated membranes on the time scale from pico- to milliseconds (138). The spin-lattice and spin-spin relaxation rates of polyunsaturated chains are lower, indicating more rapid motions of these protons. This result is surprising because polyunsaturated chains have long been perceived as rigid and bulky owing to the loss of degrees of freedom.

## DIPOLAR RECOUPLING ON-AXIS WITH SCALING AND SHAPE PRESERVATION (DROSS)

MAS NMR techniques with application of radio-frequency pulses, which are synchronized with the phase of the spinning rotor, enable recoupling of anisotropic interactions, e.g., the magnetic dipolar interaction between $^{1}$H and $^{13}$C nuclei. The strength of this interaction depends on the time-averaged orientation of the $^{1}$H-$^{13}$C bond vector with respect to the lipid bilayer normal.

In the two-dimensional dipolar recoupling on-axis with scaling and shape preservation (DROSS) experiments (Fig. 6), this technique allows assignment of order parameters to resolved $^{13}$C resonances of lipid segments (143). Conducting this experiment on $^{13}$C nuclei benefits from the much greater resolution of $^{13}$C chemical shifts. Throughout the molecule, >20 order parameters can be assigned to specific regions within the polyunsaturated lipid, without isotopic labeling.

The experiments showed unambiguously that order parameters of double bond $^{1}$H-$^{13}$C vectors are close to 0. Because of differences in bond geometry between saturated and unsaturated hydrocarbon chains, this result was not unexpected. However, the very-low-order parameters for the five $^{1}$H-$^{13}$C vectors of the methylene groups that are sandwiched between double bonds were surprising. These order parameters must be low as a result of crankshaft-like coordinated motions within the DHA chain. Such motions are likely because of lower potential barriers for rotations about the vinyl-methylene bonds compared with C-C bonds in saturated chains (144). This enables DHA chains to adapt to looped conformations that have shorter length and larger area per molecule (145).

## $^{2}$H NMR

Although the DROSS technique allows determination of assigned order parameters, for technical reasons, the precision in order parameter determination remains about one order of magnitude lower than the precision of order parameters measured by the classical approach, i.e., analysis of effective quadrupolar splittings in the $^{2}$H NMR spectra of deuterated lipids (146). Quadrupole splittings are measured with a resolution of ~50 Hz, which corresponds to a precision for order parameters of $\Delta S = \pm 0.0004$. Such small changes are of relevance, e.g., to detect the level of stress that is caused in the lipid matrix as the result of a conformational change of a membrane protein (147). We conducted preliminary $^{2}$H NMR experiments on perdeuterated DHA, incorporated at low concentration into bilayers of 18:0,18:1-PC (Fig. 7). Partial assignment of the order parameters to segments of the DHA chain was achieved by taking advantage of the small differences in chemical shift between the deuterium resonances in MAS experiments with partial recoupling of the quadrupolar interaction (Gawrisch, K., Safley, A.M., and Polozov, I.V., unpublished data). The results fully confirmed the observations from the less precise DROSS experiment. Order parameters of all methylene segments between double bonds in the hy-

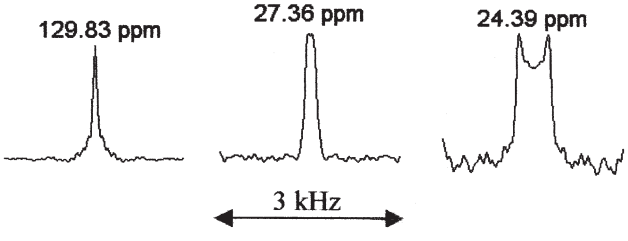


**FIG. 6.** Extracted columns of a two-dimensional dipolar recoupling on-axis with scaling and shape preservation (DROSS) spectrum corresponding to resonance signals of double bonds (129.83 ppm), methylene groups between the double bonds (27.36 kHz), and the C-17 methylene group of stearic acid (24.39 ppm). The peak doublets are the result of recoupling of dipolar interactions between the $^{1}$H and $^{13}$C nuclei. The value of order parameters is directly proportional to the magnitude of splittings.

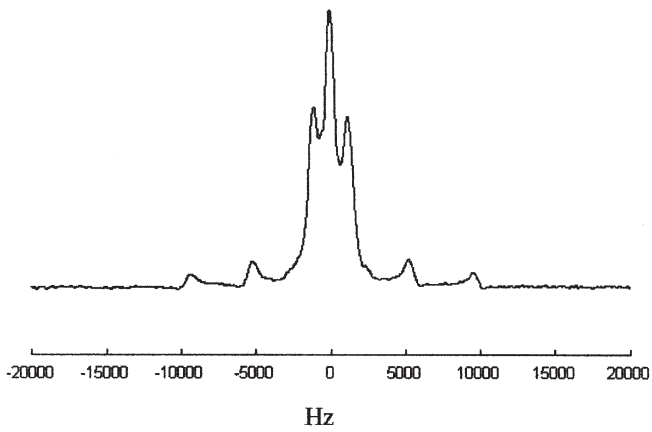


**FIG. 7.** $^{2}$H nuclear magnetic resonance (NMR) spectrum of perdeuterated docosahexaenoic acid in an 18:0,18:1-PC matrix.

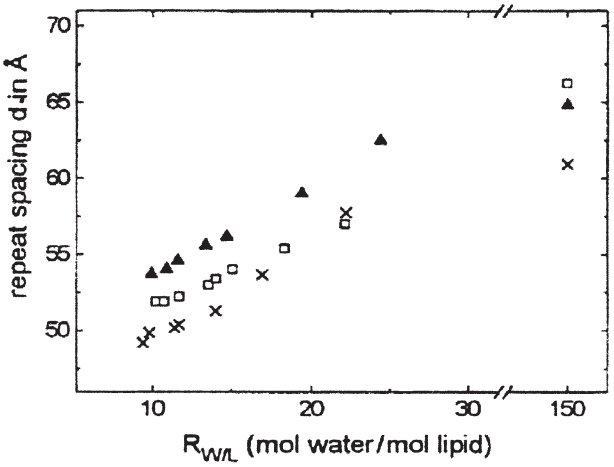


**FIG. 8.** X-ray repeat spacing of lipid bilayers as a function of water content expressed as the number of water molecules per lipid. *Source:* Reference 142.

drocarbon chain, as well as the order of the majority of the double bonds, are very low. Only the two methylene segments near the carboxyl group of DHA have order parameters that are comparable to values of more saturated chains.

## X-RAY DIFFRACTION

Results of X-ray diffraction experiments on 18:0,22:6-PC, recorded as a function of water content, were compared with the results of experiments on 18:0,18:1-PC and di14:0-PC (Fig. 8) (142). The repeat spacing of bilayers with polyunsaturated chains, i.e., the thickness of the lipid layer plus the thickness of the water layer separating two bilayers, is rather similar to the thickness of the di14:0-PC bilayers with only 14 carbon atoms per chain.

Water uptake of polyunsaturated lipids is slightly increased and does not account for this difference (142). We estimated that the polyunsaturated DHA occupies a unit cell with average dimensions of $6 \times 6 \times 14$ Å in the bilayer. Compared with saturated and monounsaturated chains, this is an increase in chain area of ~7 $Å^2$. DHA chains in an extended angle-iron or helical conformation exceed this length by up to 10 Å (148); therefore, they cannot be the only conformation that DHA chains adopt in bilayers. Consequently, we propose that looped conformations, as proposed in a paper by Dolmazon's laboratory (145), are very common in polyunsaturated chains.

We also conducted X-ray experiments as a function of osmotic stress to reduce the sample's water uptake. Controlled dehydration of membranes creates lateral tension, which compresses bilayers laterally. Using a combined NMR and X-ray approach, we followed the changes in area per molecule of saturated and polyunsaturated chains in 18:0,22:6-PC as a function of lateral tension. We observed that 75% of the increase in chain length and reduction in area per molecule was the result of changes in the average conformation of the polyunsaturated chain (142).

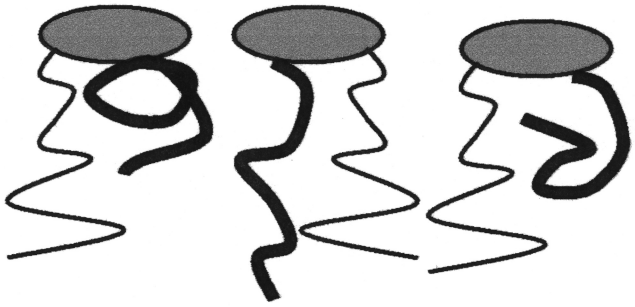

**FIG. 9.** A schematic that highlights the conformational freedom of docosahexaenoic acid (DHA) chains in mixed-chain lipids. DHA converts rapidly between looped and extended conformations. The center of mass of the DHA chain is closer to the lipid–water interface.

## CONFORMATION AND FLEXIBILITY OF THE DHA CHAIN

The alteration of elasticity of neural membranes according to the number of double bonds per fatty acid is one possible role of lipid polyunsaturation. Membranes contain a matrix of lipid molecules with hydrophilic ("water-loving") headgroups (shown in gray motif) and hydrophobic ("water-rejection") fatty acid chains in black (Fig. 9). Membranes respond to external perturbations like elastic rubber bands. With NMR spectroscopy and X-ray diffraction, the elastic deformation of membranes under tension, including the changes in structure and motions of lipid hydrocarbon chains, could be measured. The results suggest that polyunsaturated chains in membranes prefer flexible, looped, and helical structures with rapid transitions among a large number of conformers. This provides increased flexibility to receptor-rich neural membranes that contain high concentrations of DHA. A common perception of polyunsaturated chains is that they are stiff and inflexible due to the presence of motionally restricted double bonds. In contrast, our NMR studies indicate exceptionally high deformability of DHA chains in biomembranes.

## DIFFERENCE IN MEMBRANE PROPERTIES BETWEEN DHA AND DPA LIPIDS

Although there is ample evidence that membrane biophysical properties control membrane protein function to a significant extent, the idea that the loss of a single double bond from DHA to DPAn-6 in lipids is sufficient to alter membrane biophysical properties has been met with some skepticism. We compared deuterated *sn*-1 chain order parameter profiles in mixed-chain 18:0,22:6-PC and 18:0,22:5-PC by $^2$H NMR order parameter experiments.

Saturated chain order of the first half of the chain near the lipid–water interface was high and almost constant (order parameter plateau), whereas order of the second half of the chain decreased with a steep gradient toward the terminal methyl chain end. This order profile has been linked to the probability of gauche-*trans* isomerization in chains (higher at the terminal methyl end).

Changes in hydrocarbon chain length and area per molecule are reflected in order parameter changes. An increase of average chain order by $\Delta S = +0.002$, which can be easily resolved, corresponds to an increase of average bilayer thickness of 0.1 Å and a decrease of area per molecule of 0.2 Å$^2$. Compared with monounsaturated bilayers, the order of *sn*-1 chains that are paired with polyunsaturated chains in the *sn*-2 position is lower, mostly for the second half of the chain, with a maximal decrease by $\Delta S = -0.018$ near carbon atom number 13 for 18:0,22:6-PC (149). We propose that this decrease reflects formation of short looped chain conformations (*vide infra*) with a higher density of polyunsaturated chain segments near the lipid–water interface. As a result of this redistribution of DHA chain density, the lower segments of the saturated chain have more freedom for movement and lower chain order parameters in the second half of the chain. The surprising observation has been that the loss of a single double bond from DPAn-6 to DHA in 18:0,22:5-PC results in order parameters that are much closer to those of a matrix with monounsaturated oleic acid chains in position *sn*-2.

In summary, assigned order parameters and relaxation times related to molecular motions were measured using novel MAS NMR approaches and classical $^2$H NMR order parameter studies on chain perdeuterated, polyunsaturated lipids. In comparison to saturated chains, DHA order parameters were low, reflecting both a change in bond geometry and an increase in chain dynamics. The loss of a single double bond near the terminal methyl group of hydrocarbon chains has a significant influence on lipid matrix properties; therefore, membranes rich in DHA have unique properties. We speculate that the differences are of importance for the function of receptor proteins in retinal and synaptosomal membranes.

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# Metabolism and Functions of Highly Unsaturated Fatty Acids: An Update

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**ABSTRACT:** This review briefly examines the recent progress in knowledge about the synthesis and degradation of highly unsaturated fatty acids (HUFA) and their functions. Following the cloning of mammalian Δ6-desaturase (D6D), the D6D mRNA was found in many tissues, including adult brain, maternal organs, and fetal tissue, suggesting an active synthesis of HUFA in these tissues. The cloning also confirmed the long-postulated hypothesis that the same pathway is followed in n-6 and n-3 HUFA synthesis. Dietary n-6 and n-3 HUFA both induce fatty acid oxidation enzymes in peroxisomes when compared to their respective precursor polyunsaturated fatty acids. This suggests that peroxisomes may be the primary site of HUFA degradation when HUFA are supplied in excess from the diet. Peroxisome proliferators strongly induce the enzymes for the HUFA synthesis. The mechanism of this induction is currently unknown. Recent studies revealed new HUFA functions that are not mediated by eicosanoids. These functions include endocytosis/exocytosis, ion-channel modulation, DNA polymerase inhibition, and regulation of gene expression. These new discoveries will enable us to re-examine the underlying mechanisms for the classical symptoms of essential fatty acid deficiency as well as vitamin E deficiency. Progress has also been made in understanding the mechanism by which dietary HUFA reduce body fat deposition. One mechanism is induction of genes for fatty acid oxidation, which is mediated by peroxisome proliferator-activated receptor-α. Another likely mechanism is that HUFA suppress genes for fatty acid synthesis by reducing both mRNA and protein maturation of sterol regulatory element binding protein-1.

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Among the essential fatty acids shown in Figure 1, at least linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) are required from diets because mammals lack enzymes to synthesize these fatty acids from acetyl CoA. Mammals are able to synthesize other fatty acids from these precursor fatty acids. Δ6-Desaturase (D6D) catalyzes the first and rate-limiting step (1). Products of D6D [highly unsaturated fatty acids (HUFA)] can also be supplied from foods (Fig. 1). We recently succeeded in cloning mammalian D6D using the sequence of the algal D6D (2). The predicted amino acid sequence of the cloned D6D is entirely different from that of the "linoleoyl-CoA desaturase" whose purification was reported nearly 20 yr ago (3). The D6D mRNA is expressed in many human tissues, including adult brain (2,4). We also have shown that the dietary precursor 18:2n-6 (2) and products (HUFA) (4) suppress D6D activity and mRNA when substituted for oleic acid. This feedback regulation of HUFA synthesis is largely at the transcriptional level (Nakamura, M.T., Cho, H.P., and Clarke, S.D., unpublished data).

Tracer studies showed that only a small percentage of dietary precursor fatty acids were converted to HUFA (5,6). These data have raised concern that the HUFA synthesis from precursors may be insufficient, especially in rapidly growing animals. Thus, dietary HUFA supplementation may be necessary for the early stages of development. Indeed, search of the BLAST expressed sequence tag (EST) database shows the expression of the D6D mRNA in human fetus, infant, amnion, uterus, and breast, suggesting active HUFA synthesis by fetus and infant as well as maternal contribution to the HUFA supply. These data imply a possible benefit of HUFA supplementation in infant formula. Nevertheless, these results are not conclusive for the following reasons. First, the synthetic pathway of HUFA is fully activated only in deficient conditions, and the expression and activity of the synthetic enzymes drop quickly when the body HUFA is repleted (2,4). Second, tissue expression detected by EST does not necessarily mean the presence of actual HUFA synthesis in these tissues, nor does it yield any quantitative data.

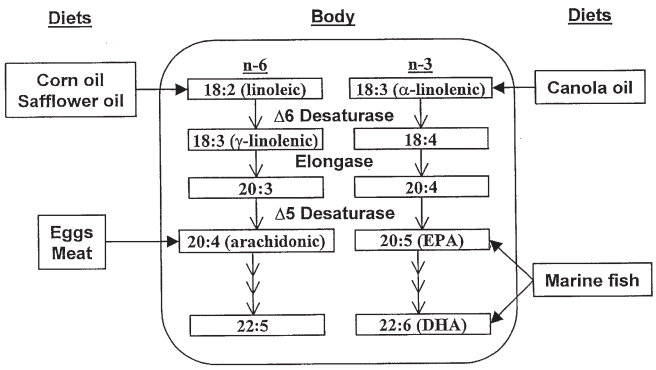


**FIG. 1.** Synthetic pathway of highly unsaturated fatty acids (HUFA). EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

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Abbreviations: D6D, Δ6-desaturase; FAS, fatty acid synthase; HUFA, highly unsaturated fatty acids; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acids; SREBP, sterol regulatory element binding protein; TG, triglyceride.

Another recent development to be noted is that our cloning study demonstrated that the synthesis of both n-6 and n-3 HUFA is catalyzed by the same Δ6- and Δ5-desaturases as shown in Figure 1 (2,4). This strongly supports the importance of a balanced supply of dietary n-3 and n-6 fatty acids to meet the requirement of both n-3 and n-6 HUFA. Also, the studies suggest that an excessive supplementation of either n-3 or n-6 HUFA in the diet may cause a deficiency in the other group because it may shut down the synthetic pathway shared by n-3 and n-6 fatty acids.

## DEGRADATION OF HUFA

There are two known degradation pathways of HUFA. One is the formation of eicosanoids, which is a unidirectional reaction. Eicosanoids are further metabolized to inactive forms and excreted mostly in urine (7). The other pathway is fatty acid β-oxidation. In noneicosanoid functions, the hydrolyzed free HUFA can be re-esterified to phospholipids after forming a thioester bond with coenzyme A (CoA). The HUFA CoA can also be oxidized *via* the β-oxidation pathway in mitochondria and peroxisomes (8,9). Both n-6 and n-3 HUFA induce acyl-CoA oxidase, the rate-limiting enzyme of peroxisomal β-oxidation, when precursor polyunsaturated fatty acids (PUFA) in the diet are replaced with the respective HUFA (10–12). These studies suggest that peroxisomes may be the primary site of oxidization of excess HUFA. Another line of studies has elucidated the molecular mechanism of this induction. Peroxisome proliferator-activated receptor-α (PPARα) plays a key role in the induction of the fatty acid oxidation enzymes in both peroxisomes and mitochondria, and the response elements have been identified in key enzymes such as acyl-CoA oxidase (13) and carnitine palmitoyltransferase 1 (14). Free fatty acids directly bind and activate PPARα (15,16). Taken together, the excess HUFA are likely to induce their own oxidation machinery *via* a feed-forward mechanism, which is mediated by PPARα. However, the quantitative contribution of peroxisomes in HUFA degradation is yet to be determined.

## INDUCTION OF D6D BY PEROXISOME PROLIFERATORS

Kawashima and colleagues (17) found that fibrates, a group of PPARα ligands known as peroxisome proliferators, are potent inducers of D6D activity. We found that Wy 14,643, a PPARα-specific ligand and another peroxisome proliferator, strongly induces the D6D mRNA and that transcriptional activation of the D6D gene largely accounts for the induction (Nakamura, M.T., Cho, H.P., and Clarke, S.D., unpublished data). The mechanism of this induction is presently unknown. The simplest explanation for the activation of D6D transcription by these compounds is that the D6D gene has the PPARα response element and is activated directly by liganded PPARα. However, this mechanism faces some difficulties in explaining results of other studies. First, although both HUFA and peroxisome proliferators are agonistic ligands of PPARα (15,16), these compounds have opposite effects on D6D gene expression: Wy 14,643 activated transcription of the D6D gene, whereas HUFA suppressed the transcription (Nakamura, M.T., Cho, H.P., and Clarke, S.D., unpublished data). Second, in spite of marked activation (16-fold) of D6D activity in rat liver after 7-d feeding of clofibric acid, HUFA in the liver did not increase (17). In contrast, when growth hormone was overexpressed for the same duration (7 d), the liver HUFA was enriched more than 50%, with a much smaller increase (<threefold) in the D6D activity (18). An alternative hypothesis to accommodate these observations is that peroxisome proliferators induce HUFA synthesis by increasing HUFA oxidation because of their ability to induce rate-limiting enzymes of fatty acid β-oxidation. Because functions of HUFA are modulated by the abundance of HUFA in cells (1,7,19,20), it is likely that many cellular functions are affected by the balance between the supply and degradation of HUFA. Therefore, the regulatory mechanism of synthesis and degradation of HUFA would be an important area for future investigation.

## PHYSIOLOGICAL ROLE OF HUFA

HUFA are required for a wide range of physiological functions. The best characterized among them are the functions of eicosanoids that are enzymatically synthesized from HUFA. Eicosanoids work as autocrine/paracrine hormones and mediate a variety of functions such as immune response, blood pressure regulation, and blood coagulation (21). In addition to these eicosanoid-mediated functions, recent studies have uncovered further involvement of HUFA in fundamental cellular functions, including endocytosis/exocytosis (22), ion-channel modulation (23), DNA polymerase inhibition (24), and regulation of gene expression (25). Schmidt *et al.* (22) showed that endophilin, a vital component in neurotransmitter recycling, is a lysophosphatidic acid acyl transferase. Their study suggests that rapid hydrolysis of phospholipids by phospholipase $A_2$ and reacylation by lysophospholipid acyl transferases change the membrane curvature and thus play a critical role in endocytosis and exocytosis. Another important role of HUFA is to help muscle contraction synchronized by slowing down the recovery of ion channels (23). This is the likely mechanism by which dietary fish oil could reduce death rates after myocardial infarction. DNA polymerase inhibition, another potential function of HUFA, is still in the early stage of investigation, and its significance in the regulation of the cell cycle and DNA repair is yet to be elucidated (24). HUFA also regulate many genes involved in lipid metabolism (25). HUFA directly bind and modulate activities of transcription factors such as PPAR (15,16) and hepatocyte nuclear factor 4 (26). Moreover, our recent study suggests that HUFA regulate gene expression by modulating the mRNA stability and proteolytic processing of steroid regulatory element binding protein 1 (SREBP-1) (27,28), which is discussed in more detail below. A common feature in these HUFA functions is that HUFA first need to be hydrolyzed

from membrane phospholipids to perform their functions. This implies phospholipases as a key regulator of these functions.

These discoveries enable us to revisit and examine the potential mechanisms of classical symptoms of essential fatty acid deficiency as well as vitamin E deficiency. For example, the main symptoms of essential fatty acid deficiency are dry skin, dermatitis, and water loss through skin (29). This may be due to the decrease of waxy compound secretion by sebaceous glands, rather than a change in the membrane structure of epidermal cells. Another example is embryo resorption in vitamin E-deficient rats. The resorption occurs on the 13th day of gestation, when nutrient transport by pinocytosis plays a critical role, and vitamin E-deficient rats seem to lose this pinocytosis function (30,31). This observation is in accordance with the role of HUFA in exocytosis/endocytosis.

## REDUCTION OF BODY FAT BY DIETARY HUFA

In a genetic model of obesity, replacing dietary 18:2n-6 with a small amount of its D6D product, 18:3n-6, reduces the development of obesity (32). This effect was observed in a dietary induced model of obesity (12). Dietary fish oil also decreased fat deposition in a dietary induced rat model (33,34) and possibly in humans (35). Taken together, dietary HUFA exert a unique effect of reducing fat deposition compared with the precursor PUFA. As reviewed in the Degradation of HUFA section above, this HUFA effect can be explained, at least in part, by increased acyl-CoA oxidase gene expression (10,11) and the resulting higher β-oxidation capacity in peroxisomes (12). Interestingly, 18:2n-6, 18:3n-6, and 20:4n-6 all activate PPARα with equal potency *in vitro* (15). Thus, the unique effect of HUFA *in vivo* may be due to the difference between these fatty acids as substrates for triglyceride (TG) synthesis. HUFA seem to be a very poor substrate for TG synthesis, whereas the precursor PUFA are readily stored in adipose tissue in humans (36), pigs (37), and rats (38), as fatty acid composition analysis indicates.

In addition to the induction of oxidation enzymes, dietary PUFA suppress hepatic lipogenic genes such as fatty acid synthase (FAS), pyruvate kinase, and S-14 at the transcriptional level (25). SREBP-1 is the likely mediator of this effect. The binding site of SREBP-1 is identified in the proximal promoter of the FAS gene (39), and overexpression of SREBP-1 activates all lipogenic genes, including the FAS gene (40). We found that PUFA suppressed the SREBP-1 mRNA, but the transcription of the SREBP-1 gene was unchanged (27). Our subsequent study has shown that PUFA do indeed increase degradation of the FAS mRNA (28). PUFA also suppressed both precursor and mature SREBP-1 proteins, with suppression of the latter being stronger (27). These results suggest that PUFA suppress the FAS gene by reducing the stability of the SREBP-1 mRNA and proteolytic processing of SREBP-1 protein. At present, it is not clear whether HUFA suppress SREBP-1 expression more strongly than precursor PUFA. Our *in vivo* study using a D6D inhibitor suggests that Δ6-desaturation is required for the suppression of the FAS gene by PUFA (41). It is yet to be determined which mechanism, the induction of fatty acid oxidation or the suppression of fatty acid synthesis, plays a greater role in the reduction of body fat by dietary HUFA.

## SUMMARY

Recent developments have brought new insights into the metabolism and function of HUFA:

(i) Expression of D6D mRNA in many tissues suggests that active synthesis of HUFA occurs in these tissues.

(ii) Cloning desaturase confirmed the postulated shared pathway for n-6 and n-3 HUFA synthesis.

(iii) Peroxisomes may be the primary site of HUFA degradation when HUFA are supplied in excess from the diet.

(iv) Peroxisome proliferators strongly induce enzymes for the HUFA synthesis by a currently unknown mechanism.

(v) Recently found HUFA functions include endocytosis/exocytosis, ion-channel modulation, DNA polymerase inhibition, and regulation of gene expression.

(vi) The likely mechanisms by which dietary HUFA reduce body fat deposition are the induction of genes for fatty acid oxidation and suppression of genes for fatty acid synthesis by reducing both the SREBP-1 mRNA stability and maturation of the protein.

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# Stable Isotope Approaches, Applications, and Issues Related to Polyunsaturated Fatty Acid Metabolism Studies

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**ABSTRACT:** The use of stable isotope tracers for investigating fatty acid metabolism in human subjects has increased substantially over the last decade. Advances in analytical instrumentation, commercial availability of labeled substrates, and safety considerations are major reasons for this increased use of stable isotope tracers. Several experimental design options are available for using either deuterium or carbon-13 as tracers for fatty acid and lipid studies. Options include feeding a pulse dose of labeled fat or a mixture containing two or more labeled fats. Multiple doses of the labeled fat can be fed at timed intervals to increase enrichments. Administration by injection or continuous intravenous infusion is an alternative. Another option is to use diets containing foods from plants that have slightly higher natural carbon-13 enrichment. Each basic experimental design has its specific strengths, and the best choice of experimental design depends on the study objectives. Stable isotope studies have been used to address a variety of questions related to unsaturated fatty acid metabolism in humans. Examples are provided that illustrate the use of stable isotopes to investigate oxidation of docosahexaenoic acid, desaturation of linoleic and linolenic acids in infants and adults, incorporation of long-chain n-6 and n-3 fatty acids, bioequivalency of linolenic acid in primates, $^{13}C$ nuclear magnetic resonance spectra of arachidonic acid in living rat brain, and effect of triacylglycerol structure on absorption. Radioisotope and stable isotope tracer studies in animals and humans are responsible for much of our understanding of fatty acid and lipid metabolism. However, tracer studies have limitations, and there are some unresolved issues associated with isotope studies. Examples of unresolved issues are quantification of isotope data, validity of *in vivo* fatty acid metabolite results, kinetic modeling, subject variability, and use of blood lipid data as a reflection of tissue lipid metabolism. Resolving these issues, developing novel methodology, and applying stable isotope tracer methods to questions related to PUFA metabolism are broad areas of interesting and challenging research opportunities.

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Abbreviations: DOB, delta $^{13}C$ values over baseline; dpm, decompositions per minute; %en, percent energy; GC, gas chromatography, GC–C–IRMS, gas chromatography–combustion–isotope ratio mass spectrometry; IRMS, isotope ratio mass spectrometry; High22:6, high docosahexaenoic-acid diet; High20:4, high arachidonic acid diet; LC-PUFA, long-chain polyunsaturated fatty acid; Low20:4, low-arachidonic acid diet; Low22:6, low-docosahexaenoic acid diet; MIDA, mass isotopomer distribution analysis; MS, mass spectrometry; NCI, negative chemical ionization; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PCI, positive chemical ionization; PUFA, polyunsaturated fatty acid; SIM, selected-ion monitoring; TAG, triacylglycerol; VLDL, very low density lipoprotein.

The theory and application of isotope tracer methods for metabolic studies were validated by radioisotope studies in animals, and the results from these early metabolic tracer studies have provided much of our current knowledge about fatty acid metabolism (1). More recently, stable isotopes have emerged as practical alternatives to radioisotopes. The increased use of stable isotopes has been prompted by advances in analytical instrumentation, increased availability of stable isotope-labeled fats, and the proliferation of misguided regulations and concerns about the safety of radioisotope tracers. A number of good reviews detail the history, theory, methodology, and applications of stable isotopes in biological studies (2–8). The focus of this review is to discuss briefly study design options, associated analytical methods, and recent studies that illustrate use of deuterium and carbon-13 isotope approaches for addressing questions relevant to fatty acid metabolism in infants and mothers. Some issues and research opportunities related to the use of stable isotopes to study polyunsaturated fatty acid (PUFA) metabolism are discussed.

## STUDY DESIGN OPTIONS

Various stable isotope experimental approaches are available, but there is no one best study design. The final choice of the approach to use depends on the information needed, sensitivity and accuracy requirements, and availability of labeled tracers and analytical equipment. The single-labeled experimental design is the most widely used approach and involves administration of a pulse oral dose of one labeled fatty acid. A superior approach is the multiple-labeled design that involves administration of a pulse oral dose of a mixture containing two or more labeled fatty acids (1). These types of tracer experiments are often incorporated into an experimental design that consists of a control and an experimental or treatment group. The advantage of a multiple-labeled experimental design compared to a single-labeled design is that the metabolic fate of two or more fatty acids can be directly compared in the same subject under identical experimental conditions. This design feature enhances confidence in the results because all experimental variables affect each labeled fatty acid equally, and it reduces some interpretation problems due to subject variability. Interpretation of the tracer data is simplified because data for the labeled fatty acids are directly comparable to each other. Other advantages of the multiple-

labeled design are that a labeled fatty acid, such as oleic acid (18:1n-9), can be used as an internal standard or control and each subject can serve as his or her own control. Use of an internal standard fatty acid in a series of experiments provides a common denominator for comparing results from different studies with different fatty acids. Feeding a mixture containing three or four differently labeled fatty acids is the equivalent of combining three or four single-isotope experiments into one, but more information is obtained for essentially the same effort as required for one single-isotope experiment. We typically use labeled 18:1n-9 as an internal control because 18:1n-9 is easy to label with two, four, six, or eight deuterium labels. Using fatty acids with different numbers of deuterium labels is advised because it avoids questions about what fatty acid was the precursor of any fatty acid metabolite detected. Employing a multiple-isotope design is uncomplicated with deuterium-labeled fatty acids and conventional gas chromatographic–mass spectrometric (GC–MS) analysis. It is more complex if a combustion–isotope ratio mass spectrometry (C–IRMS) approach is used because the advantage of different labeling patterns is lost when samples are combusted.

A double-labeling approach is useful for following the metabolic fate of different parts of the same molecule. For example, this approach can be used to determine if a fatty acid acyl group remains attached to the glycerol backbone of a triacylglycerol (TAG) or a phospholipid. Labeling both acyl and double-bond carbons provides an approach to detect specific desaturases, to investigate metabolic pathways, and to measure isotope effects.

Multiple-pulse oral-dose studies involve administering several doses of a labeled fatty acid during an appropriate time interval. This approach can be used to increase the concentration of labeled fatty acid metabolites in lipid pools that are difficult to enrich and to increase the enrichment of metabolites present at low concentrations. The increased enrichment enhances both detection and quantification.

The constant-infusion isotope-dilution technique consists of continuously infusing a labeled fatty acid at a constant rate until an isotopic steady state is achieved. The fatty acid turnover rate is calculated from the isotope dilution data at steady state (9). A bolus injection of labeled fatty acid can also be used to calculate plasma fatty acid turnover rate from the decrease in enrichment of the labeled substrate over time (10). Administration of a pulse-oral dose of a tracer combined with continuous intravenous infusion of a tracer containing a different number of labels is a variation that has been used to measure cholesterol absorption (11). The ratio of the tracers in plasma is determined and used to calculate percent absorption. This design should be applicable to a variety of fatty acid absorption and metabolism problems.

Mass isotopomer distribution analysis (MIDA) is a stable-isotope approach for investigation of *de novo* lipogenesis and is a modification of traditional radioisotope methods used to measure fatty acid synthesis. A good review of the fundamental concepts, applications, and limitations has been published (12). The general experimental approach involves infusion of carbon-13-labeled acetate, extraction of plasma samples, and determination by mass spectrometry of the distribution pattern of newly synthesized fatty acid isotopomers.

## ANALYTICAL METHODS FOR STABLE ISOTOPES

The two most common analytical methods used in biological studies to measure stable isotope-labeled fatty acids are GC–MS and GC–C–IRMS). The GC–MS method uses a conventional mass spectrometer operated in a positive chemical-ionization (PCI) mode to analyze fatty acid methyl esters and in a negative chemical-ionization (NCI) mode to analyze fatty acid pentafluorobenzyl esters. Both chemical-ionization methods normally use conventional selected-ion monitoring (SIM) of masses corresponding to the appropriate fatty ester molecular ions. Natural carbon-13 background is about 2.5% for the $m + 2$ ion of an 18-carbon methyl ester. This background must be subtracted if the molecular ion for the labeled fatty acid is 2 atomic mass units higher than the tracee fatty acid. Errors inherent in this correction can substantially decrease the accuracy of results obtained with dideuterated fatty acids if enrichments are less than about 0.5%. Samples from both deuterium and carbon-13 studies can be analyzed by either NCI or PCI MS methods using SIM. The typical amount of tracer used in a pulse-oral-dose *in vivo* experiment is 30–50 mg/kg body weight of 95% enriched $^{2}$H-labeled fatty acid. Both methods provide molecular-ion data to confirm fatty acid identification based on GC retention times. NCI is about 500 times more sensitive than PCI and is the clear choice when the sample size is less than 0.2 mg of total fatty acid. When sample size is not a limiting factor, both methods have an accuracy of about $0.2 \pm 0.02$% enrichment.

GC–C–IRMS instrumentation has become the method of choice for analysis of carbon-13-labeled fatty esters. Isotope-ratio mass spectrometers have high sensitivity and precision. The accuracy of the $^{13}C/^{12}C$ ratio data is about $0.01 \pm 0.001$% enrichment. Differences between the accuracy of GC–MS and GC–C–IRMS methods may or may not be a major issue because the standard error for the MS data is much smaller than the standard error for the biological part of the study. However, detection limits for $^{13}$C-labeled fatty acids are lower than for deuterium-labeled fats and allow $^{13}$C-labeled fatty acids to be followed for many days. The typical amount of tracer used in a pulse-oral-dose *in vivo* experiment is 2–10 mg/kg body weight of a 99% enriched uniformly labeled $^{13}$C fatty acid. Proportionally more $^{13}$C-labeled fatty acid is fed when enrichments are lower or if only one or two carbons are labeled with $^{13}$C. A disadvantage of GC–C–IRMS is that molecular-ion data are not obtained for confirmation of fatty acid structure. Subtraction of baseline $^{13}$C enrichment is necessary because baseline $^{13}$C levels can vary significantly between subjects owing to the natural variation in the $^{13}$C content of foods. The data also need to be examined to ensure that a significant amount of [$^{13}$C]acetyl CoA is not recycled back into fatty acids by *de novo* synthesis.

Analysis of $^{2}$H- and $^{13}$C-labeled compounds by nuclear magnetic resonance (NMR) methods has been used for

decades to obtain exact structure information for fatty acids extracted from biological samples. However, the relatively low sensitivity of NMR methods compared to MS methods has limited their application for monitoring the metabolism of stable isotope-labeled fatty acids in biological studies. A $^{13}C$ NMR spectrometer equipped with a surface coil has been used in an *in vivo* method to measure incorporation of $^{13}C$-labeled fatty acid into tissues of live animals, but the method is much less sensitive than *in vitro* NMR methods.

## APPLICATIONS OF STABLE ISOTOPE METHODS

Stable isotope tracer studies provided a powerful approach to address questions relevant to PUFA studies in infants and mothers. For example, a variety of stable isotope experimental designs have been used to investigate the conversion of labeled n-6 and n-3 PUFA to their respective n-6 and n-3 long-chain polyunsaturated fatty acid (LC-PUFA) metabolites. These studies with adults, infants, and animal models have provided considerable information about the *in vivo* conversion and PUFA metabolism. The following examples illustrate the general utility of stable isotope tracer studies to investigate desaturation–elongation of linoleic and linolenic acids, TAG absorption, and kinetic parameters related to lipid synthesis, fatty acid oxidation, and *de novo* fatty acid synthesis.

### Synthesis of n-6 and n-3 LC-PUFA by Adults

*(i) Single-labeled pulse-dose design.* The origin of 18:2n-6 and n-6 LC-PUFA in milk lipids and the effect of lactation duration were determined by feeding [U-$^{13}C$]18:2n-6 (1 mg/kg body weight) to six mothers at 4, 6, and 12 wk after starting lactation (13). Milk and breath samples were collected for a 108-h period. The results showed that incorporation into milk lipids and oxidation of [$^{13}C$]18:2n-6 were not related to dietary 18:2n-6 intake. This finding suggests that these processes are separately regulated and noncompetitive. A multiple compartmental model was used to calculate cumulative recoveries of $^{13}CO_2$ (17.7–24%) in breath and [$^{13}C$]18:2n-6 (11.7–13.1%) in milk lipid samples. Accretion of 20:3n-6 was 3–25% of total 20:3n-6 in milk, suggesting a wide variation in 18:2n-6 conversion among lactating mothers. Oxidation, transfer, and conversion of [$^{13}C$]18:2n-6 were not influenced by lactation duration. Conversion of dietary 18:2n-6 to n-6 LC-PUFA was estimated to provide about 11% of the 20:3n-6 and 1.2% of the 20:4n-6 in milk lipids.

*(ii) Single-labeled pulse-dose design.* The effect of dietary n-3 fatty acids on the conversion and oxidation of 18:3n-3 was investigated in male and female adults (21–66 yr) (14). Subjects were fed diets containing different levels of oleic, linolenic, or eicosapentaenoic + docosahexaenoic acids. At the end of the 7-wk diet period, [U-$^{13}C$]18:3n-3 (45 mg) was fed as the methyl ester using an oral pulse dose of a labeled fatty acid. The maximal amounts of [$^{13}C$]20:5n-3 and [$^{13}C$]22:6n-3 incorporated into total plasma lipid were each about three times lower in the 18:3n-3 diet group than in the 18:1n-9 diet group. These results suggest that dietary 18:3n-3 decreased conversion of 18:3n-3 to 20:5n-3 and 22:6n-3 compared to the oleic acid diet. Conversion of [$^{13}C$]18:3n-3 to [$^{13}C$]22:6n-3 was not detected in plasma total lipid of subjects fed the 20:5n-3 + 22:6n-3 diet. Oxidation of [$^{13}C$]18:3n-3 was 24.8% for the 20:5n-3 + 22:6n-3 diet group, 20.4% for the 18:3n-3 diet group, and 15.7% for the 18:1n-9 diet group. The combined results suggest that conversion of 18:3n-3 to LC-PUFA is decreased and oxidation of 18:3n-3 is increased by dietary n-3 fatty acids.

*(iii) Single-labeled pulse-dose design.* The question of retroconversion of 22:6-n-3 to 20:5n-3 and 22:5n-3 was investigated in adult human subjects and rats by feeding a single oral dose of [U-$^{13}C$]22:6n-3 as the TAG (15). Time-course data were presented for plasma and high-density lipoprotein lipid classes, platelet phospholipids, and red cell phosphatidylcholine (PC). Overall, total retroconversion of [U-$^{13}C$]22:6n-3 was 1.4% in humans and 9% in rats. No increase in 20:4n-6 and 22:4n-6 carbon-13 enrichment was observed, which suggests that recycling of [$^{13}C$]acetyl CoA into elongated PUFA products was negligible. Interestingly, a precursor–product relationship for the expected sequence 22:6n-3 $\rightarrow$ 20:5n-3 $\rightarrow$ 22:5n-3 was supported by the rat data but not by the human data. The results provide several other interesting insights into the metabolic fate of 22:6n-3 and show that rats are not a good model for 22:6n-3 metabolism in humans.

*(iv) Multiple-labeled pulse-dose design.* The effect of dietary 22:6n-3 on distribution of 18:2n-6 and 18:3n-3 between lipid classes and on conversion to n-6 and n-3 LC-PUFA metabolites was investigated in male subjects (age 28–39 yr) (16). The subjects were fed a typical U.S. diet (Low22:6) or a typical U.S. diet supplemented with 6.5 g of 22:6n-3 (High22:6) for 90 d. At the end of the 90-d diet period, a mixture of deuterated 18:1n-9 (39–48 mg/kg), 18:2n-6 (29–35 mg/kg), and 18:3n-3 (27–36 mg/kg) TAG were fed. This approach allowed the metabolism of the three deuterated fats to be directly compared within each subject. The relative percentages of 18:1-$d_6$, 18:2-$d_2$, and 18:3-$d_4$ incorporated into plasma lipid classes were similar for all subjects, showing that the selectivity of the various acyltransferases was not influenced by dietary 22:6n-3. Plasma total lipid time-course data for [$^{2}H$]22:6n-3 accretion was 88% lower and [$^{2}H$]20:4n-6 accretion was 71% lower for the 22:6n-3–supplemented diet group compared to the Low22:6 diet group. Overall, dietary 22:6n-3 supplementation reduced the total amount of [$^{2}H$]LC-PUFA metabolites synthesized from 18:2-$d_2$ and 18:3-$d_4$ by about 70%. Based on total plasma lipid data from subjects fed the Low22:6 diet, *ca.* 4% of the 18:2-$d_2$ and *ca.* 5% of the 18:3-$d_4$ were converted to their respective LC-PUFA. The results suggest that inhibition of 18:2n-6 conversion to 20:4n-6 contributes to the physiological effects produced by dietary 22:6n-3 supplementation.

*(v) Multiple-pulse oral-dose design.* Enrichment of a fatty acid in plasma lipids can be increased by administering multiple doses of a labeled fatty acid at timed intervals. Higher enrichments enhance both detection and measurement of

labeled fatty acid metabolites because the increased isotope enrichment of a precursor fatty acid increases the enrichment of the fatty acid metabolites. This multiple-pulse dose approach was used to measure the effect of dietary arachidonic acid on conversion of 18:2n-6 to n-6 LC-PUFA metabolites in adult subjects (17). Male subjects (age 20–39 yr) were provided a typical U.S. diet (Low20:4) or a typical U.S. diet supplemented with 1.5 g of 20:4n-6 (High20:4) for 50 d. The stable isotope experiment consisted of feeding a 3.5 g dose of 18:2-$d_2$ at 8:00 A.M., 12:00, and 5:00 P.M. The deuterated fat was fed as a TAG and included as part of the subject's normal meal. Comparison of n-6 fatty acid metabolite data from the two diet groups showed that in plasma total lipid, 1.5 g/d of additional dietary 20:4n-6 reduced accumulation of both deuterated 20:3n-6 and 20:4n-6 by about 50%. For subjects fed the Low20:4 diet, about 3% or 325 mg of [$^2$H]20:4n-6 was synthesized from the 10.4 g of [$^2$H]18:2n-6 fed.

## Synthesis of n-6 and n-3 LC-PUFA by Infants

*(i) Single-labeled pulse-dose design.* Metabolism of an oral pulse dose of [U-$^{13}$C]18:2n-6 (1 mg/kg body weight) was studied in breast-fed full-term infants during the first week after birth (18). The results show that newborn infants have the capacity to desaturate and elongate 18:2n-6, but the amount of 18:2n-6 converted to 20:3n-6 and 20:4n-6 was low (1.5 and 1.2%, respectively). The authors suggest that conversion of 18:2n-6 may have been inhibited by dietary LC-PUFA since breast milk provides sufficient LC-PUFA to meet requirements. This suggestion is consistent with the observation that dietary 20:4n-6 and 22:6n-3 supplementation (16,17) reduced conversion of 18:2n-6 and 18:3n-3 in adults by 50–70%.

*(ii) Multiple-labeled pulse-dose design.* Premature infants were studied 30 d after birth by using an elegant combined dual-labeled and oral multiple-pulse experimental design (19). This study was the first to use this approach with $^{13}$C-labeled fatty acids. Formula containing about a 10:1 mixture of uniformly $^{13}$C-labeled 18:2n-6 and 18:3n-3 was continuously administered by intragastric infusion for 48 h. This dual-labeled approach with $^{13}$C-labeled fatty acids is possible because the plasma n-6 and n-3 fatty acids can be separated by GC before C–IRMS analysis. Time-course area data were used to calculate the amount of $^{13}$C-labeled 20:4n-6 and 22:6n-3 synthesized. The results provide clear evidence that low-birth-weight premature infants are able to synthesize 20:4n-6 and 22:6n-3. Estimates based on plasma phospholipid data indicate that about 6% of the total [$^{13}$C]18:2n-6 dose was converted to 20:4n-6, and about 14% of the [$^{13}$C]18:3n-3 dose was converted to 22:6n-3.

*(iii) Natural carbon-13-enriched diets.* The average natural abundance of $^{13}$C in foods is about 1.1%, but the percentage varies depending on the food source. Carbon-13 enrichment is about 1.081% for plants that use the $C_3$ photosynthesis pathway (e.g., soybean), and about 1.0975% for plants that use the $C_4$ photosynthesis pathway (e.g., corn). Diets containing mainly carbohydrate, protein, or oil from $C_4$ plants will produce a detectable increase in the $^{13}$C enrichments of breath and tissue samples. This "natural carbon-13 diet approach" has rarely been utilized for PUFA metabolism studies, but the feasibility has been demonstrated by feeding full-term infants a corn oil-based formula for 4 d in place of breast milk or commercial formula (20). By day 4, the $^{13}$C enrichments for 18:2n-6 and 20:4n-6 in plasma total lipid increased by comparison with baseline $^{13}$C enrichments. The increase in 20:4n-6 enrichment showed that these infants (mean age 18 d) were actively synthesizing 20:4n-6 from dietary 18:2n-6.

## Synthesis of n-6 and n-3 LC-PUFA in Animals

*(i) Single-labeled pulse-infusion design.* Accretion in fetal tissue lipids of synthesized 22:6n-3 was compared to that of preformed 22:6n-3 by infusion of [U-$^{13}$C]18:3n-3 (3.9–16 mg total) or [U-$^{13}$C]22:6n-3 (1.7–3.7 mg total) into pregnant baboons over a 1-h period (21). The baboons were in the third trimester of pregnancy. Incorporation of preformed [$^{13}$C]22:6n-3 in fetal brain lipid was about 20 times greater than incorporation of 18:3n-3–derived [$^{13}$C]22:6n-3. This result suggests that dietary 22:6n-3 has about 20 times the bio-equivalency of dietary 18:3n-3 and that an estimated dietary intake of 0.45% energy (%en) 18:3n-3 is needed to meet fetal requirements for 22:6n-3. This tracer study provided a wealth of other information about the incorporation, conversion, and transfer of n-3 and n-6 PUFA between maternal and fetal organs. The information is considered relevant to humans because the studies were conducted with primates.

*(ii) Single-labeled oral-pulse design.* Newborn baboons were fed human infant formula containing a 10:1 ratio of 18:2n-6 to 18:3n-3. At age 4 wk, they were dosed with [U-$^{13}$C]18:3n-3 (7.5 mg) or [U-$^{13}$C]22:6n-3 (4.4 mg) to determine the bioequivalency of dietary 18:3n-3 and 22:6n-3 (22). Two weeks after dosing (6 wk of age), brain, retina, retinal pigment epithelium, liver, plasma, and erythrocytes total lipids were analyzed by GC–C–IRMS. Accretion of preformed [$^{13}$C]22:6n-3 in brain lipids was 1.7% of the total dose, which suggests that 22:6n-3 was not highly conserved. The ratio of preformed [$^{13}$C]22:6n-3 to 18:3n-3-derived [$^{13}$C]22:6n-3 in these tissue lipids ranged from 7 to 51 and was lowest in brain lipid. The results indicate that dietary 22:6n-3 has about seven times the bioequivalency of dietary 18:3n-3.

*(iii) Single-labeled pulse-infusion design.* A single intravenous dose of [U-$^{13}$C]18:2n-6 (*ca.* 20 mg) was used to investigate the metabolism of 18:2n-6 and its LC-PUFA metabolites in maternal plasma and fetal organ lipids of baboons during the third trimester of pregnancy (23). Diets contained no LC-PUFA and an 18:2n-6 to 18:3n-3 ratio of 10:1. Maternal plasma samples were collected hourly for 8 h and daily for 20 d. Fetal samples were collected at eight time points between 1 and 29 d. The maximum amount of [$^{13}$C]20:4n-6 in fetal brain occurred at day 21 and was equal to 0.025% of the total [$^{13}$C]18:2n-6 dose. As a percentage of total label, accretion of [$^{13}$C]20:4n-6 in all

major fetal organs (liver, brain, heart, kidney, lung) continued to increase during most or all of the 29-d study period. Concentration of total n-6 [$^{13}$C]-labeled fatty acids and the percentage of 22-carbon [$^{13}$C]-labeled fatty acids were higher in fetal brain than in other organs. These tracer data suggest that about 50% of the fetal brain requirement for 20:4n-6 is provided by conversion of 18:2n-6 to 20:4n-6 and 50% is supplied by maternal stores.

*(iv) Dual-labeled pulse-dose design.* NMR was used to compare the accretion of 20:4n-6 synthesized from 18:3n-6 and preformed 20:4n-6 in suckling rats administered a mixture of [3-$^{13}$C]18:3n-6 (9.6 mg) and [1-$^{13}$C]20:4n-6 (10.4 mg) ethyl esters (24). Accretion of preformed 20:4n-6 was 7 times higher in liver total-lipid extracts than synthesized 20:4n-6 and 10 times higher in total brain lipid extracts. The results suggest that the source of most of the 20:4n-6 in brain lipids is from diet, tissue stores, and its synthesis by liver. A second part of this study involved injecting about 10 mg/d of [1-$^{13}$C]20:4n-6 for four consecutive days into the stomach of the rat pups. After 3 d, *in vivo* NMR spectra (obtained with a surface coil) of the intact brain and liver of the living rat pups detected [1-$^{13}$C]20:4n-6 in liver but not in brain. This *in vivo* NMR approach is attractive, but sensitivity is a limiting factor at this time.

## TAG Structure

A multiple-labeled oral-pulse-dose design was used to investigate the effect of TAG structure on fatty acid absorption and metabolism in middle-age male subjects fed native or randomized lard diets (25). A mixture of TAG (93–130 mg /kg) that contained both 1,3-dideuteriolinoleoyl-2-tetradeuteriopalmitoyl-*rac*-glycerol (L$d_2$P$d_4$L$d_2$) and 1,3-hexadeuteriopalmitoyl-2-tetradeuteriolinoleoyl-*rac*-glycerol (P$d_6$L$d_4$P$d_6$) was fed. The results shows that [$^2$H]16:0 and [$^2$H]18:2n-6 were equally well absorbed, and [$^2$H]-labeled fatty acids in the *sn*-2 TAG position were 85% retained in chylomicron TAG. After absorption, [$^2$H]16:0 in the chylomicron *sn*-2 TAG position migrated to the *sn*-1,3 positions, and [$^2$H]18:2n-6 in the *sn*-1,3 TAG positions migrated to the *sn*-2 acyl position. Migration was minimal when the labeled fatty acids were initially incorporated at the preferred or usual chylomicron TAG acyl position. The only effect of TAG acyl position on distribution in plasma lipid classes was less (30%) incorporation of [$^2$H]16:0 in the 1-acyl position of PC when [$^2$H]16:0 was fed as a *sn*-2 TAG. TAG structure did not influence conversion of [$^2$H]18:2n-6 to [$^2$H]20:3n-6 and [$^2$H]20:4n-6.

## Kinetic Studies

Radioisotope tracers have been used for more than 30 yr to obtain kinetic data for estimating turnover rates, half-lives, and fractional synthesis rates for TAG, very low density lipoprotein (VLDL), and fatty acids in human subjects. Kinetic information, obtained by continuous infusion and pulse injection of radioisotope-labeled fatty acids, has been reported for palmitic, stearic, oleic, linoleic, and arachidonic acids (26–28). Current stable isotope kinetic methods and theory are patterned after radioisotope methods (2,29,30). Many issues related to the experimental designs, assumptions, and various models used for interpretation of stable isotope kinetic tracer data have been critically reviewed (31). The following examples illustrate some approaches and results obtained using stable isotope tracers to study lipid synthesis, fatty acid synthesis, and fatty acid oxidation.

*(i) Lipid class synthesis.* Continuous infusion of [U-$^{13}$C]16:0 and [U-$^{13}$C]18:2n-6 over a 24-h period was used to determine the kinetics of surfactant PC synthesis in lungs of eight critically ill infants (age 11–149 d) with respiratory failure (32). The fractional synthesis rates for surfactant PC-16:0 and PC-18:2n-6 ranged from 0.4 to 3.4%/h, and from 0.5 to 3.8%/h, respectively. Mean times for maximal enrichments in surfactant PC were 49.2 h for 16:0 and 45.6 h for 18:2n-6. The half-lives of PC-16:0 and PC-18:2n-6 varied widely, and a shorter half-life correlated with the severity of respiratory failure. The results showed that synthesis of lung surfactant is a relatively slow process and demonstrated the application of $^{13}$C-labeled fatty acids for investigation of lipid class synthesis in lung tissue.

*(ii) Fatty acid oxidation in infants and adults.* A carbon-13 breath test was developed as an alternative to the [$^{14}$C]-triolein breath test for assessing fat malabsorption and gastric emptying. The carbon-13 breath test is used as a safe and noninvasive research tool for measuring fatty acid oxidation in children and healthy adults. IRMS methods are used to measure changes in the $^{13}CO_2/^{12}CO_2$ ratios of breath samples and can accurately detect small changes if appropriate corrections for variation in the natural $^{13}$C background are made (33). The "$^{13}CO_2$ Breath Test" has been used in many studies to measure factors that affect fatty acid oxidation. The following recent applications illustrate its general utility.

*(iii) Oxidation of 22:6n-3 and 18:2n-6.* The effect of dietary 22:6n-3 supplementation on oxidation of [$^{13}$C]22:6n-3 was determined in lactating women (34). An oral dose of $^{13}$C-enriched oil (2 mg/kg body weight) containing 50% 22:6n-3 was fed to the 22:6n-3-supplemented (0.3 g/d) and nonsupplement diet groups. Kinetic and cumulative oxidation rate data for the two diet groups were not different. Time-course data from this study were compared with similar data for lactating women fed a pulse dose of [U-$^{13}$C]18:2n-6 (1 mg/kg body weight) after 2, 6, and 12 wk of lactation (13). The 0–4 h time-course data showed that the [$^{13}$C]22:6n-3 oil was absorbed more quickly than [$^{13}$C]18:2n-6 (1 h vs. 3.5 h for maximum absorption) and oxidized much faster. After 4 h, the percent enrichment data for breath $CO_2$ were similar for both fatty acids. These results suggest that increased dietary 22:6n-3 intake did not result in higher oxidation of 22:6n-3.

*(iv) Oxidation of 16:0 and 18:1n-9.* A multiple-pulse-dose approach was used to estimate the relative oxidation for 18:1n-9 vs.16:0 in adult subjects (35). The tracer fatty acids were incorporated into a liquid formula diet containing 16%en each of 16:0 and 18:1n-9. A steady-state plateau in

$^{13}CO_2$ breath samples was achieved by feeding an oral dose (0.6 mg/kg) of [$^{13}$C]18:1n-9 or [$^{13}$C]16:0 at 20-min intervals for 7 h. The $^{13}$C enrichment in chylomicron samples was used to correct $^{13}CO_2$ data for differences in percent absorption of the labeled fatty acids. The $^{13}CO_2$ enrichment data for [$^{13}$C]18:1n-9 were 1.21 ± 0.02 (range 1.01–1.55) times higher than for [$^{13}$C]16:0. These results suggest that the fractional oxidation rate for 18:1n-9 was 21% higher than for 16:0 and may be related to health benefits reported for 18:1n-9.

*(v) Fat digestion.* The fat digestion capabilities of formula-fed term (age 1–234 d) and preterm (age 10–57 d) infants were investigated by measuring the oxidation of an oral pulse dose (10–20 mg/kg body weight) of 1,3-distearoyl-2-[$^{13}$C-carboxy]-octanoyl-glycerol (36). Carbon-13 enrichment did not increase in breath $CO_2$ from three of five preterm infants younger than 20 d. This result suggests impaired fat digestion capability in the preterm infants. For infants 30 d or older, $^{13}CO_2$ enrichment data were within normal ranges for adults and showed that the capacity of infants to digest fat is developed to normal levels by about 1 mon of life.

*(vi) Fatty acid synthesis.* The MIDA approach involves measurement of the stable isotope isomer distribution pattern in the fatty acids synthesized following continuous infusion of [$^{13}$C]acetate. This approach provides a general method for determining the effect of diet and physiological changes on *de novo* lipogenesis. For example, the approach was used in humans to show that the percentage of VLDL-16:0 and VLDL-18:0 produced by *de novo* hepatic lipogenesis is small, 0.9 and 0.37% respectively (37). Carbohydrate and glucose refeeding increased the percentage of newly synthesized VLDL-16:0 and VLDL-18:0 to 1.64 and 0.64%, respectively. This small increase was considered physiologically insignificant. The MIDA approach was used in rats to measure the effect of insulin and supplementation with glucose and fructose on the fractional synthetic rate of VLDL-TAG. Both insulin administration and sugar supplementation were shown to increase greatly the fractional synthetic rate of VLDL-TAG (38).

Injection of $^{3}$H-labeled glycerol is a traditional approach used to measure synthesis of triacylglycerols. As an alternative, use of deuterated glycerol was validated recently as a stable-isotope method for estimating VLDL-TAG fractional catabolic rates (39). The method used a single-pulse infusion of a bolus dose of [$^{2}H_5$]-glycerol. VLDL-TAG was isolated from plasma samples collected over a 12-h period and percent enrichment was measured by mass spectrometry. A fractional catabolic rate (pools/h) of 0.301 to 0.559 (mean 0.378 ± 0.092) was calculated from the monoexponential decrease in VLDL-[$^{2}H_5$]-glycerol for six normolipidemic men.

*(vii) Fractional conversion rates.* Term infants were fed formula containing 0.4, 1.0, and 3.2% of fat as 18:3n-3 from birth until 3 wk old (40). Each infant was then fed 25–40 mg/kg body weight of [U-$^{13}$C]18:2n-6 and 20–25 mg/kg body weight of [U-$^{13}$C]18:3n-3. The tracer/tracee ratios in plasma total phospholipid were determined, and a precursor–product compartmental model was used to calculate fractional conversion rates for synthesis of [$^{13}$C]20:4n-6 and [$^{13}$C]22:6n-3 and fractional incorporation rates. The fractional conversion rate for 18:3n-3 → 22:6n-3 increased 39% and that for 18:2n-6 → 20:4n-6 decreased 64% when the 18:3n-3 content of formula was increased from 0.4 to 3.2% of total fat. Fractional incorporation rates increased 2.4-fold for 22:6n-3 and decreased 55% for 20:4n-6. The fractional conversion rate decrease for 18:3n-3 was not statistically significant because of large variability within and between diet groups. However, the combined results showed that dietary 18:3n-3 influenced both n-6 and n-3 LC-PUFA metabolism. The relevance of these plasma phospholipid results to organ tissue lipids remains to be determined.

## ISSUES

A major issue concerning stable isotope studies is whether plasma lipid data provide a valid quantitative estimate for accretion and synthesis of PUFA. Concerns about the validity of qualitative results are usually not an issue. Examples of some typical questions are:

(i) Is it valid to use human plasma data as a reflection of PUFA incorporation and conversion in organ lipids?

(ii) Do the different methods for expression of isotope data [e.g., percent enrichment, tracer to tracee ratio, weight, percent of total tracer, percent delta over baseline (% DOB)], result in equally valid quantitative conclusions?

(iii) How large is normal subject variability and does it invalidate results from studies with a small number of subjects?

(iv) Is it valid to extrapolate results from animal studies to humans?

(v) Is it possible to obtain valid *in vivo* kinetic data?

These are controversial and complicated questions that have not been satisfactorily resolved. Whether isotope data provide valid quantitative results depends on many factors. Conclusions based on isotope tracer results can lead to misconceptions, and the results from other types of studies should always be considered. The following comments summarize some nuances related to the above questions, but an in-depth discussion of these questions is beyond the scope of this review.

*Validity of stable-isotope results.* Does accretion of n-6 and n-3 LC-PUFA in human plasma lipids provide a valid quantitative measure of LC-PUFA synthesis from 18:2n-6 and 18:3n-3 precursors? The answer is yes, no, and maybe. The presence of labeled n-6 and n-3 LC-PUFA metabolites in human plasma lipids is qualitative evidence that desaturation and elongation of 18:2n-6 and 18:3n-3 have occurred. All recent studies have shown that *in vivo* conversion of 18:2n-6 and 18:3n-3 to LC-PUFA is not trivial. It is clear also from many lines of evidence that increased synthesis of LC-PUFA can increase accumulation in plasma lipids but not necessarily in proportion to the amount synthesized. The reason is that accretion of n-6 and n-3 LC-PUFA metabolites in lipid classes is controlled or regulated by several metabolic pathways. The main pathways are desaturation–elongation, acylation–deacylation–reacylation, and fatty acid oxidation. The amounts and ratios of n-6 and n-3 fatty acids in diet can alter accretion of synthesized LC-PUFA by competing for

acylation of the 2-acyl position of phospholipids and increasing turnover rates. Mechanisms responsible for the transport or transfer of lipids from plasma to other organ lipids are also important. An additional major complication is that most of the enzymes involved in these pathways have a different affinity or selectivity for different fatty acid structures. The result is that the relationship between synthesis and accretion is complex and probably not linear for LC-PUFA.

Stable isotope studies provide quantitative conversion data that are sufficiently accurate for some purposes and not for others. For example, plasma total lipid data are a valid indicator of the minimum amount of 22:6n-3 synthesized from 18:3n-3, but these data are probably not an accurate measure of the total amount synthesized. Comparison of the relative amounts of n-6 and n-3 LC-PUFA in plasma total lipids from subjects fed a mixture of labeled 18:2n-6 and 18:3n-3 provides an accurate reflection of relative synthesis rates for LC-PUFA. However, the results are valid only for the actual set of experimental conditions. The bottom line is that tracer data for human plasma LC-PUFA can provide a valid reflection of LC-PUFA accretion and a useful estimate of total synthesis.

*Expression of isotope data.* The answer to the question of "what is the best or most valid way of expressing stable isotope data?" depends on the experimental design and objective of the study. Stable isotope tracer data can be presented in a variety of ways. Examples are percentage enrichment, tracer/tracee ratio, weight, percentage of total tracer, and change in DOB. Each approach is valid and has advantages, but percentage data are the most likely to be misleading because the numbers are unitless or relative. Exceptions are the percentage total accumulation used to quantify the total amount of fatty acid oxidation and percentage data calculated from concentration data. Percentage of total tracer is useful because the numbers are relative to the total amount of tracer and are not influenced by the amount of tracee present. The standard used in radioisotope studies is specific activity [decompositions per minute (dpm)/unit weight]. If an internal standard is added to the lipid extract, stable isotope data can be expressed in an analogous way. Examples are microgram/mole and microgram/milligram of lipid.

*Subject variability.* Variation between results from different subjects raises questions about the validity of the stable isotope results from studies with a small number of subjects. For most stable-isotope studies in humans, the time and effort involved preclude the use of a large number of subjects. The use of a small number of subjects is not necessarily a major problem if there is reasonable assurance that the subjects reflect the normal distribution within a specific population. Variation between results from different subjects that appear to represent the same population is disconcerting. For control vs. experimental treatment studies, a large variability between data for subjects within the same group prevents conclusions from being drawn unless there are large differences in the stable isotope data for the two groups. When the reason for variation between data for different subjects cannot be explained, it is commonly referred to as "subject variability." However, subject variability is the sum of many experimental variables that either were not controlled or cannot be controlled. When an internal control fatty acid is simultaneously fed along with one or more experimental fatty acids, the variability of the relative data is less because uncontrolled experimental variables influence each fed fatty acid equally. This multiple-labeled isotope approach also facilitates comparisons of metabolic data for different fatty acids because each subject serves as his or her own control and experimental variables affect each fatty acid equally.

Our experience has been that stable isotope tracer data from subjects confined to a metabolic ward are less variable than data from free-living subjects. This observation suggests that experimental variables other than inherent metabolic differences contribute substantially to variation between data from different subjects. Differences in plasma lipid concentration, body weight, and the amount of tracer fed are responsible for much of the variability between data for individual subjects. Adjusting tracer data for these differences usually reduces variability greatly.

*Use and relevance of animal studies.* Can animal metabolism studies be used as valid indicators of human metabolism? Animal studies usually provide valid qualitative information, but extrapolation of quantitative results from animal models to humans without validating the model should be avoided. Small rodents are an example of a poor human model, and quantitative results from PUFA studies with rodents should not be extrapolated to humans. Extrapolation of results from some species of primates may be valid, but additional research is needed in this area. However, PUFA metabolism studies in animals can be very useful because experimental conditions can be rigorously controlled and organs can be sampled. Well-designed animal studies have provided valuable information that could not be obtained with human studies.

*Kinetics.* An elusive goal has been finding an answer to the question of how to use fatty acid isotope tracer data to obtain valid *in vivo* kinetic data in humans. Various approaches that range from classical enzyme kinetic models to multiple compartmental modeling have been used. The real question is whether the kinetic model and assumptions used to simplify the calculations are valid. The problem is that fatty acid metabolism *in vivo* involves interactions between several lipid pools or compartments, and a number of simplifying assumptions must be made that may or may not be totally correct. However, even simple kinetic treatments appear to provide useful information on the relative differences in the metabolism of different fatty acids. Kinetic data for PUFA metabolism in animal models are likely to be valid because the various organs and fatty acid pools can be sampled.

## RESEARCH OPPORTUNITIES

Stable isotope studies can be used to address many questions related to PUFA metabolism in infants and mothers. Each concern or question discussed in the previous section would be a challenging research opportunity. Other obvious examples of questions that provide opportunities for using stable isotope tracers are:

(i) Does percent conversion of 18:2n-6 and 18:3n-3 to LC-PUFA change during pregnancy?

(ii) What factors are responsible for the apparent differences in capacity of individuals to synthesize and accumulate LC-PUFA?

(iii) Does the n-6/n-3 ratio influence PUFA metabolism more than the actual amount of dietary 18:2n-6 and 18:3n-3?

(iv) Are estimates for the bioequivalency of 18:3n-3 and 22:6n-3 in primates valid predictors for humans?

(v) Can blood lipid markers be used to estimate accretion of PUFA metabolites in organs that cannot be sampled?

(vi) How important is dietary TAG structure?

(vii) What is the relative importance of desaturation–elongation and acyltransferase reactions with respect to synthesized LC-PUFA accretion in tissue lipids?

Development of new analytical methodology is another general area of opportunity. Recently developed atmospheric-pressure chemical-ionization MS methods provide molecular-ion information for intact TAG and phospholipids (41). Combining this analytical method with stable isotope tracer studies provides a new approach to investigate the affect of dietary TAG structure and fatty acid acyl position on absorption and metabolism. This approach also could be used to study lipid class synthesis and selective pairing of LC-PUFA with other fatty acids in phospholipids. Quadrupole ion-trap MS systems have potential for use in fatty acid tracer studies because of their inherent high sensitivity, but they have not been used to analyze samples from stable isotope-labeled fatty acid studies. Affordable internal and external ion-trap MS systems are available that should provide sensitivity and accuracy equal to those of NCI methods.

A noninvasive and sensitive analytical method that would provide a real-time quantitative measure of isotope-labeled PUFA metabolism in human subjects is an exciting and fascinating concept. Developments in NMR instrumentation and technology provide hope that this concept can be realized. Early studies showed that $^{13}C$ NMR could detect natural $^{13}C$-labeled fatty acids in liver and adipose tissue of living rats by using a surface coil. These studies showed that the influence of dietary PUFA could be detected in organs of live rats (42). Recent studies have shown that incorporation of $^{13}C$-labeled PUFA in the liver of live rats could be measured by $^{13}C$ NMR with the use of a surface coil (23,43). Continued refinement of this approach provides an intriguing research opportunity.

## SUMMARY

Stable isotope studies are clearly a powerful tool for investigating a variety of questions related to PUFA metabolism in mothers and infants. Several stable isotope experimental study designs are available, and they have been used to provide considerable information related to *in vivo* conversion of n-6 and n-3 PUFA, fatty acid oxidation, TAG absorption, and lipid synthesis. Many other questions could be addressed, but some issues about the validity of stable isotope results need to be resolved. New approaches will be developed as new and improved analytical instrumentation becomes available.

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# Application of New Methods and Analytical Approaches to Research on Polyunsaturated Fatty Acid Homeostasis

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**ABSTRACT:** New methods and analytical approaches are important to challenge and/or validate established beliefs in any field including the metabolism of polyunsaturated fatty acids (PUFA; polyunsaturates). Four methods that have recently been applied toward obtaining a better understanding of the homeostasis of PUFA include the following: whole-body fatty acid balance analysis, magnetic resonance imaging (MRI), $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy, and gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS). Whole-body balance studies permit the measurement of both the percentage of oxidation of linoleate and α-linolenate and their conversion to long-chain PUFA. This method has shown that β-oxidation to $CO_2$ is normally the predominant metabolic fate of linoleate and α-linolenate. Furthermore, models of experimental undernutrition in both humans and animals show that β-oxidation of linoleate and α-linolenate markedly exceeds their intake, despite theoretically sufficient intake of linoleate or α-linolenate. Preliminary results suggest that by using MRI to measure body fat content, indirect whole-body linoleate balance can be done in living humans. $^{13}C$ NMR spectroscopy provided unexpected evidence that linoleate and α-linolenate were metabolized into lipids synthesized *de novo*, an observation later quantified by tracer mass balance done using GC–C–IRMS. This latter method showed that within 48 h of dosing with $^{13}C$-α-linolenate, >80% underwent β-oxidation to $CO_2$ by suckling rats, whereas 8–9% was converted to newly synthesized lipids and <1% to docosahexaenoate. Further application of these recently developed methods in different models should clarify the emerging importance of β-oxidation and carbon recycling in PUFA homeostasis in mammals including humans.

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Progress in scientific research depends on a mixture of new ideas and new methods. Sometimes new ideas can be tested using existing, and even old, methods. Often, however, a new method may be required to undertake the appropriate experiment. Applying new methods to an old problem can sometimes reveal the information that was hoped for. Unanticipated results that perhaps contradict prevailing concepts may also occur in the process. It takes originality, patience, and luck to optimize experimental and analytical methods to research objectives.

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Abbreviations: GC, gas chromatography; GC–C–IRMS, GC–combustion–IRMS; IRMS, isotope ratio mass spectrometry; LC, long chain; MRI, magnetic resonance imaging; NMR, nuclear magnetic resonance spectroscopy; PUFA, polyunsaturated fatty acids.

Research on the metabolism of polyunsaturated fatty acids (PUFA; polyunsaturates) has benefited from new methods almost since the inception of this field in the original studies of Burr and Burr in 1929–1930. Of any single analytical method, gas chromatography (GC) has arguably made the greatest contribution to the fields of fatty acid and sterol analysis. The application of deuterium isotope ratio mass spectrometry (IRMS) to problems in lipid metabolism is older than GC, but refinements in the 1990s have pushed this method into the forefront of PUFA research. Nuclear magnetic resonance (NMR) spectrometry was considered to be a tool of chemists until people began to use it to measure tracer metabolism in isolated tissues and live animals in the 1980s. These methods are by no means the only analytical developments that have contributed to an improved understanding of PUFA homeostasis.

## WHOLE-BODY LINOLEATE AND α-LINOLENATE BALANCE

In a nutritional context, balance methodology refers to comparing "what goes in to what comes out." It has been applied extensively to the metabolism of nutrients that cannot be synthesized, i.e., minerals and indispensable amino acids. Because there is no endogenous synthesis of "essential" nutrients in mammals, the difference between what goes in (dietary intake) and what comes out (excretion and β-oxidation) represents the amount accumulating in some form or other in the organism. If animals are used, what accumulates can be measured directly and compared with intake or excretion under the experimental conditions in question.

This method can be applied to two important questions in PUFA metabolism, i.e., (i) How much dietary linoleate (18:2n-6) or α-linolenate (18:3n-3) is converted to the respective long-chain (LC) PUFA? (ii) How much linoleate or α-linolenate undergoes β-oxidation, i.e., what is their true bioavailability under normal conditions? The only apparent condition on such fatty acid balance studies is that, because linoleate and α-linolenate can both be chain-elongated from their respective 16-carbon precursors, which are present in edible green vegetables, and because each of these precursors can be converted to several LC-PUFA, the dietary PUFA in a whole-body fatty acid balance study should be only linoleate and α-linolenate. This is not usually a difficult condition to meet as long as semipurified diet ingredients are used. PUFA accumulation would then have two components, (i) accumulation of linoleate or α-linolenate and (ii) accumulation of all

n-6 or n-3 LC-PUFA, namely, the percentage of conversion from linoleate or α-linolenate.

If the analysis is done correctly, the difference between intake minus the sum of both excretion and the combined accumulation of linoleate or α-linolenate plus their respective LC-PUFA represents the disappearance of linoleate or α-linolenate from the whole-body pool of n-6 or n-3 PUFA, i.e., their β-oxidation. In growing animals, a balance period of only a few days is sufficient, but it can be several weeks if it is desirable. The advantages of this analytical approach to studying both net desaturation-chain elongation and β-oxidation of PUFA are as follows: (i) No tracers are involved; thus the data are not dependent on isotope equilibration with various pools. (ii) β-Oxidation exceeding intake can be measured, which is a surprisingly common phenomenon (see below). (iii) Whole-body rather than single compartment synthesis of LC-PUFA can be measured. (iv) The analytical method (quantitative fatty acid extraction and analysis by capillary GC) is relatively simple and well established. The single main disadvantage is that LC-PUFA also undergo β-oxidation, although to a lesser extent than linoleate or α-linolenate. This component of the equation can be estimated from published values (1) but cannot be measured directly; thus, there is an inherent although modest error in its estimation.

The whole-body fatty acid balance method provided the first clear indication of the overall capacity to synthesize LC-PUFA in rats. Subcellular preparations, tracer conversion, or changes in precursor to LC-PUFA ratios simply cannot provide this information because they are isolated from the integrated influences on PUFA homeostasis in the whole body. Predictable changes such as increased LC-PUFA synthesis during pregnancy or impaired LC-PUFA synthesis in nutritional deprivation such as moderate zinc deficiency (Table 1) confirm the plausibility of the results obtained using whole-body fatty acid balance methodology. Perhaps most surprising are the relatively low levels of conversion [3–4% to arachidonate (20:4n-6) and 1–2% to docosahexaenoate (22:6n-3)], especially relative to β-oxidation of the parent PUFA.

**TABLE 1**
**Various Nutritional Manipulations Probing Key Aspects of Whole-Body Homeostasis of Linoleate and α-Linolenate, i.e., Conversion to Long-Chain Polyunsaturated Fatty Acids (LC-PUFA) or β-Oxidation**

| | Linoleate | α-Linolenate | Reference |
|---|---|---|---|
| | (% of intake) | | |
| Conversion to LC-PUFA[a] | | | |
| Normal, young rats | 3–4 | 1–2 | 2 |
| Pregnant rats | 8 | 45 | 3 |
| Pregnant + mild zinc deficiency | 4 | 24 | 3 |
| β-Oxidation | | | |
| Normal, young rats | 70 | 80 | 2 |
| Pregnant rats | 62 | 35 | 3 |
| Fasted, refed pregnant rats | 106 | 142 | 4 |
| Weight-cycled male rats | 81 | 89 | 5 |
| Linoleate-deficient male rats | 485 | ND[b] | 6 |

[a]18- to 22-carbon LC-PUFA in each family.
[b]ND, not determined.

This method also shows that whole-body β-oxidation of linoleate and α-linolenate is generally in the range of 70–80% (Table 1), values that are in broad agreement with whole-body tracer data (1). Predictably, β-oxidation of PUFA is reduced by pregnancy and increased by a variety of conditions involving nutritional deprivation (Table 1). One important advantage of this method is that because whole-body analysis is done at two time points to determine accumulation, a net loss over the balance period (negative accumulation) can be measured. Hence, unlike with tracer methods, depletion of PUFA from body stores is easily quantified. A significant degree of whole-body PUFA depletion occurs with weight loss, fasting-refeeding, and dietary linoleate deficiency (Table 1).

Indirect approaches using whole-body fatty acid balance methodology can also be used to estimate requirements for certain PUFA such as docosahexaenoate (7). Docosahexaenoate accumulation at 10 mg/d in the human neonate was estimated from a collation of tissue data. Taking into consideration an estimate that 50% of dietary docosahexaenoate undergoes β-oxidation in neonates (8) left an apparent requirement for docosahexaenoate intake and/or synthesis of 20 mg/d. Although breast milk almost invariably provides at least 60 mg/d docosahexaenoate, no data obtained by balance or tracer methods in an animal or human model suggest that α-linolenate can be converted to docosahexaenoate at the necessary rate of 20 mg/d, i.e., a conversion rate of 5% (7). These results, although preliminary and potentially in need of revision, demonstrate the applicability of whole-body fatty acid balance to important questions in PUFA research in both infants and animal models.

## MAGNETIC RESONANCE IMAGING (MRI) AND *IN VIVO* WHOLE-BODY FATTY ACID BALANCE

One significant limitation to applying whole-body fatty acid balance to understanding PUFA metabolism in humans is the need for an accurate measure of the whole-body content of the fatty acids in question, i.e., the need for tissue analysis. Under some circumstances, MRI may be able to provide the necessary information to estimate the whole-body pool of PUFA such as linoleate in living humans (9). The key requirements of this method can be met without direct lean tissue PUFA analysis, at least under the following experimental conditions: (i) Whole body MRI scans provide accurate lean and fat tissue volumes for the whole body. (ii) Lean and fat tissue densities and water content are known; thus, the actual mass of individual fatty acids in these compartments can be determined. (iii) Linoleate is predominantly in body fat and because fat is the only tissue (besides plasma) that can be routinely biopsied; fatter individuals reduce the error of having only a sample of subcutaneous fat with which to analyze linoleate levels. (iv) Linoleate levels seem to be the same at all fat locations (10), allowing one to generalize that the data obtained from one biopsy site is valid across all fat depots. (v) Studying linoleate β-oxidation during energy deficit and moderate weight loss essentially eliminates the need to

measure linoleate conversion to n-6 LC-PUFA because energy deficit impairs desaturation (11). (vi) Moderate energy deficit leading to <15% weight loss does not change lean tissue linoleate levels in animals (12); thus, only the size of the lean tissue compartment but not a change in its PUFA content must be known.

In our initial experiments with this *in vivo* version of the whole-body fatty acid balance method, it became clear that β-oxidation of linoleate during weight loss of 13 kg in obese men can markedly exceed its intake, despite a nominally adequate linoleate intake (9). This was not evident in the PUFA content of plasma fatty acid profiles. Because of their already large linoleate stores and ongoing "normal" linoleate intake, this whole-body loss exceeding intake by two- to threefold was unlikely to be a significant risk, at least immediately. However, the point is that indirect whole-body linoleate balance can be done in living humans under minimally invasive and ethically acceptable circumstances. This method appears to provide plausible estimates of linoleate β-oxidation and indicates for the first time that under conditions of apparent linoleate adequacy, linoleate β-oxidation can considerably exceed its intake. This may lead to assessment of conditions in which linoleate sufficiency is less certain, i.e., during pregnancy, lactation, early infancy, or chronic risk of undernutrition.

In one such future application, we are beginning an evaluation of fetal MRI to assess the feasibility of determining fat accumulation during the third trimester *in utero*. Fetal adipose tissue accumulates uniquely during the third trimester. Growth retardation *in utero* or premature birth are both associated with a higher risk of compromised neurological development postnatally and with lower fetal fat accumulation. At birth, adipose tissue contains more docosahexaenoate than α-linolenate (7); thus, this depot is potentially important in both normal postnatal development and early postnatal PUFA homeostasis. The PUFA balance methodology is analogous to that used in living adults (9) in which the changes in total fetal fat volumes will be combined with PUFA profiles obtained from separate autopsy samples. In principle, this should allow the estimation of PUFA accumulation in fetal adipose tissue.

## $^{13}$C NMR SPECTROSCOPY: APPLICATION TO A NEW PATHWAY IN PUFA METABOLISM

NMR spectroscopy has several applications in PUFA metabolism, particularly in determining the physiological properties of fatty acids such as docosahexaenoate (13). Our particular interest in this method was in the potential application of $^{13}$C NMR to the noninvasive measurement of LC-PUFA synthesis in organs such as brains of living animals and humans. As detailed elsewhere (14), technical limitations still prevent useful information on this potentially valuable application of NMR to studying PUFA metabolism. However, in the process of attempting to measure LC-PUFA synthesis noninvasively from uniformly $^{13}$C-labeled linoleate and α-linolenate in live neonatal rat pups, we made the serendipitous observation that the tracer was appearing in substantial amounts in brain lipids containing saturated carbons, i.e., cholesterol, saturated, and monounsaturated fatty acids. This was clearly evident in the *in vivo* $^{13}$C NMR spectra because peaks for saturated carbons of all lipids cluster in one region of the spectrum, whereas the peaks containing unsaturated carbons cluster in a separate region distinct from the saturated carbon peaks.

At the time, we were unaware of Andrew Sinclair's seminal work with $^{14}$C-α-linolenate showing essentially the same thing in suckling rats (15); skeptical then of our *in vivo* NMR data, we proceeded with $^{13}$C NMR analysis of brain total lipid extracts (16). Spectra of these extracts unequivocally confirmed the $^{13}$C enrichment in saturated fatty acids and cholesterol that appeared in the poorly resolved *in vivo* $^{13}$C NMR spectra (16). More sophisticated NMR methods, including generating double quantum, two-dimensional spectra, demonstrated the carbon pairing of telltale satellite peaks that indicated exactly which cholesterol carbons came into the molecule as intact $^{13}$C-enriched carbon pairs directly from the dosed $^{13}$C-PUFA (17). Further analysis by continuous flow IRMS confirmed the occurrence of substantial "carbon recycling" from 18-carbon PUFA, probably through ketones, into brain lipids synthesized *de novo* (16).

The point of this example is that our goal, i.e., to demonstrate synthesis of LC-PUFA in an *in vivo* model, had been quite different from the main results it produced. Had we used routine methods to examine LC-PUFA synthesis, the cholesterol fraction would not have been retained and we would not have learned about "carbon recycling" from PUFA into *de novo* lipid synthesis. This is also one of the drawbacks to GC/MS with selected ion monitoring, i.e., the investigator selects the ions, and therefore the molecules of interest, and discards or ignores the others regardless of their information content. In light of the data to be presented in the following section, unfortunately, this is a lost opportunity to evaluate a pathway of PUFA metabolism that appears to be, quantitatively, more important than LC-PUFA synthesis.

Carbon recycling from PUFA into *de novo* lipid synthesis appears to occur *via* β-oxidation of the PUFA, incorporation of the PUFA carbons into ketones, and use of the labeled ketones for *de novo* lipid synthesis in the brain (18). This pathway has not been investigated but would be an ideal subject for $^{13}$C NMR analysis because this method is well suited to identifying the water-soluble products (ketones, but probably other metabolites as well) through which the $^{13}$C enrichment is transferred.

## GAS CHROMATOGRAPHY–COMBUSTION–ISOTOPE RATIO MASS SPECTROMETRY (GC–C–IRMS): APPLICATION TO CARBON RECYCLING OF PUFA

IRMS is the most sensitive and precise way to measure the increase in $^{13}$C (or other tracer) above background during a tracer experiment. Because this method requires that the isotope in question be gaseous, it is limited to low-molecular-weight isotopes, mostly of hydrogen, oxygen, nitrogen, and

carbon. Gaseous samples such as breath require a purification step but can otherwise be sent straight to the mass spectrometer for analysis. Nongaseous samples must be combusted, a process that used to be done manually, with the gaseous sample then being purified and fed through a vacuum system to the mass spectrometer. In the past two decades, "continuous flow" systems have combined automated combustion and purification of the samples with the subsequent MS, thereby greatly reducing the need for manual input. In the past decade, the further refinement of adding a gas chromatograph ahead of the combustion system has been commercialized.

In principle, any organic compounds that can be separated by GC can have isotopic enrichments analyzed by GC–C–IRMS. For instance, it is now reasonably common to measure, in both animal models and humans, synthesis of $^{13}$C-labeled n-3 LC-PUFA after dosing with $^{13}$C-α-linolenate (8,19–22). Following on from our preliminary data showing that carbon recycling into *de novo* lipid synthesis appeared to capture a significant amount of the carbon skeleton of a mixture of linoleate and α-linolenate (16), we applied GC–C–IRMS to quantifying the fate of a physiologic oral dose of $^{13}$C-α-linolenate in suckling rat pups. Total lipids of brain, liver, gut, lung, and remaining carcass were extracted; the $^{13}$C enrichment in fatty acids and sterols was analyzed separately; tissue fatty acid and sterol levels were quantified; and the data were combined so as to determine the distribution of $^{13}$C from α-linolenate in body lipids. In addition, the total recovery of $^{13}$C in body lipids would indicate by mass balance how much $^{13}$C was missing and therefore lost through β-oxidation.

Results for the brain, liver, and gut have already been reported (23), from which it was clear that recycling of α-linolenate carbon into *de novo* lipid synthesis exceeded that going into docosahexaenoate by 5- to 40-fold, depending on the tissue and time of sampling after dosing with the tracer. More recently, we confirmed these data in the lung and are compiling the carcass and whole-body data. A nearly complete whole-body estimate showed that 48 h after dosing, <10% of $^{13}$C-α-linolenate remained in total body lipids in any form, i.e., that ~90% underwent complete β-oxidation. Of the remaining 10%, <5% stayed as $^{13}$C-α-linolenate, and <5% was converted to n-3 LC-PUFA. Less than 1% of the dosed $^{13}$C-α-linolenate appeared to be going into docosahexaenoate in the whole body of the suckling rat. If our remaining analyses and those of others working in this field confirm these preliminary conclusions (Fig. 1), these data will corroborate our whole-body balance data showing that 1–2% of dietary α-linolenate is normally used by the growing rat to make n-3 LC-PUFA (2).

The influence on n-3 LC-PUFA synthesis of preformed docosahexaenoate in the milk consumed by the suckling rats will still have to be assessed; the point remains, however, that IRMS is a reliable and sensitive analytical method for quantitatively assessing the bioavailability and whole-body homeostasis of PUFA.

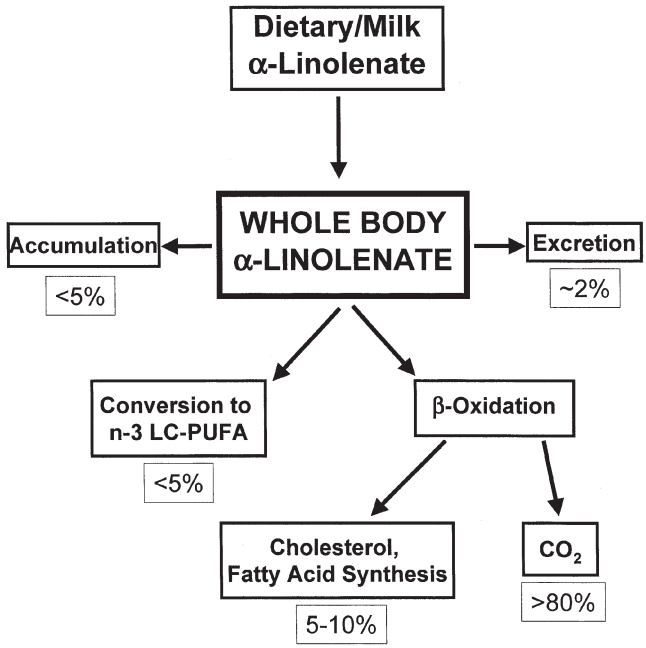


**FIG. 1.** Scheme outlining α-linolenate homeostasis in suckling rats based on metabolism of an oral dose of $^{13}$C-α-linolenate 48 h after dosing in 6-d-old rat pups. The values are derived from published (23) and unpublished data. LC-PUFA, long-chain polyunsaturated fatty acids.

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# A New Concept of Cellular Uptake and Intracellular Trafficking of Long-Chain Fatty Acids

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**ABSTRACT:** Fatty acids are the main structural and energy sources of the human body. Within the organism, they are presented to cells as fatty acid:albumin complexes. Dissociation from albumin represents the first step of the cellular uptake process, involving membrane proteins with high affinity for fatty acids, e.g., fatty acid translocase (FAT/CD 36) or the membrane fatty acid–binding protein ($FABP_{pm}$). According to the thus created transmembrane concentration gradient, uncharged fatty acids can flip-flop from the outer leaflet across the phospholipid bilayer. At the cytosolic surface of the plasma membrane, fatty acids can associate with the cytosolic FABP ($FABP_c$) or with caveolin-1. Caveolins are constituents of caveolae, which are proposed to serve as lipid delivery vehicles for subcellular organelles. It is not known whether protein ($FABP_c$)- and lipid (caveolae)-mediated intracellular trafficking of fatty acids operates in conjunction or in parallel. Channeling fatty acids to the different metabolic pathways requires activation to acyl-CoA. For this process, the family of fatty acid transport proteins (FATP 1-5/6) might be relevant because they have been shown to possess acyl-CoA synthetase activity. Their variable N-terminal signaling sequences suggest that they might be targeted to specific organelles by anchoring in the phospholipid bilayer of the different subcellular membranes. At the highly conserved cytosolic AMP-binding site of FATP, fatty acids are activated to acyl-CoA for subsequent metabolic disposition by specific organelles. Overall, fatty acid uptake represents a continuous flow involving the following: dissociation from albumin by membrane proteins with high affinity for fatty acids; passive flip-flop across the phospholipid bilayer; binding to $FABP_c$ and caveolin-1 at the cytosolic plasma membrane; and intracellular trafficking *via* $FABP_c$ and/or caveolae to sites of metabolic disposition. The uptake process is terminated after activation to acyl-CoA by the members of the FATP family targeted intracellularly to different organelles.

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## THE OVERALL VIEW

Long-chain fatty acids are the major energy source in the mammalian organism, supplying ATP *via* mitochondrial β-oxidation. As an example, cardiomyocytes represent a cell type that is essentially dependent on efficient and constant energy flow provided by fatty acids. Any extra fatty acid not used for ATP production is stored as triglyceride, preferentially in lipid droplets at the endoplastic reticulum (ER) membrane. All cells are able to synthesize triglycerides for storage of excess fatty acids as well as for rapid recruitment of fatty acids if required. Adipocytes are "professionally" dedicated to fat storing, thus serving as energy providers in times of demand and as banks of triglycerides in times of surplus supply.

In addition to adipocytes, there is one other cell type in the mammalian organism that represents a professional triglyceride producer, i.e., the intestinal mucosal cell. All long-chain fatty acids entering the mucosal cell have to be esterified to triglycerides by the ER before they are bound to apolipoproteins in the Golgi compartment and leave the cell by exocytosis as very low density lipoproteins (VLDL) or chylomicron particles. These are released to the systemic circulation *via* the lymphatic system and thoracic duct (1,2). At the surface of endothelial cells, they are exposed to the action of endothelial lipoprotein lipase for cleavage to unesterified fatty acids. Consecutively they are bound to albumin, the major fatty acid transporting molecule in blood serving to keep fatty acids in solution for sufficient cellular delivery.

Another cell type that is of special interest to fatty acid utilization is the endothelial cell. In the heart, there is a tight barrier of endothelial cells separating blood [where fatty acids are tightly bound to albumin ($K_D$, $10^{-8}$)] from cardiomyocytes. The impervious interendothelial junctions do not allow any paracellular permeation of fatty acids (3) (Fig. 1). Because endothelial cells use only a small fraction of fatty acids for their own energy requirements (4), they channel the bulk of fatty acids unmetabolized from the blood to the subendothelial space from which they are taken up by cardiomyocytes. For uptake and translocation across endothelial cells, it was recently shown that fatty acids enter vesicular compartments that might shuttle unmetabolized fatty acids into the subendothelial space (Ring, A., Pohl, J., Völkl, A., and Stremmel, W., unpublished results). The vesicles represented caveolae as well as clathrin-coated vesicles. This vesicular uptake route of endothelial cells is fast and efficient, and requires dissociation of fatty acid:albumin complexes at the cell surface (see below). In addition, a rather slow pinocytotic transport system could operate with low-affinity albumin binding sites, allowing transcellular translocation and delivery of fatty acid:albumin complexes to the subendothelial space (5). Here the albumin molecules accept fatty acids translocated through the endothelium and present them to the

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Abbreviations: ACBP, acyl-CoA binding proteins; ER, endoplasmic reticulum; $FABP_c$, cytosolic fatty acid binding protein; $FABP_{pm}$, plasma membrane fatty acid binding protein; FAT, fatty acid translocase; FATP, fatty acid transport protein; mAspAT, mitochondrial aspartate aminotransferase; PPAR, peroxisome proliferator-activated receptor; VLDL, very low density lipoproteins.

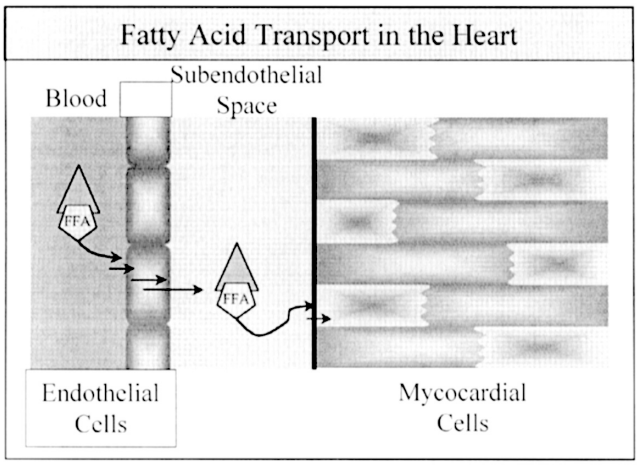


**FIG. 1.** From the vascular system to cardiomyocytes, fatty acids have to pass various barriers. After dissociation from albumin, they enter the endothelial cell layer by specific plasma membrane fatty acid uptake systems. This is followed by transcytosis and release to the subendothelial space. Here, albumin serves again as acceptor and delivery molecule to plasma membranes of cardiomyocytes. Specific membrane transport systems control the entry into parenchymal cells for metabolic disposition in the heart, particularly to β-oxidation for ATP production.

adjacent parenchymal cells for uptake. An alternative hypothesis postulates direct cell–cell contact to shuttle fatty acids from endothelial cells directly to parenchymal cells (6).

## THE MOLECULAR VIEW

The molecular mechanism by which fatty acids enter cells has long been controversial. Theories of passive diffusion across the phospholipid membrane were contrasted by experimental observations compatible with a catalyzed membrane translocation process (7,8). The passive diffusion theory is valid as long as the distribution spaces are lipid compartments, e.g., the inner and outer leaflet of phospholipid bilayers or fused intracellular membranes. Once a fatty acid has entered the outer layer of a phospholipid bilayer, it may be distributed between both membrane compartments according to concentration gradients (9). The solubility of fatty acids in aqueous solutions is very low. Thus only a tiny amount of unbound (free) fatty acids is released from the lipophilic environment into aqueous media. Proteins with defined affinity for fatty acids [e.g., albumin, cytosolic fatty acid binding protein ($FABP_c$)] accumulate fatty acids, thus establishing distribution gradients from lipophilic media toward the aqueous solution. FABP embedded in a membrane can create concentration gradients across phospholipid bilayers and fatty acids can flip-flop accordingly. The presence and concentration of these proteins on both sides of the plasma membrane determine uptake rates in different organs. In the isolated perfused rat heart, single-pass extraction rates of 50% were observed at physiologic fatty acid concentrations (10), whereas in brain, only 1% was extracted.

Because albumin enters cells to a lesser degree than fatty acids, a previous hypothesis postulated high-affinity albumin receptor sites on the plasma membrane, facilitating dissociation of bound fatty acids followed by passive movement across the phospholipid bilayer (11). Several lines of experimental evidence have ruled out this possibility (12,13).

Therefore, the question concerning how fatty acids pass through plasma membranes was of particular interest. The issue was addressed experimentally in different cell types with similar results (14–21). The time course of fatty acid accumulation in hepatocytes revealed a rapid linear initial uptake phase during the first 30 s (22) (Fig. 2). This was followed by a transition phase with declining uptake rates, and a late period characterized by almost linear but low-rate accumulation. The initial uptake phase represents unidirectional (vectorial) fatty acid influx (23). This initial uptake rate was determined as a function of the (calculated) unbound fatty acid concentration applied to the incubation medium (Fig. 3) (22). Modulation of the unbound oleate concentration was achieved by varying the molar ratios of oleate/albumin, leaving either oleate or albumin constant. With increasing unbound oleate concentrations, saturation kinetics were observed with a $K_m$ of 90 nmol/L and a $V_{max}$ of 835 pmol/(min·mg protein) (22). Additional criteria arguing in favor of a carrier-mediated uptake process were as follows: (i) competitive inhibition of uptake by other long-chain fatty acids (24,25) and (ii) inhibition of uptake by protease pretreatment of cells (16). Comparable results were obtained in hepatocytes, cardiomyocytes, intestinal mucosal cells, type II pneumocytes, keratinocytes, and adipocytes (14–20).

For further determination of fatty acid influx, the responsible driving forces were analyzed. In hepatocytes and cardiomyocytes, it was shown that influx was stimulated by $Na^+$ in the medium (16,26,27). Studies using hepatocellular plasma membrane vesicles revealed accelerated fatty acid influx in the presence of a negative intravesicular charge (cation exchange; valinomycin induced $K^+$ depletion) (Fig. 4) (27). Furthermore, it was shown that acidification of the cell inte-

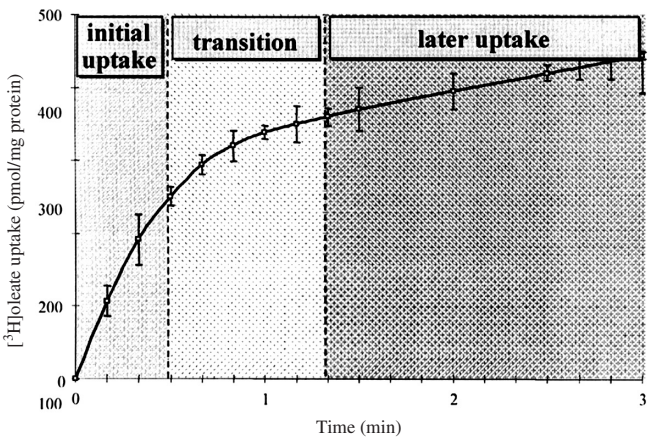


**FIG. 2.** Time course of fatty acid uptake in hepatocytes. Short-term cultured hepatocytes were incubated with [$^3$H]oleate bound to albumin in a molar ratio of 1:1 at 37°C. At the times indicated, the reaction was stopped and uptake determined (22). The initial rapid uptake phase represents cellular influx. This is followed by a transition phase with declining uptake rates. The late uptake period is again almost linear and represents slow intracellular accumulation of fatty acids. (*Source:* Ref. 22.)

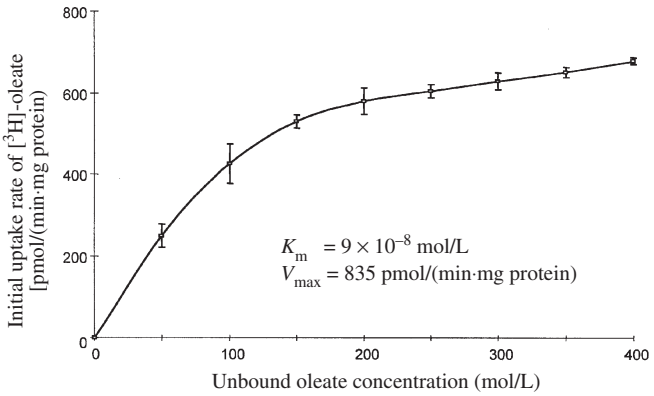


**FIG. 3.** Initial uptake rates of [$^3$H]oleate by short-term cultured hepatocytes as a function of the external unbound oleate concentration. Initial uptake velocities were plotted against the calculated unbound oleate concentration. Saturable influx kinetics indicate the presence of a membrane fatty acid carrier system (22). (*Source:* Ref. 22.)

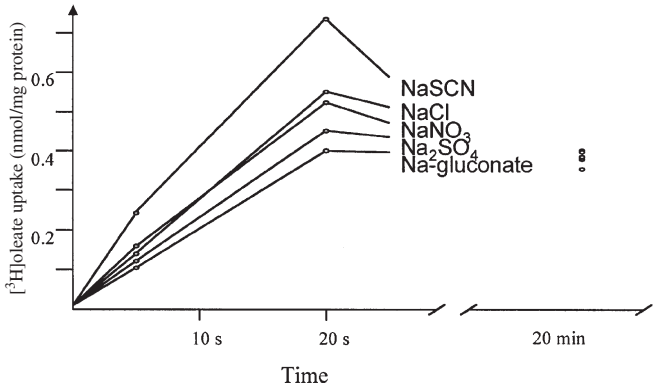


**FIG. 4.** Effect of anion substitution on fatty acid uptake. Hepatocyte plasma membrane vesicles were incubated with 173 µmol/L [$^3$H]oleate:albumin (1:1) at 37°C in the presence of various indicated salts in the medium. In the presence of more permeable accompanying anions ($SCN^- > Cl^- = NO_3^- > SO_4^{2-} >$ gluconate$^-$), $Na^+$-dependent vesicular influx is accelerated (27). It suggests that the translocation of fatty acids across the plasma membrane is stimulated by a relatively more negative intravesicular charge. (*Source:* Ref. 27.)

rior by the $NH_4Cl$ prepulse technique decreased influx velocity, whereas cellular alkalinization stimulated uptake (28). These results are compatible with a $Na^+/H^+$ fatty acid-cotransport system by which fatty acids enter the cell as uncharged or positively charged molecules (Fig. 5).

The next important question was how fatty acids are delivered from the plasma membrane to sites of fatty acid metabolism. Apart from the theory of movement along an intracellular membrane continuum (6), it was shown that they can bind to $FABP_c$ with high affinity (29).Thus, an interaction-mediated transfer of long-chain fatty acids between the cytosolic leaflet of the plasma membrane phospholipid bilayer and $FABP_c$ can be assumed (30). The interactions may occur *via* specific phospholipids and/or membrane proteins, particularly fatty acid translocase (FAT)/CD 36 (30,31). Although only 1–2 mol of fatty acids can be trapped by 1 mol $FABP_c$, the high intracellular concentration of $FABP_c$ seems to be sufficient for operation as a protein-mediated intracellular delivery system (29). This pathway may also play a role in the regulation of intracellular fatty acid metabolism

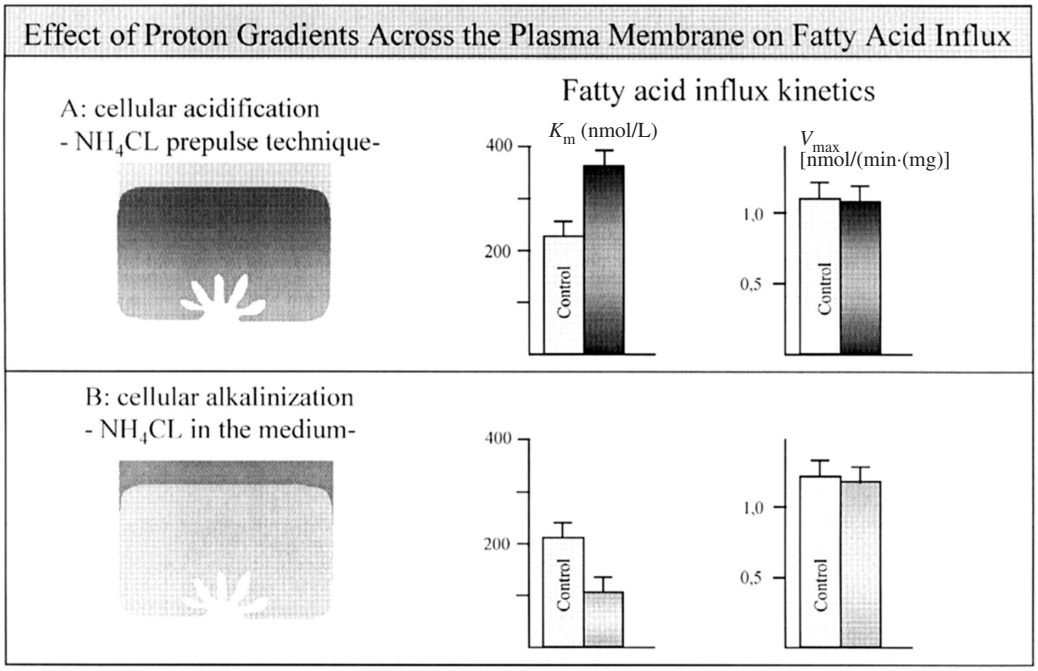


**FIG. 5.** Cellular acidification by the $NH_4Cl$ prepulse technique resulted in a significant $K_m$ increase of fatty acid influx kinetics at unchanged $V_{max}$ values, indicating reduced transporter activity. In contrast, cellular alkalinization decreased $K_m$ at unchanged $V_{max}$ values, indicating increased transport velocity (28). The data are compatible with the hypothesis of a $Na^+/H^+$-fatty acid$^-$ cotransport system by which fatty acids enter the cells as uncharged or positively charged molecules.

(30–32). Indeed, $FABP_c$ can enter the nucleus like the peroxisome proliferator-activated receptor (PPAR) family, which is the main regulator of cellular lipid metabolism (33). As a sensor of the intracellular fatty acid concentration, $FABP_c$ may be involved directly or indirectly *via* PPAR in the transcriptional regulation of responsible genes. Alternatively it was shown that fatty acids bind with high affinity to caveolin-1 at the cytosolic surface of the plasma membrane (34). Caveolins (types 1–3) have been described as integral "hairpin"-like proteins facing the cytosol. They possess an intramembrane region of 33 amino acids anchoring in phospholipid bilayers. Caveolins are essential components of caveolae, which are specific membrane microdomains rich in cholesterol and sphingolipids. Caveolae form typical electron microscopically visible invaginations in the plasma membrane or intracellular vesicular structures, which are functionally involved in important cellular transport processes such as endo- and transcytosis as well as signal transduction pathways (35,36). Their role in cellular cholesterol transport is well established (37). Recently we found that fatty acids taken up by Hep G2 hepatoma cells travel within a vesicular compartment staining positive for caveolin-1. This suggests fatty acid transport by caveolae (Pohl, J., Ring, A., and Stremmel, W., unpublished data). Previously, vesicular uptake was visualized in native rat hepatocytes (38) (Fig. 6). We suggest that fatty acids accumulate within the caveolar membrane and are subsequently delivered to subcellular compartments for further metabolism. Such a caveolin-mediated intracellular delivery system would allow a high number of fatty acids to be transported within the cell.

How fatty acids are incorporated into the caveolae membrane (bound to caveolin-1) remains unclear. The notion that caveolin-1 is anchored within the cytosolic leaflet of the plasma membrane could imply that it may serve as an immediate acceptor protein for membrane translocated fatty acids. This may lead to membrane asymmetry and could eventually induce budding of caveolae from the plasma membrane and formation of vesicles. Alternatively, membrane translocated fatty acids could be incorporated into preformed caveolar vesicles by membrane collision and/or binding to caveolin-1. Most intriguing is the thought that caveolae are loaded with unmetabolized fatty acids *via* direct interaction with $FABP_c$, serving as an intermediate delivery pool.

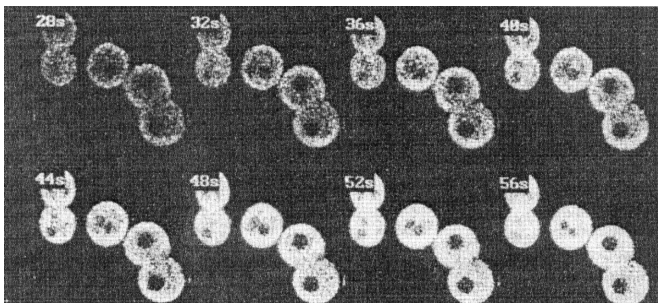


**FIG. 6.** Confocal visualization of 12-*N*-methyl-7-nitrobenzo-2-oxa-1,3-diazol amino stearate (12-NBD stearate) uptake into hepatocytes. Short-time cultured hepatocytes were superfused with the incubation medium containing 50 µmol/L 12-NBD stearate and 25 µmol/L bovine serum albumin. Illustrated is the sequence of pictures obtained between 12 and 56 s of incubation using the frame mode of the confocal laser scanning microscopy system. Uptake into hepatocytes revealed a granular staining pattern starting from the area of the plasma membrane toward the cytosol. It suggests that the bulk of fatty acid uptake is mediated by a vesicular uptake route. (*Source:* Ref. 38.)

The mechanism of interaction between intracellular fatty acids and subcellular organelles is unclear. It is probably controlled by the metabolic demand of the cell, directing fatty acids for ATP production to mitochondria or for storage as triglycerides to the ER. Activation to acyl-CoA is required for any kind of metabolic disposition. It has been long believed that acyl-CoA does not pass biological membranes.

Therefore, it was postulated that native fatty acids first enter the subcellular compartment of destination before undergoing acyl-CoA activation and further metabolism. An alternative hypothesis postulates activation at the outer membrane surface of the organelle. From there, acyl-CoA can be taken up *via* acyl-CoA membrane translocating systems, e.g., by the carnitin shuttle into mitochondria for β-oxidation, or by the ABC transporter assembly into peroxisomes for synthesis of specialized lipids (39). Triglyceride and other complex lipid synthesis can occur at the outer leaflet of the ER membrane, contributing to the formation of lipid droplets. Whether the latter process may also take place at the cytoplasmic site of the plasma membrane is not yet established. Lipid droplet formation can be associated with accumulation of caveolins in their periphery, serving in intracellular trafficking of lipids *via* caveolae (37).

According to the facts presented above and to theoretical considerations, cellular uptake of fatty acids requires a continuous flow down a concentration gradient starting from the amount of fatty acids presented to the plasma membrane until acyl-CoA activation for final metabolic disposition by subcellular compartments. Thus, uptake is eventually regulated by the cellular metabolic demand. The steps in the uptake cascade are as follows: fatty acid:albumin complexes presented to the plasma membrane; dissociation of the complex and binding to plasma membrane proteins with high affinity for fatty acids; flip-flop to the cytosolic leaflet facilitated by an interphospholipid bilayer concentration gradient; binding to $FABP_c$ and caveolin followed by incorporation into caveolae; discharge from the plasma membrane and delivery to subcellular compartments; and activation to acyl-CoA. This overall flow rate represents the total uptake velocity, which is described by saturation kinetics (Fig. 3).

## THE PLAYERS

In the last two decades, most attention has focused on the identification of the responsible membrane fatty acid carrier protein. This question has been difficult, subject to controversies, and may finally be insoluble owing to the complex characteristics of the overall uptake described above. In the following section, we focus briefly on only three of the proposed candidate players (for more details please refer to the cited literature).

*(i) FAT/CD36.* FAT/CD 36 is a 80-kDa protein with two proposed transmembrane spanning domains, very short N- and C-terminal intracytoplasmic domains, and an extracellular glycosylated loop (Fig. 7) (40,41). It may accelerate fatty acid dissociation from albumin and catalyze integration of protonized fatty acids into the outer phospholipid bilayer of the plasma membrane. This accumulation of fatty acids creates a diffusional gradient across the plasma membrane, which is followed subsequently by a flip-flop of fatty acids to the inner leaflet (FAT-catalyzed facilitated diffusion). Expression of this translocase in fibroblasts lacking CD 36 revealed a phloretin-sensitive, very high affinity, saturable fatty acid uptake component ($K_m$, 4 nmol/L) (42). For translocation across the plasma membrane and further on to the cytosol, an interaction with $FABP_c$ has been proposed (30,31).

Also of interest is the observation that the lipoprotein receptor SR-B1, which shares homologies with FAT/CD 36, was shown to co-localize with caveolin (43). This would suggest that FAT might interact with the caveolae-linked uptake route described above. For FAT/CD 36, an intracellular vesicular recruitment compartment has been suggested (44). At least in muscle cells, the protein was shown to reside at the sarcolemma as well as in an intracellular ("endosomal") pool; upon muscle contraction, FAT/CD 36 is redistributed toward the sarcolemma with a concomitant increase in the rate of muscular fatty acid uptake (44). Moreover, hormones such as insulin also can trigger the translocation of FAT/CD 36 to the cell surface (30). These observations indicate that FAT/CD 36–mediated fatty acid uptake can actively be regulated by translocation of the protein from intracellular stores to the plasma membrane.

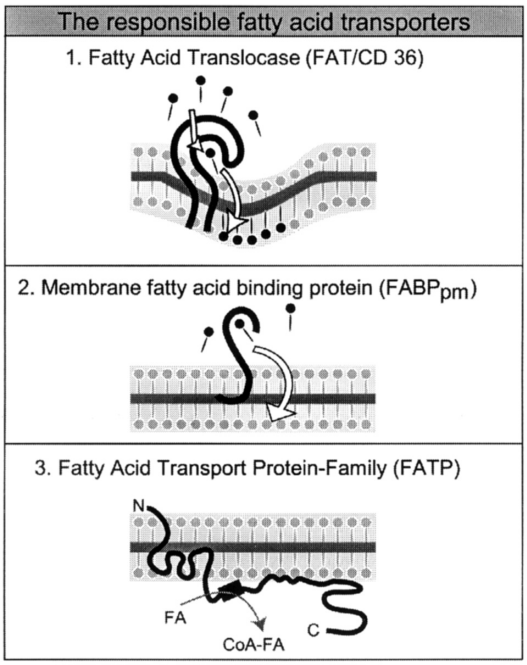


**FIG. 7.** Illustration of three major membrane fatty acid (FA) transport systems. Fatty acid translocase (FAT)/CD 36 is a 80-kDa protein characterized by two transmembrane spanning domains, short intracytoplasmatic tails, and an extracellular glycosylated loop. It translocates native long-chain FA with high affinity across the plasma membrane. Plasma membrane fatty acid-binding protein ($FABP_{pm}$) is a 40-kDa protein, loosely associated with plasma membranes. It represents the membrane-bound form of mitochondrial aspartate aminotransferase (mAspAT). It has an affinity for long-chain FA, and is involved in cellular uptake of long-chain FA. The FA transport protein (FATP) family represents a group of membrane-associated proteins involved in the overall FA uptake process by activating them to acyl-CoA (ACS-activity) at a conserved cytosolic AMP binding site. FATP 1-5/6 have varying N-termini associated with a single transmembrane spanning domain representing different intracellular targeting signals or binding domains for specified proteins.

FAT is not present in hepatocytes but is found at high levels in colon and spleen (40). This differential expression indicates that it is not the only carrier for fatty acids throughout the organism and that it may also be involved in other cellular functions. CD 36–deficient mice reveal impaired fatty acid uptake in the heart, adipose tissue, and muscle, but show normal uptake in liver and intestine (45).

*(ii) $FABP_{pm}$.* is a 40-kDa protein anchored to the outer leaflet of the plasma membrane with its hydrophobic tail (Fig. 7) (46,47). The implication of its involvement in the overall uptake process of fatty acids was documented by antibody inhibition experiments (14–16,20) as well as transfection studies (48,49). The protein is identical to mitochondrial aspartate aminotransferase (mAspAT), thus revealing two different functions in two separate cell compartments (46). It is conceivable that on its passage to the vascular system, the protein is briefly trapped in the outer plasma membrane leaflet with its lipophilic tail binding fatty acids with high affinity, thus facilitating dissociation from albumin (47). After protonization, fatty acids can accumulate in the outer leaflet of the phospholipid bilayer (28). According to the resulting intramembrane concentration gradient, they flip-flop to the inner leaflet. Here they may be bound by $FABP_c$ or caveolin-1 and enter the cells as proposed above. There is a differential distribution of $FABP_{pm}$ throughout the organism, indicating that it does not represent an exclusive fatty acid transport system. It is even possible that other proteins with mAspAT-like properties exist and also operate as FABP on the outer leaflet of the plasma membrane. The requirements are as follows: high abundance, plasma membrane passage, hydrophobic tails for retainment in the outer phospholipid bilayer, and high affinity for fatty acids to mediate dissociation from albumin. Apparently, not too many different proteins meet these criteria because an antibody to $FABP_{pm}$ alone resulted in a 65% inhibition of fatty acid uptake in certain cell types (14–17,21). Nevertheless, the specificity of $FABP_{pm}$ as a fatty acid carrier has to be questioned in view of the rather unspecific characteristics mentioned above. If in the future, proteins with similar characteristics should be identified, this would indicate that the essential function of cellular fatty acid delivery is functionally highly conserved by the option of mutual substitution.

*(iii) FATP family.* The FATP family represents a group of membrane proteins with a molecular weight of 63 kDa. The name was originally created when two proteins leading to cellular enrichment of a fluorescent fatty acid were identified using expression cloning from a murine adipocyte cDNA library (50). One was the known long-chain fatty acid

acyl-CoA synthetase; the other was unknown and was named FATP. In fact, fatty acid uptake studies with stably transfected 293 cells revealed a three- to fourfold increase of fatty acid accumulation over controls.

To date, five members of this family have been described in mice and six in humans (FATP 1-5/6) (51). By hydropathy plot analysis, it was originally proposed that FATP have six transmembrane domains and it was assumed that the N- and C-termini face the cytosolic site of the phospholipid bilayer (50). A more recent detailed analysis of the membrane topology of FATP 1 revealed only one transmembrane domain (52). In addition, the molecule is preferentially oriented toward the cytoplasm with two membrane loops within the inner leaflet, peripheral membrane–associated domains, and two large stretches of nonmembrane-associated residues carrying the highly conserved AMP binding site and the C-terminus (52) (Fig. 7). Only a very short segment of the N-terminus faces the luminal/extracellular site of the membrane bilayer. The N-terminal 51 amino acids of the protein family members are variable (51), and they may represent specific targeting signals for different subcellular compartments. The protein is not glycosylated and does not resemble a typical transmembrane transport protein (52). Binding sites for fatty acids are not defined. The most interesting region of this membrane-anchored protein family is the highly conserved AMP binding region (53). Carefully conducted studies by a number of investigators demonstrated CoA-synthetase activity of FATP 1, 2, 4, and 5 toward various lipid compounds, particularly fatty acids (54–60). Although some substrate specificity was demonstrated, e.g., for very long-chain fatty acids by FATP 1, 2, and 4, this does not rule out the general capability of activating long-chain fatty acids and other related compounds as well. This enzymatic activity could be linked to the conserved AMP binding site of FATP. It is challenging that facilitation of cellular fatty acid uptake may be due to this long-chain acyl-CoA synthetase activity, stimulating fatty acid flow through membranes as described above. In fact, its putative function as direct fatty acid carrier is not well supported by its structural conformation and membrane topology. Another challenging thought relates to the differential subcellular distribution of this protein family. Apart from the initial report localizing FATP 1 to the plasma membrane (50), FATP 2 was found in peroxisomes (55) and the ER (57). FATP 1 (52) and FATP 4 (Herrmann, T., Rost, D., Pohl, J., and Stremmel, W., unpublished observation) were also demonstrated in the ER. This distinct subcellular localization might be mediated by the variable amino termini of the different FATP. From a common origin in the ER as delivery compartment, it could be assumed that the N-terminal assembly with other proteins is capable of directing the complex to subcellular compartments for incorporation into the specific metabolic disposition pathways (61). Accordingly, different cell types with different metabolic requirements could reveal a specific pattern of FATP. By this mechanism, a tuned adaptation and regulation of fatty acid metabolism according to the demands of a cell appears plausible. For FATP 1, a preferential localization at the plasma membrane was shown (50). Its capability to activate fatty acids to acyl-CoA could be utilized for complex lipid synthesis at this site or binding to acyl-CoA binding proteins (ACBP) for intracellular transport. In addition, the preferential activation of very long-chain fatty acids (54) could indicate a role in the intracellular signaling pathways by transfer of fatty acids to PPAR. FATP 4 is preferentially, but not exclusively, detectable in the intestinal mucosal cells and is essentially involved in fatty acid absorption (62,63). Triglyceride and phospholipid synthesis in the ER is a prominent feature of these cells. Therefore, it is conceivable that FATP 4 might play a role in activation of absorbed fatty acids to acyl-CoA for triglyceride synthesis at the ER. In these cells, the ER is closely attached to the microvillous plasma membrane. Such a proposed involvement of FATP 4 in triglyceride formation would indicate a vital function of this protein throughout the organism.

## THE CONCEPT

According to the facts described above and to theoretical considerations, the following working hypothesis can be derived (Fig. 8). At the outer leaflet of the plasma membrane, fatty acids are dissociated from albumin. In different cell types, a concert of different membrane-associated proteins with affinity for long-chain fatty acids facilitates this dissociation process. The best-described candidates of such membrane proteins are FAT (CD 36) and $FABP_{pm}$. Fatty acids dissociated from albumin accumulate in the vicinity of these proteins on the surface of the plasma membrane. Here, fatty acid anions are protonized and rapidly integrate into the external phospholipid bilayer as uncharged molecules. Thus, a concentration gradient toward the inner leaflet is created and fatty acids flip-flop across (passive diffusion component). Here they can be transferred to $FABP_c$.

An interaction of $FABP_c$ with anionic phospholipids and/or FAT/CD 36 has in fact been proposed (30,31,64–67). This could promote the vectorial transport of fatty acids across the plasma membrane. Holo-$FABP_c$ could then serve in intracellular distribution of unmetabolized fatty acids. Because it can also enter the nucleus, a regulatory function has been suggested (see above). It is also quite conceivable that holo-$FABP_c$ can provide fatty acids to preformed caveolar vesicles by protein–phospholipid or protein–protein interaction with caveolins.

Alternatively or complementarily, membrane-translocated fatty acids could bind with high affinity to caveolins (34) embedded in the inner leaflet of the plasma membranes or in the outer leaflet of preformed caveolae colliding with the plasma membrane. A caveolin positive-vesicular uptake component could be visualized in hepatoma as well as in endothelial cells (see above). Caveolae thus may serve as high-capacity intracellular lipid transport vehicles, delivering the bulk of fatty acids to different subcellular compartments for further metabolic disposition. A comparative quantification of both complementary routes of intracellular fatty acid transport ($FABP_c$- and caveolae-mediated) cannot be estimated at present.

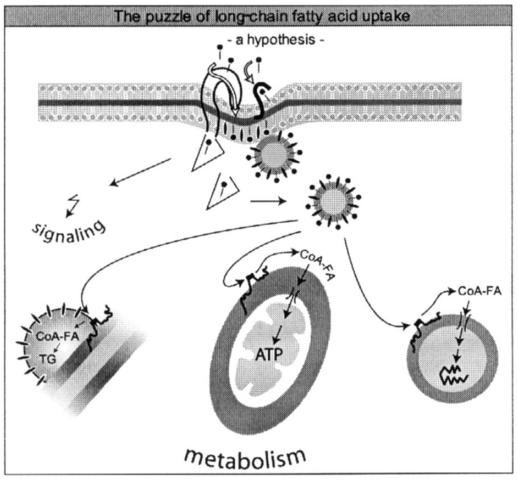


**FIG. 8.** The overall cellular uptake of fatty acids represents a continuous flow from fatty acid:albumin complexes at the cell surface to acyl-CoA formation as the first step in metabolic disposition of fatty acids. At the surface of the plasma membrane, FAT and $FABP_{pm}$ mediate fatty acid dissociation from albumin. After protonization, fatty acids flip-flop across to the inner leaflet of the membrane. Here they are transferred to cytosolic fatty acid binding protein ($FABP_c$) serving as protein-mediated intracellular fatty acid delivery system as well as signaling molecules for fatty acid metabolism. Interaction with caveolins allows incorporation into caveolae. Intracellular trafficking within these vesicles may represent the pathway for uptake of the bulk of fatty acids. At the cytosolic surface of different subcellular compartments, different FATP activate the fatty acids presented to acyl-CoA for channeling to further metabolic disposition. Plasma membrane localized FATP may be responsible for generation of acyl-CoA, which can be loaded onto cytosolic binding proteins (ACBP). Moreover, binding to peroxisome proliferator-activated receptors (PPAR) for signaling or involvement in complex lipid synthesis at this site is a further option. Endoplastic reticulum (ER)-associated FATP may be responsible for lipid droplet formation (triglyceride synthesis), which serves as an intracellular energy storage compartment. At the mitochondria and peroxisomes, FATP may provide acyl-CoA for translocation into the organelles for oxidation and synthesis of specialized lipids (symbols are illustrated in Figure 7). See Figure 7 legend for other abbreviations.

Up to this point, the uptake pathway described apparently does not require energy. It represents a continuous flow of fatty acids, involving membrane binding proteins, rapid flip-flop across the membrane, transfer to $FABP_c$, and possibly integration into caveolin-associated membrane structures. The uptake flow ends at subcellular compartments in which the metabolic disposition of fatty acids is determined. Here the family of FATP enters the stage. By their acyl-CoA synthetase activity, fatty acids are activated as the first step of any kind of metabolism. This could occur exclusively at the plasma membrane level from which acyl-CoA bound to cytosolic binding proteins (ACBP) is transported to sites of metabolic demand. Alternatively it could occur directly at subcellular compartments, which would require special targeting signals of FATP themselves or association with certain proteins directing FATP to the required functional location. The last-mentioned options appear more plausible due to the variable N-terminus of different FATP (signaling sequence) constituting the single transmembrane region of this protein. After physical linking to the site of metabolic disposition, the protein could act as a membrane-associated enzyme facing the cytosol with its catalytic end. Plasma membrane-associated FATP 1 could activate fatty acids for loading of ACBP, or for transfer to PPAR serving the signaling pathways, or for complex lipid synthesis at the cytosolic plasma membrane leaflet. At the ER, FATP might be involved in lipid droplet formation (37) (e.g., for synthesis of triglycerides). Mitochondrial and peroxisomal FATP could activate fatty acids for translocation *via* the carnitine shuttle into mitochondria and for a proposed acyl-CoA transporter in the peroxisomal membrane, respectively. Whether the distinct patterns of FATP distribution in different cell types represent a physiologic adaptation to their respective metabolic demand or are due to an organ-specific, genetically determined transcriptional control of FATP synthesis is unresolved.

The proposed concept relates to the principal mechanism of fatty acid uptake and does not cover specificities of certain cell types, e.g., endothelial cells. It represents hypotheses in addition to a summary of experimentally proven facts and certainly reflects our personal view.

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# Polyunsaturated Fatty Acid Supply with Human Milk

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**ABSTRACT:** Polyunsaturated fatty acids in human milk may derive from diet, liberation from maternal body stores, or endogenous synthesis from precursor fatty acids. The contribution of each of these sources has not been studied in detail. Although maternal diet is a key factor affecting human milk composition, other factors such as gestational age, stage of lactation, nutritional status, and genetic background are known to influence the fat content and fatty acid composition in human milk. Both linoleic and α-linolenic acids, the essential fatty acids, are present in human milk, as are several other n-6 and n-3 longer chain polyunsaturated fatty acids that are required for optimal growth and development of infants. The fatty acid profile of human milk from lactating women of different countries is remarkably stable, but there is variability in some of the components, such as docosahexaenoic acid, which is mainly due to differences in dietary habits. Tracer techniques with stable isotopes have been valuable in assessing the kinetics of fatty acid metabolism during lactation and in determining the origin of fatty acids in human milk. Based on these studies, the major part of polyunsaturated fatty acids in human milk seems not to be provided directly from the diet but from maternal tissue stores.

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Human milk is a unique mixture of lipids that delivers several polyunsaturated fatty acids (PUFA) to the breast-fed infant. With a fat content of about 4% in mature milk, most of these fatty acids are found in triglycerides (98%) and only a minor portion in phospholipids (1%) and other components (1). The amounts of saturated and monounsaturated fatty acids in human milk are large when compared to PUFA. In contrast to saturated and monounsaturated fatty acids, which can be derived from endogenous synthesis, the major part of PUFA in human milk is essential fatty acids, which cannot be synthesized endogenously by mother or infant and, therefore, have to be provided with the diet. The essentiality of both linoleic acid (LA), the precursor of the n-6 series, and α-linolenic acid (ALA), the precursor of the n-3 series, has been known for many years (2,3). Interestingly, both fatty acids compete for the same enzymes for desaturation and elongation (see Fig. 1). Some of their products, including arachidonic acid (AA) and docosahexaenoic acid (DHA), play important roles in infant growth and neurodevelopment (4,5). Furthermore, PUFA in human milk may serve as precursors for eicosanoids, which are potent modulators of processes such as vasoconstriction and immunoreactivity (6). Many studies have focused on human milk fatty acid composition and the effects of different diets. Here we report on different aspects influencing the PUFA composition in human milk and on results of dietary supplementation studies. Furthermore, we present some newer studies using tracer techniques with stable isotopes to assess the origin and metabolism of PUFA during lactation.

## PUFA IN HUMAN MILK

Some 214 different fatty acids have been characterized in human milk, of which 40 are polyunsaturated (7). Usually, only 35 to 40 different fatty acids, including 10 to 15 PUFA, are reported in studies on human milk composition, because they represent the major portion and can be analyzed by the commonly available chromatographic methods. Fatty acids are not randomly distributed within the triglyceride molecules. It is well known that palmitic acid, for example, is preferentially found in the *sn*-2 position and that lipases hydrolyze more of the fatty acids localized at the *sn*-1 and *sn*-3 positions (8). This has been demonstrated to affect absorption of fat and calcium in infants (9). For LA and ALA preferential binding to the *sn*-2 and *sn*-1 hydroxyl groups of the glycerol backbone has been described. Long-chain polyunsaturated fatty acids (LC-PUFA) such as AA and DHA have been found primarily in the *sn*-2 and *sn*-3 positions (8).

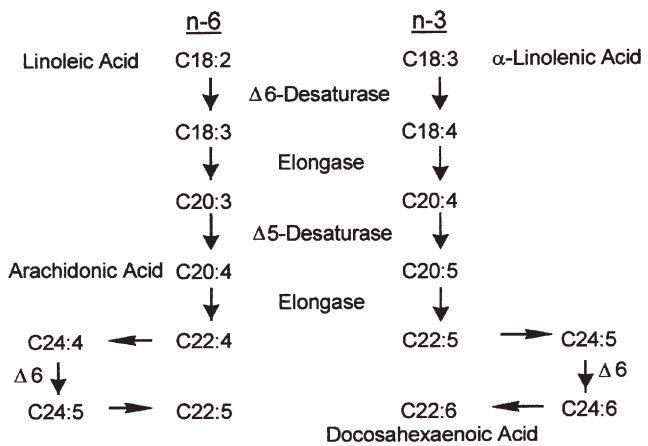


**FIG. 1.** Revised pathways of polyunsaturated fatty acids [based on data from Sprecher *et al.* (50)].

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Abbreviations: ALA, α-linolenic acid, 18:3n-3; AA, arachidonic acid, 20:4n-6; DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; LA, linoleic acid, 18:2n-6; LC-PUFA, long-chain polyunsaturated fatty acids; PUFA, polyunsaturated fatty acids.

*Changes with lactational stages.* Even during a single feeding, the amount of fat secreted into human milk changes. At the beginning, in foremilk, a fat content of about 1–3% is present, whereas in the last portion of human milk, the hindmilk, the fat content is increased to about 5–8% (10). This phenomenon is related to fat synthesis and its excretion *via* the fat globules in the mammary gland. During breast-feeding, the size of these fat globules increases. In neonatal care units, hindmilk has been used with success for enhancing weight gain in low-birth-weight infants (11). Also with advancing lactation, the fat content increases in colostrum, transitional, and mature milk from about 2% on day 3 postpartum to more than 4% at around 3 mon (12). For single fatty acids, relative changes have also been observed. In a study by Genzel-Boroviczeny *et al.* (13) over the first 30 d postpartum, LA and ALA increased slightly, whereas AA and DHA decreased significantly. No differences were observed between preterm and term mother's milk in that study. A study by Makrides *et al.* (14) that followed lactating mothers until 30 wk postpartum demonstrated a similar decrease of AA and DHA over time. However, because total fat content increases with advancing lactation, the absolute amounts of LC-PUFA excretion remain relatively stable.

*Essential fatty acids.* The most abundant PUFA in human milk is LA. The content of LA in human milk seems to have increased twofold over the last decades (7), which may reflect a shift in the Western world's diet toward higher consumption of n-6 fatty acids. An imbalance of the precursors LA and ALA in the diet might lead to an imbalance of product fatty acids of the n-6 and n-3 series. The influence of the LA/ALA ratio (or different intakes of ALA) on endogenous synthesis of LC-PUFA has been studied in infants (15) and adults (16) with the use of stable isotopes. In the study in infants, a lower LA/ALA ratio (or higher intake of ALA) resulted in a higher incorporation of DHA into plasma phospholipids and, at the same time, in a decreased incorporation of AA into plasma phospholipids. In the study in adults, high vs. low intake of LA also had effects on endogenous LC-PUFA synthesis. Further, an imbalanced ratio of LA and ALA is thought to interfere with an appropriate functioning of the immune system (6,17). The LA/LA ratio is on the order of 5:1 to 15:1 in most milks (18). This ratio has also been proposed by the European Society for Paediatric Gastroenterology and and Nutrition (ESPGAN) for infant formula feeding (19). The LA/ALA ratios found in different studies of human milk composition are presented in Table 1, with some extreme values observed in South Africa (20).

*LC-PUFA.* AA and DHA, the two predominant LC-PUFA in human milk, are known to be required for normal development of infants (4). The amounts of LC-PUFA in human milk in most samples are at least 10-fold lower than those of LA. The most abundant LC-PUFA of the n-6 and n-3 series in human milk and normally reported in compositional studies are 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6, and 20:5n-3, 22:5n-3, and 22:6n-3. These fatty acids can also be formed endogenously from precursors, even in the very premature infant (21,22). The absolute amounts of endogenous synthesis and the definite amounts required for optimal development in infants remain to be determined. The amounts of AA and DHA in human milk have been used as some guidelines for infant formula contents of LC-PUFA. PUFA and LC-PUFA profiles in human milk are to some extent remarkably stable (18). On the other hand, there are also quite variable amounts of LC-PUFA, especially DHA, the amount of which varies about 20-fold under certain dietary circumstances (23). Levels reported for DHA in human milk in various countries are presented in Figure 2. Low levels of DHA are found in human milk in the United States (24), and very high levels are found in human milk of mothers consuming marine diets in China (23).

**TABLE 1**
**Polyunsaturated Fatty Acids in Samples of Mature Human Milk from Different Countries (wt% of total, mean, or median values)[a]**

| | China Chulei *et al.*, 1995 (23) | U.K. Sanders *et al.*, 1978 (25) | Canada Chen *et al.*, 1995 (43) | Hungary Sas *et al.*, 1986 (44) | Sweden Jansson *et al.*, 1981 (45) | Japan Idota *et al.*, 1991 (46) |
|---|---|---|---|---|---|---|
| Number of women | 39 | 4 | 198 | 13 | 24 | 351 |
| LA | 20.6 | 6.9 | 10.5 | 11.0 | 12.9 | 13.3 |
| AA | 0.9 | 0.5 | 0.4 | 0.5 | 0.4 | 0.4 |
| ALA | 3.0 | 0.8 | 1.2 | 1.2 | <1.4 | 1.4 |
| DHA | 0.9 | 0.6 | 0.1 | 0.1 | 0.3 | 1.0 |
| LA/ALA ratio | 6.9 | 8.6 | 9.0 | 9.2 | >9.2 | 9.3 |

| | Spain de Lucchi *et al.*, 1988 (47) | Germany Koletzko *et al.*, 1988 (48) | Gambia Prentice *et al.*, 1988 (49) | United States Putnam *et al.*, 1982 (24) | South Africa van der Westhuizen *et al.*, 1988 (20) |
|---|---|---|---|---|---|
| Number of women | 28 | 15 | 23 | 9 | 12 |
| LA | 14.7 | 10.8 | 13.0 | 15.8 | 16.2 |
| AA | 0.8 | 0.4 | 0.3 | 0.6 | 0.6 |
| ALA | 1.3 | 0.8 | 0.8 | 0.8 | 0.4 |
| DHA | 0.4 | 0.2 | 0.4 | 0.1 | 0.2 |
| LA/ALA ratio | 11.3 | 13.3 | 15.5 | 18.8 | 40.5 |

[a]Abbreviations: LA, linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexenoic acid.

*Human milk samples from different countries.* Most mean or median values for PUFA in human milk in different countries are quite similar. Reported LA values range from a value of 7% (wt% of total) in the United Kingdom (25) to a value of 21% in China (23), with values in most countries falling in the range of 10–15% (see Table 1). Reported ALA values range from 0.4% in South Africa (20) to 3.0% in China (23). Although methodologies might also influence to some extent the detection of LC-PUFA, a remarkably similar content of AA in human milk is found worldwide, with values ranging from 0.3 to 0.9%. More variation is found in the content of DHA (see Fig. 2); as noted above, a 20-fold variation was found in a study conducted within China (23).

*Maternal diet.* Maternal diet is an important factor influencing human milk fatty acid composition. The differences observed in human milk of mothers from different countries therefore appear to reflect primarily different diets. The long-term effects of vegan/vegetarian, high-fish, and omnivorous diets are well known. Because vegans/vegetarians have high intakes of LA and ALA, the levels of these fatty acids in their milk are among the highest values observed in human milk (26). Fish intake is related to eicosapentaenoic acid (EPA) and DHA levels in human milk, with highest values observed in Inuit women (27) and in Chinese women consuming high amounts of marine foods (23). The rapidity of dietary influences on human milk fatty acids has recently been demonstrated in 14 lactating women drinking test formulas containing different fats (28). Increases in human milk of certain marker fatty acids were observed within 6 h of consumption of the test formulas. Maxima were reached at 10 h for ALA (safflower oil), at 14 h for EPA (menhaden oil), and at 24 h for DHA (herring and menhaden oil).

## SHORT- AND LONG-TERM STUDIES OF FATTY ACID SUPPLEMENTATION

The first studies on the influence of dietary alterations on human milk fatty acid composition were reported by Söderhjelm in 1953 (29) and Insull *et al.* in 1959 (30). The methods available at that time allowed only limited detection of individual PUFA. In the study of Insull *et al.*, from one lactating woman the sum of LA and ALA in the milk changed from 10% to well over 40% after a switch to a diet rich in corn oil. Fish oil in a dose of 5, 10, or 47 g was used in a supplementation study by Harris *et al.* (31) and led to increases in the n-3 fatty acids. DHA values increased in that study after a maximum of 4 wk of supplementation from 0.1% at baseline to 0.5, 0.8, and 1.9% of total fatty acids, respectively. Similarly, in another study, fish oil supplementation in a dose of 6 g/d over 3 wk resulted in an increase of DHA from 0.37 to 0.70% at the end of the study (32). In recent studies single-cell oils and DHA-enriched eggs have also been used as sources for DHA supplementation (33,34). Makrides *et al.* (33) reported breast-feeding mothers supplementing with a placebo or 0.2, 0.4, 0.9, or 1.3 g DHA/d (33). After about 11 wk of supplementation, the DHA levels were 0.21, 0.35, 0.46, 0.86, and 1.13 wt% of total fatty acids, respectively, and increased in a dose-dependent manner. Fatty acids other than DHA were not influenced by the dietary intervention. In a recent study, we found about the same effect on human milk DHA levels as reported above for a supplementation with 200 mg DHA/d (35). In the study of Jensen *et al.* (34), three different dietary sources of DHA (single-cell oil, fish oil, and DHA-enriched eggs) were given to lactating mothers, and fatty acid composition of human milk lipids and maternal and infant plasma phospholipids was measured (34). DHA supplementation from 2 to 8 wk postpartum with 170–260 mg/d increased DHA levels in human milk by about 0.07–0.21 mol%. Significant correlations were observed in that study between DHA contents of maternal plasma and human milk and of milk and infant plasma phospholipids

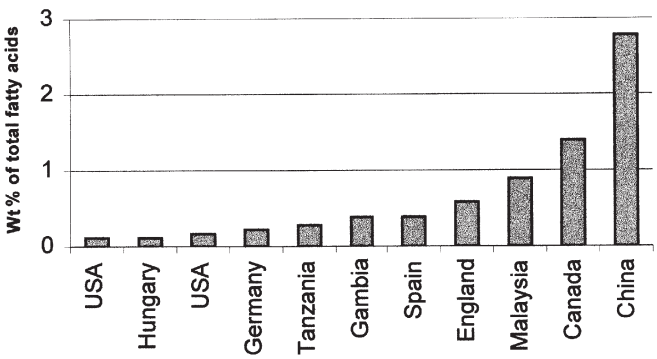


**FIG. 2.** Docosahexaenoic acid (DHA) content in human milk samples of different countries (wt% of total fatty acids; mean or median values): USA (24), Hungary (44), USA (12), Germany (48), Tanzania (51), Gambia (49), Spain (47), England (25) , Malaysia (52), Canada (Inuit) (27), China (23).

## *IN VIVO* STUDIES OF FATTY ACID METABOLISM DURING LACTATION

Methods using stable-isotope-labeled substrates have been increasingly used in the recent past for diagnostic and research purposes (36). The availability of adequate tracers has also contributed to the feasibility of studying metabolic processes *in vivo* during pregnancy, lactation, and the neonatal period. Studies in such individuals with radioactive isotopes would obviously be unethical. In contrast, the use of stable isotopes as tracers has been shown to be safe and without adverse effects.

There are only a few studies investigating the *in vivo* metabolism of fatty acids in lactating mothers. The first report appeared more than 10 years ago and described the transfer of deuterated dietary palmitic, oleic, and linoleic acid into milk in three breast-feeding women (37). There were no differences in the secretion of the individual fatty acids. Tracer fatty acids provided as triglycerides with a defined diet were transported primarily by triglycerides within the chylomicrons and very low density lipoproteins. A delay of about 6 h was observed between maximal enrichments in plasma and human milk. From the tracer transfer into human milk, it was

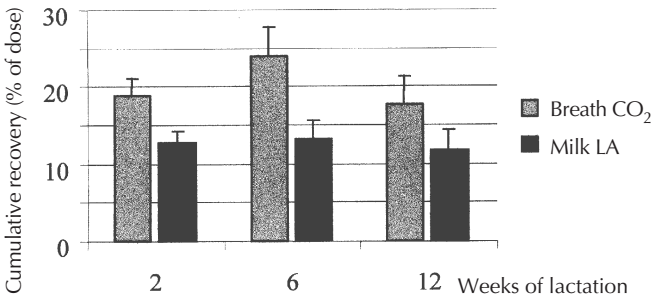


**FIG. 3.** Cumulative recovery of [$^{13}$C]linoleic acid in breath $CO_2$ and in human milk linoleic acid (LA) 5 d after tracer administration in six lactating women at different weeks postpartum (% of dose; mean ± standard error of the mean; adapted from data of Ref. 38)

concluded that long-chain fatty acids such as palmitic, oleic and linoleic acids from the diet contributed to about 30% of the fat in milk.

In a study of six lactating women on their usual diets, we measured the metabolic disposal of orally given $^{13}$C-labeled linoleic acid (38). These women were studied again in the 2nd, 6th, and 12th wk of lactation. No differences were observed between the three time points in oxidation and transfer of linoleic acid to human milk (see Fig. 3). About 20% of $^{13}$C-linoleic acid was recovered in breath $CO_2$ within 5 d. However, the amount of oxidation might even be somewhat underestimated, because some retention of $CO_2$ in the bicarbonate body pools is known to occur but was not taken into account (39). Transfer of dietary linoleic acid into human milk was estimated to reach 13%. From the data, it was calculated that about 30% of linoleic acid in human milk originates from direct dietary transfer and about 70% from maternal tissue stores. Endogenous synthesis of LC-PUFA such as AA occurred only to a low extent. Only about 3% of the AA in human milk was estimated to be derived from endogenous synthesis from LA.

In another group of 10 lactating women, we investigated the metabolism of $^{13}$C-labeled myristic, palmitic, oleic, and docosahexaenoic acids simultaneously at 6 wk postpartum (35). The labeled fatty acid mixture was similar in fatty acid content to the DHA supplement DHASCO™ (Martek Biosciences Corp., Columbia, MD). Oxidation of the fatty acid mixture and transfer from dietary intake to human milk were

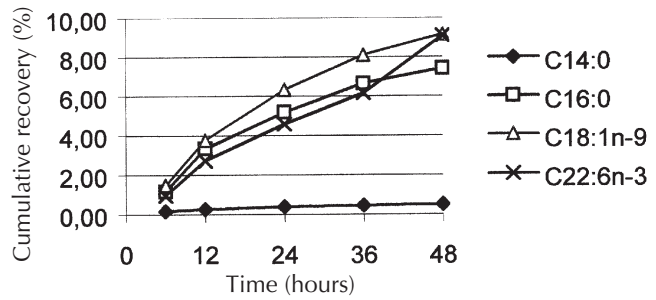


**FIG. 4.** Cumulative recovery of [$^{13}$C]-labeled myristic (14:0), palmitic (16:0), oleic (18:1n-9), and docosahexaenoic acid (22:6n-3) in human milk 48 h after oral tracer administration in 10 lactating women (median values; adapted from data of Ref. 35).

measured over a period of 48 h. Cumulative oxidation of the fatty acid mixture was comparable to our previous observation of linoleic acid oxidation in lactating women (40). The transfer and cumulative recovery of palmitic, oleic, and docosahexaenoic acids in human milk were also comparable and reached about 8% within 48 h. In contrast, the recovery of myristic acid was severalfold lower (see Fig. 4). Medium-chain fatty acids such as myristic acid are readily oxidized, which might explain part of the low direct dietary transfer of this fatty acid (41). Furthermore, extensive *de novo* synthesis of medium-chain fatty acids from carbohydrates has been demonstrated in the mammary gland (42). Our newer studies with $^{13}$C-labeled fatty acids are in good agreement with the earlier study of Hachey *et al*. using deuterated fatty acids (37). Overall, the numerical values obtained from these studies elucidate the flux of different dietary fatty acids during lactation and demonstrate the importance of maternal tissue stores for human milk fatty acid composition. However, long-term dietary intakes affect the tissue fatty acid composition. Therefore, diet remains the key factor in determining the supply of PUFA with human milk.

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# Isomeric Fatty Acids: Evaluating Status and Implications for Maternal and Child Health

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**ABSTRACT:** "Isomeric fatty acids" is a term that refers to the *trans*- and positional isomers formed during hydrogenation of naturally occurring oils. The purposes of this paper are as follows: (i) to summarize potential exposure of infants to isomeric fatty acids by reviewing estimates of isomeric fatty acids in the maternal diet, in human milk, and in infant formula/infant foods, and (ii) to evaluate the evidence for adverse effects of isomeric fatty acids on infant development with respect to growth and essential fatty acid status. Estimates of the intake of *trans*-fatty acids vary widely both within and across populations. Current estimates of *trans*-fatty acids in the North American population are 4–11% of total fatty acids or 3–13 g/(person·d), whereas in Mediterranean countries in which olive oil is the primary fat and in Far Eastern countries in which little commercially hydrogenated fat is consumed, per capita consumption of *trans*-fatty acids is <1–2 g/d. The *trans*-fatty acid content of human milk reflects the cross-cultural variation in the maternal diet, with *trans*-fatty acids in human milk samples ranging from 6 to 7% in North America to <0.5% in Hong Kong. *Trans*-fatty acids are transferred from the maternal diet through the placenta to the developing fetus or through milk to the breast-fed infant. In some studies, plasma *trans*-fatty acids are inversely related to birth weight and head circumference. The hypothesis that dietary *trans*-fatty acids could inhibit biosynthesis of long-chain polyunsaturated fatty acids with 20 and 22 carbon atoms and thus affect infant development is supported by studies demonstrating an inverse correlation of plasma *trans*-fatty acids with n-3 and n-6 long-chain polyunsaturated fatty acids in infants. However, no such relationship has been observed in human milk. A definitive answer concerning a potentially adverse effect of dietary *trans*-fatty acids on infant development awaits future studies.

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The polyunsaturated fatty acids (PUFA) found naturally in vegetable oils are modified in structure during the hydrogenation process used in the manufacture of margarine, shortening, and other commercial fats. Similarly, dairy products and meats from ruminant animals contain fat that has been modified by biohydrogenation in the rumen. The term "isomeric" in reference to dietary fat generally refers to those fatty acids in which the double bonds originally present are modified in either conformation (*cis*-/*trans*-) or position by partial hydrogenation. In both the scientific literature and the popular press, however, these fatty acids often are termed "*trans*-fatty acids" with no distinction being made between geometric and positional isomers.

The predominant source of dietary isomeric fatty acids in most Western countries is commercial hydrogenation of vegetable and marine oils (1,2). Although the amount of these fatty acids in products made from commercially hydrogenated fat is highly variable, frying fats can contain up to 40–50% of total fatty acids as *trans*-fatty acids (2). In comparison to commercially hydrogenated fats, meat and dairy products typically contain 1–8% of total fatty acids as *trans*-fatty acids and therefore contribute a smaller proportion of isomeric fatty acids to the Western diet.

Possible detrimental effects of isomeric fatty acids include an unfavorable lipid profile, promotion of cardiovascular disease in adults (3,4), and inhibition of essential fatty acid (EFA) metabolism, influencing infant development (5,6). The purposes of this paper are as follows: (i) to summarize potential exposure of the infant to isomeric fatty acids by reviewing estimates of isomeric fatty acids in the maternal diet, in human milk, and in infant formula/infant foods; and (ii) to evaluate data addressing the effects of isomeric fatty acids on infant development with respect to influences on growth and EFA status.

## EXPOSURE OF THE INFANT TO ISOMERIC FATTY ACIDS

*Estimates of isomeric fatty acids in the maternal diet.* Several methods have been used to estimate isomeric fatty acid intake. These methods include estimates based on "food disappearance" or market share data, analysis of dietary consumption data of a representative population, laboratory analysis of duplicate portion or composite diets, and estimates based on the *trans*-fatty acid content of biological tissues. The advantages and disadvantages of each of these methods have been reviewed (1,2). Briefly, many of the estimates based on food disappearance or market share data do not account for waste and therefore tend to be somewhat larger than estimates obtained by other methods. Analysis of dietary consumption data of a representative population is subject to the errors and uncertainties inherent in performing diet recalls and records, as well as the extreme variability in the isomeric fatty acid content of food items within a single category in composition tables (7). Laboratory analysis of duplicate portion or composite diets can yield accurate values, but the values obtained by this method may not be representative of the population as a whole. Both adipose tissue and human milk samples have been

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EFA, essential fatty acid; PUFA, polyunsaturated fatty acids.

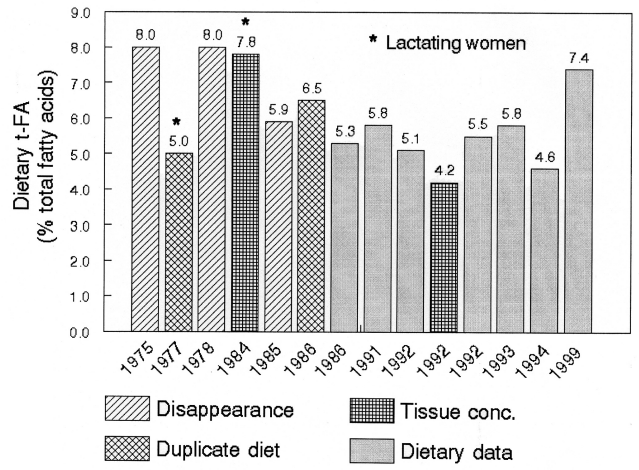


**FIG. 1.** *Trans*-fatty acid (*t*-FA) intake (% of total fatty acids) in the United States classified by method used to obtain the data. In some studies, values are reported as dietary 18:1*t*; in others, values represent total *trans*-fatty acids. References are as follows: 1975 (71); 1977 (9); 1978 (72); 1984 (8); 1985 (73); 1986 (74,75); 1991 (76); 1992 (77,77,78); 1993 (79); 1994 (80); 1999 (81).

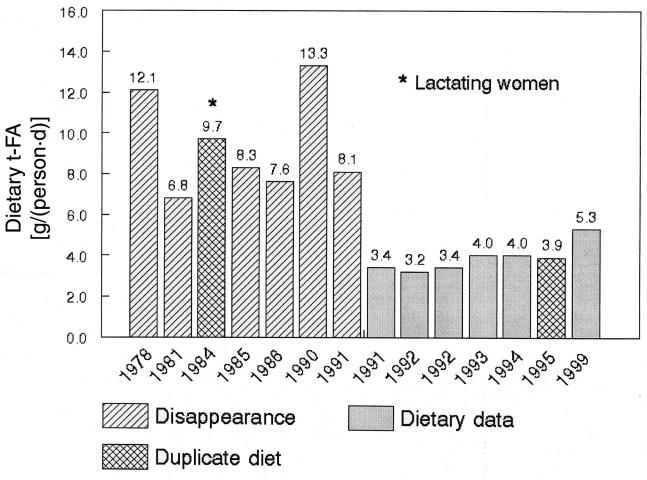


**FIG. 2.** *Trans*-fatty acid (*t*-FA) intake [g/(person·d)] in the United States classified by method used to obtain the data. In some studies, values are reported as dietary 18:1*t*; in others, values represent total *trans*-fatty acids. References are as follows: 1978 (72); 1981 (82); 1984 (8); 1985 (73); 1986 (83); 1990 (84); 1991 (76,85); 1992 (77,78); 1993 (79); 1994 (80); 1995 (86); 1999 (81).

used to estimate isomeric fatty acid consumption; however, this methodology is dependent upon equations expressing the relationship between isomeric fatty acids in the diet and the amount deposited in adipose tissue or secreted in human milk. The efficiency of this "transfer" can be influenced by many factors, most notably, energy intake. Nevertheless, estimates of dietary isomeric fatty acids obtained by all methods taken collectively can give us a measure of the relative values in the maternal diet across populations and thus an idea of the potential exposure to the unborn or breast-fed infant.

Isomeric fatty acid consumption appears to be greatest in North America compared with other parts of the world. In the United States, estimates of *trans*-fatty acid intake range from 4.2 to 8.0% of total fatty acids (Fig. 1) or from 3.2 to 13.3 g/(person·d) (Fig. 2), depending on the year of the study and the methodology used. Isomeric fatty acid consumption specific for lactating women was estimated to be 5.0–7.8% of total fatty acids (8,9). "Consensus" estimates by ILSI expert panels or an ASCN/AIN Task Force are summarized in Table 1. Consumption of *trans*-fatty acids in Canada appears to be just as great, if not greater, compared with the United States, with values ranging from 6.9 to 11.1 g/(person·d) (Fig. 3). For Canadian lactating women, estimates range from 6.9 to 10.6 g/(person·d) (10,11). It is worthy of note that the estimates by Chen *et al.* (10) were based on data obtained using 100-m capillary columns and included a number of minor isomers not generally reported in earlier estimates. According to Innis and King (11), the major sources of *trans*-fatty acids in the diet of Canadian lactating women were bakery products and breads (32%), snacks (14%), fast foods (11%), and margarines and shortenings (11%).

In the United Kingdom (Fig. 4), values reported for *trans*-fatty acid intake are somewhat less than those for North America. For the most part, estimates in the United Kingdom generally range from ~3 to >7 g/(person·d). The estimate of 12.0 g/(person·d) (12) was based on disappearance data and is notably larger than the other values.

*Trans*-fatty acid consumption in European countries is summarized in Figure 5. The southern European and Mediterranean countries, such as Spain, Portugal and Italy, have consumption values less than those in northern European countries. This is probably due to the fact that olive oil, which contains very small amounts of isomeric fatty acids compared with hydrogenated fats, is the major dietary fat used in these

**TABLE 1**
**"Consensus" Estimates of Dietary *trans*-Fatty Acids in the United States**

| | % of total energy | % of fatty acids | g/d |
|---|---|---|---|
| ILSI Expert Panel on CHD[a] | 2–4 | 4–12 | |
| ASCN/AIN Task Force[b] | 2–4 | | 8.1–12.8 |
| ILSI Expert Panel on Development[c] | | 8 | 6.4 |

[a]International Life Sciences Institute Expert Panel on *trans* Fatty Acids and Coronary Heart Disease (3).
[b]American Society for Clinical Nutrition/American Institute of Nutrition [now named American Society for Nutritional Sciences (ASNS)] Task Force (4).
[c]ILSI Expert Panel on *trans* Fatty Acids and Early Development (5).

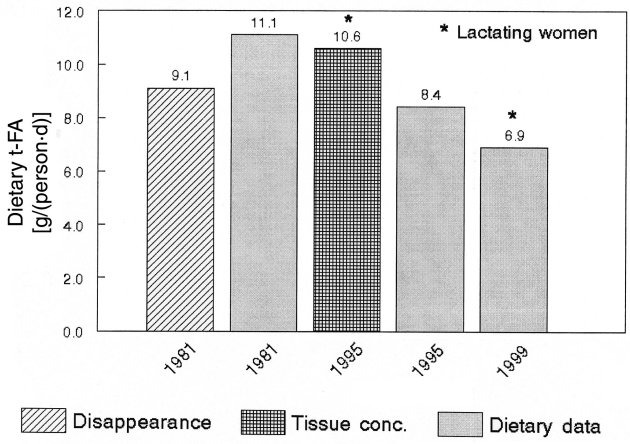


**FIG. 3.** *Trans*-fatty acid (*t*-FA) intake [g/(person·d)] in Canada classified by method used to obtain the data. In some studies, values are reported as dietary 18:1*t*; in others, values represent total *trans*-fatty acids. References are as follows: 1981 (87,87); 1995 (10,88); 1999 (11).

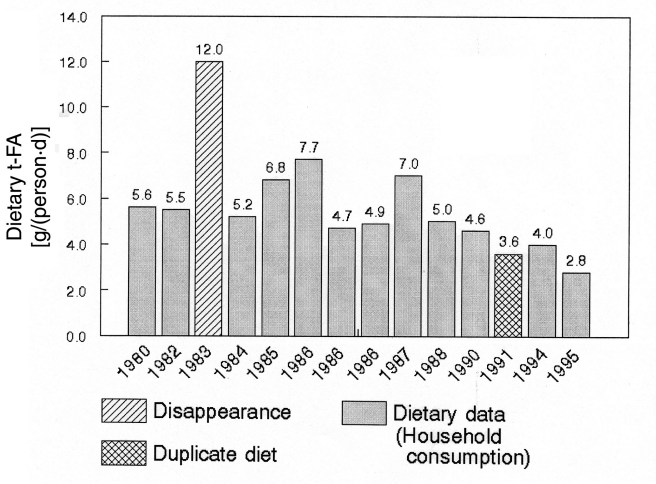


**FIG. 4.** *Trans*-fatty acid (*t*-FA) intake [g/(person·d)] in the United Kingdom classified by method used to obtain the data. In some studies, values are reported as dietary 18:1*t*; in others, values represent total *trans*-fatty acids. References are as follows: 1980 (89); 1982 (89); 1983 (12); 1984 (89); 1985 (90); 1986 (89,91); 1987 (92); 1988 (89); 1990 (89); 1991 (89); 1994 (89); 1995 (13).

countries. The values illustrated toward the front of the graph in Figure 5 are, for the most part, the recent values obtained in the TRANSFAIR study (13). In comparing these values with earlier estimates illustrated toward the back of the graph, it appears that consumption of *trans*-fatty acids in many European countries has decreased. This trend is particularly evident for the Netherlands in which estimates of the consumption of *trans*-fatty acids have decreased from 17 to 4.3 g/(person·d). Thus, exposure of European infants to *trans*-fatty acids appears to be only moderate at the present time. This same trend toward a somewhat lower consumption of *trans*-fatty acids is also evident in all of the Nordic countries except Iceland (Fig. 6).

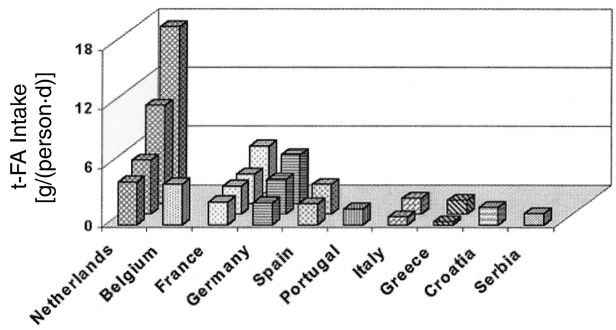


**FIG. 5.** *Trans*-fatty acid (*t*-FA) consumption [g/(person·d)] in European countries. In some studies, values are reported as dietary 18:1*t*; in others, values represent total *trans*-fatty acids. References are as follows: Netherlands (13,86,93,94); Belgium (13); France (13,24,95,96); Germany (13,97–100); Spain (13,25); Portugal (13); Italy (13,86); Greece (13,86); Croatia (86); Serbia (86).

Australia's consumption of *trans*-fatty acids is traditionally less than that of North American and some northern European countries (Fig. 7). This is probably due to the greater availability and use of tropical oils in spreads.

In Korea and Japan, isomeric fatty acid consumption is <1–2 g/(person·d) (Fig. 7). Thus, if the people of these countries continue to consume traditional diets, which contain very little hydrogenated fat, exposure of infants in these countries to isomeric fatty acids would not be a problem.

In summary, there is wide variation among various countries in the amount of isomeric fatty acids consumed. Recent changes in the formulation of fats in the food supply in some European countries have resulted in lower consumption of isomeric fatty acids. At the present time, consumption of isomeric fatty acids due to products containing commercially hy-

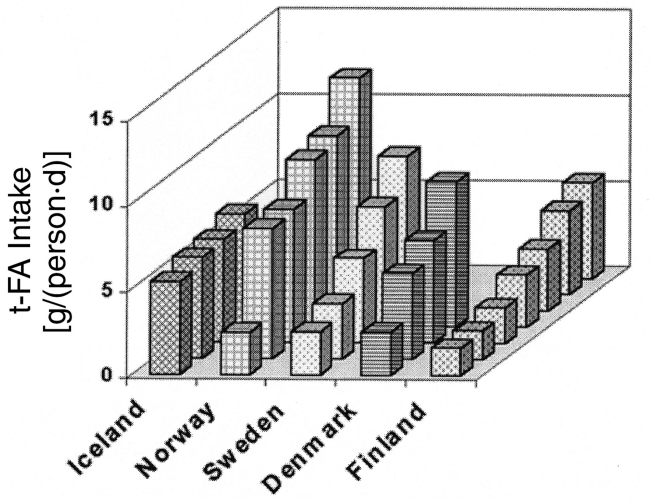


**FIG. 6.** *Trans*-fatty acid (*t*-FA) consumption [g/(person·d)] in Nordic countries. In some studies, values are reported as dietary 18:1*t*; in others, values represent total *trans*-fatty acids. References are as follows: Iceland (13,101,102); Norway (13,103); Sweden (13,101,104–106); Denmark (13,107); Finland (13,108–111).

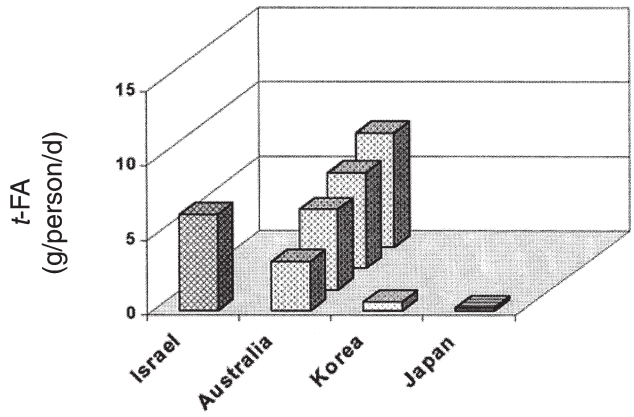


**FIG. 7.** *Trans*-fatty acid (*t*-FA) consumption [g/(person·d)] in other countries. In some studies, values are reported as dietary 18:1*t*; in others, values represent total *trans*-fatty acids. References are as follows: Israel (112); Australia (95,113,114); Korea (115); Japan (86).

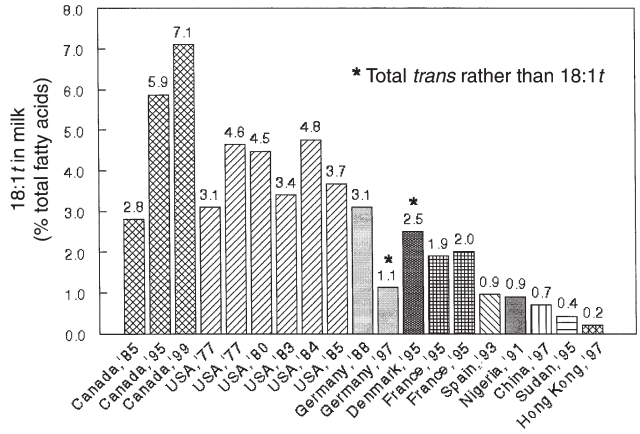


**FIG. 8.** *Trans*-fatty acid (*t*-FA) content of human milk samples from various countries. References are as follows: Canada, 1985 (15); Canada, 1995 (10); Canada, 1999 (11); United States, 1977 (9,16); United States, 1980 (17); United States, 1983 (18); United States, 1984 (8); United States, 1985 (19); Germany, 1988 (20); Germany, 1997 (21); Denmark, 1995 (22); France, 1995 (23,24); Spain, 1993 (25); Nigeria, 1991 (26); China, 1997 (27); Sudan, 1995 (28); Hong Kong, 1997 (27).

drogenated fat appears to be greatest in North America. *Trans*-fatty acids from the maternal diet can cross the placenta (14) or be secreted in human milk (8,9). Thus, developing fetuses and nursing infants in the United States and Canada would be subject to greater exposure to these fatty acids than would infants in other parts of the world.

*Estimates of isomeric fatty acids in human milk*. Isomeric fatty acids are transferred rapidly from the maternal diet to human milk (8,9). The *trans*-fatty acid content of human milk reflects the cross-cultural variation in the maternal diet (Fig. 8). Human milk samples collected from Canada (10,11,15) and the United States (8,9,16–19) contain greater amounts of *trans*-isomers of octadecenoic acid (18:1*t*) than do milk samples from lactating women in Germany (20,21), Denmark (22), and France (23,24). As expected, human milk samples from Spain (25), Nigeria (26), China (27), Sudan (28), and Hong Kong (27) have low amounts of 18:1*t*, reflecting the lack of commercially hydrogenated fat in the maternal diet in these countries. Thus, *trans*-fatty acids in human milk samples range from an average of 6–7% of total fatty acids in North America to <0.5% in Hong Kong.

Chen *et al*. (10) published one of the most complete analyses of the isomeric fatty acid content of human milk. Using a combination of capillary gas chromatography and silver nitrate thin-layer chromatography, these investigators analyzed the geometric and positional isomer distribution of 198 human milk samples collected in 1992 from nine provinces of Canada. Although 18:1*t* was the most prevalent *trans*-isomer group with a mean of 5.87 wt% of total fatty acids, smaller amounts of other *trans*-fatty acids were found. These included 18:2Δ9*c*,13*t*/8*t*,12*c* at 0.36%; 18:2Δ9*c*,12*t* at 0.29%; 18:2Δ9*t*,12*c* at 0.24%; 16:1Δ9*t* at 0.18%; total 18:3*t* at 0.11%; 14:1Δ7*t* at 0.09%; and 18:2Δ9*t*,12*t* at 0.05%. In addition, small amounts (<0.2%) of unusual positional *cis*-isomers were identified.

*Estimates of isomeric fatty acids in infant formula/infant foods.* Small amounts of isomeric fatty acids can be found in infant formula as a result of processing. Published values for the *trans*-fatty acid content of infant formula are given in Table 2. In the United States and Canada (16,29–32), samples of infant formula analyzed contained 0.1–3.1% of total fatty acids, except for one formula analyzed by Hanson and Kinsella (29) which contained 15.7%. In Europe, the content of *trans*-fatty acids in infant formula ranged from 0.2 to 5.0% of total fatty acids (22,33–36).

Infant foods can also contain *trans*-fatty acids (Table 3). In earlier reports (29,37,38), the range of *trans*-fatty acids in baby foods was given as 0.2–7.6%. Holub (39), however, recently reported that Canadian baby biscuits contain up to 37% of fatty acids as *trans*-isomers and that Canadian infant cereals can contain up to 23% *trans*-fatty acids. It is important to note that one must take into account the total fat content and usual consumption of these products before "exposure" to *trans*-fatty acids can be calculated.

*Isomeric fatty acid status based on analysis of blood samples.* Isomeric fatty acids in the diet are reflected in serum/plasma fatty acid profiles (40–43). The concentrations of *trans*-fatty acids have been determined in the plasma of pregnant women, lactating women, newborn infants, and children. Koletzko and Müller (14) were among the first to assess the isomeric fatty acid status of pregnant women. These investigators found that maternal plasma collected from 30 German women at term delivery (38–42 postmenstrual weeks) contained 1.99 ± 0.14% of total fatty acids as *trans*-fatty acids. The major component of this was 18:1*t* at 1.21 ± 0.10%, with smaller amounts of the *trans*-isomers of 18:2 (0.37 ± 0.07%), 16:1*t* (0.35 ± 0.04%) and 14:1*t* (0.06 ± 0.01%). In a more recent study (44), the *trans*-fatty acids in maternal plasma of 41 German women were 0.83% by weight of total fatty acids in triacylglycerols, 0.42% in the phospholipids, 0.28% in the cholesterol esters, and 1.19% in the non-esterified fatty acid fraction. These values are much less than

**TABLE 2**
***Trans*-Fatty Acid Content of Infant Formulas in North America and Europe**

| | | *trans*-Fatty acids | |
|---|---|---|---|
| | Samples (*n*) | Average (%) | Range |
| United States | | | |
| Ref. 16 | 3 | 0.5 | (0.1–1.3) |
| Ref. 29 | 11 | 1.2 | (0.8–2.0) |
| | 1 | 15.7 | |
| Ref. 31 | 10 | 0.8 | (0.2–1.3) |
| Ref. 30 | 1 | 1.9 | |
| Canada | | | |
| Ref. 32 | | | |
| Liquid formula | 12 | 1.9 | (0.9–3.1) |
| Powdered formula | 14 | 1.4 | (0.6–2.5) |
| Spain | | | |
| Ref. 36 | 28 | 2.42 | (0.2–4.5) |
| Ref. 34 | 20 | 2.3 | — |
| Germany | | | |
| Ref. 35 | 21 | <2.0 | (0.2–1.8) |
| (28 total samples) | 7 | 2.0 | (2.4–4.6) |
| Denmark | | | |
| Ref. 22 | | | |
| Standard formula | 6 | 2.7 | (1.4–4.2) |
| Special formula | 7 | 1.5 | (0.3–3.8) |
| France | | | |
| Ref. 33 | | | |
| Premature formula | 2 | 1.9 | (1.3–2.5) |
| 0–5 mon formula | 9 | — | (0.4–3.1) |
| 6–10 mon formula | 8 | — | (0.4–5.0) |
| <10 mon formula | 1 | 3.0 | |

**TABLE 3**
***Trans*-Fatty Acid Content of Infant Foods in North America**

| | | *trans*-Fatty acids | |
|---|---|---|---|
| | Samples (*n*) | Average(%) | Range |
| Ref. 37, U.S. baby food | 3 | 3.2 | (0.2–7.6) |
| Ref. 29, U.S. dry infant cereal | 8 | 0.5 | (0.3–0.6) |
| Ref. 38, Strained vegetable and beef | 2 | 5.1 | (4.7–5.6) |
| Ref. 39, Canadian baby biscuits | | Up to 37% | |
| Canadian infant cereals | | Up to 23% | |

those in Canadian women studied by Elias and Innis (45) who reported the concentrations of *trans*-fatty acids in maternal plasma lipid fractions of 58 Canadian women at 35 wk of gestation as 3.99 ± 0.21% of total fatty acids with a range of 1.26–7.90% in the triacylglycerol fraction, 2.37 ± 0.10% (1.12–4.47) in the phospholipid fraction, and 1.57 ± 0.10% (0.64–3.70) in the cholesteryl ester fraction. The concentrations of *trans*-fatty acids in all three plasma lipid fractions were significantly related to dietary intake of *trans*-fatty acids adjusted for total energy intake ($r$ = 0.33–0.39).

*Trans*-fatty acids in the maternal plasma of women in the United States have also been reported. In a study of 43 pregnant women in Memphis, Tennessee, Carlson and colleagues (46) demonstrated that intake of *trans*-fatty acids correlated with maternal phospholipid 18:1*t*. The women in this study had relatively high plasma *trans*-fatty acid values, i.e., 18:1*t* was 2.57 ± 1.7% (1.0–7.8) in maternal plasma triglyceride and 1.44 ± 0.50% (0.4–2.6) in maternal plasma phospholipid. As a percentage of *cis*-18:1n-9, 18:1*t* was 8% in maternal plasma triglyceride and 14.2% in the maternal plasma phospholipid. As a percentage of all-*cis*-18:2, the *trans*-isomers (*t*/*t*, *t*/*c*, and *c*/*t*) of 18:2 were 5.4% in maternal plasma triglyceride and 1.7% in maternal plasma phospholipid. Carlson and colleagues (47) also studied 50 pregnant women in Kansas City, Missouri, and found that maternal plasma triglyceride contained 3.81% of total fatty acids as 18:1*t*, a value even greater than that in the Memphis women. In addition, Lammi-Keefe and Makhoul (48; personal communication) analyzed blood collected from pregnant Connecticut women at 24–28 wk gestation ($n$ = 10), 32–35 wk gestation ($n$ = 16), and 36–39 wk gestation ($n$ = 14) and reported that total *trans*-fatty acids were 1.73 ± 0.14, 1.61 ± 0.13, and 1.88 ± 0.13%, respectively, as gestation progressed. Thus, the amount of *trans*-fatty acids in the plasma of women in the United States is roughly comparable to that in Canada but greater than that in German women.

Maternal dietary *trans*-fatty acids can be transferred to the developing fetus (14), and umbilical cord plasma can be used to assess the isomeric fatty acid status of the infant at birth. The amount of *trans*-fatty acids in infant phospholipids correlates with that in maternal phospholipids (45,46). *Trans*-fatty acids were found in umbilical cord plasma at roughly the same percentages as those in maternal plasma by Koletzko and Müller (14). These investigators reported 1.66 ± 0.14 wt% of total fatty acids as *trans*-fatty acids in infant plasma compared with 1.99 ± 0.14 wt% in maternal plasma for 30 German newborn term infants and their mothers. In later studies (44–46), infant plasma *trans*-fatty acids were significantly less in triacylglycerol and phospholipid fractions compared with values in maternal plasma. In contrast, the cholesterol ester fraction of the infant plasma contained a greater concentration of *trans*-fatty acids than did the maternal plasma (44,45).

For Canadian infants ($n$ = 70) (45), values for total *trans*-fatty acids in lipid fractions of umbilical cord plasma ($n$ = 70) were triacylglycerol, 2.83 ± 0.19 (0.63–12.79); phospholipid, 0.67 ± 0.03 (0.11–1.33); and cholesterol ester, 2.04 ± 0.01 (0.86–4.24). A somewhat similar distribution of *trans*-fatty acids in plasma lipid classes was observed by Koletzko (49) who studied 29 preterm German infants on day 4 of life. Total *trans*-isomers were 1.62 ± 0.10 wt% in the triacylglycerols, 1.08 ± 0.07% in phospholipids, and 1.95 ± 0.17% in sterol esters. In a more recent study, Koletzko and colleagues (44) reported values for *trans*-fatty acids in infant plasma that were quantitatively lower, but similar in distribution with respect to lipid class, to the earlier values. For the 41 German term infants in this study, total *trans*-fatty acids in cord plasma were 0.45% in triacylglycerol, 0.21% in phospholipid, and 0.78% in cholesterol esters. The lower *trans*-fatty acid content in the plasma of German infants reported in 1998 compared with 1992 probably reflects a decrease in the overall *trans*-fatty acid content of the German food supply during this time. In the work of Carlson and colleagues (46), 18:1*t* in cord blood was 1.15 ± 0.50% (range, 1.0–7.8%) in triacylglycerols and 0.52 ± 0.25% (range, 0.1–1.2%) in phospholipids. In general, *trans*-

fatty acids appear to be incorporated into the triacylglycerol fraction to a greater extent than into the phospholipid fraction.

If one examines the role of *trans*-fatty acids as inhibitors of EFA metabolism, it is important to express the concentration of *trans*-isomers as percentages of their corresponding *cis*-fatty acids. Carlson and colleagues (46) reported that 18:1*t* isomers, expressed as a percentage of 18:1*c*, were 4 and 7.1% in triacylglycerol and phospholipid, respectively, in cord blood. The *trans*-isomers of 18:2, relative to all-*cis*-18:2, were 6.8% in triacylglycerol and 4.0% in phospholipid. These values were greater in cord blood than in maternal blood, and the investigators suggested that the transfer of *trans*-isomers of 18:2 from mother to fetus was restricted less than the transfer of linoleic acid.

Breast-fed infants receive isomeric fatty acids proportional to these fatty acids in the milk, which reflects the maternal diet (8). Innis and King (11) measured *trans*-fatty acids in the plasma lipids of 62 exclusively breast-fed Canadian infants at 2 mon of age. *Trans*-fatty acids in the triacylglycerol fraction were 6.5 ± 0.33% with a range of 1.9–15.6% and in the plasma phospholipid fraction, 3.7 ± 0.16% of total fatty acids with values ranging from 1.7 to 8.3%. *Trans*-fatty acids in plasma triacylglycerols and phospholipids of the breast-fed infants were significantly correlated ($r = 0.82$ and 0.67, respectively) with *trans*-fatty acids in the milk.

*Trans*-fatty acids in plasma triacylglycerol and phospholipid were found to be less in infants than in young adults (50). Specifically, the *trans*-fatty acid content of plasma at birth in German infants was 1.91% of total fatty acids in triacylglycerols and 0.69% in phospholipids compared with 2.25 and 1.50%, respectively, in young adults (20–26 yr of age). This relationship did not hold, however, for the cholesterol ester fraction. Collective values for German children, aged 1–15 yr, were 1.78, 2.72, and 1.66% for total *trans*-fatty acids in phospholipids, triacylglycerol, and cholesterol esters, respectively. These values were reported in 1994, and on the basis of current estimates of *trans*-fatty acid intake in Germany (13), values would be expected to be lower at the present time.

## EFFECTS OF ISOMERIC FATTY ACIDS ON INFANT DEVELOPMENT

It is clear that in many parts of the world, significant amounts of isomeric fatty acids are being consumed by pregnant and lactating women and that these fatty acids are transferred from the mother to the developing fetus and the nursing infant. The physiologic implications for infants exposed to isomeric fatty acids, however, are not as clear.

Arachidonic acid (AA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3) are important in perinatal growth and neural development (51), and the formation of these long-chain EFA from their shorter-chain precursors is believed to be inhibited by *trans*-fatty acids (52–54). Investigations on the effects of *trans*-fatty acids in development have focused on two general areas, i.e., growth and EFA status. However, because AA status may influence infant growth (55,56), these two areas are not distinct.

*Isomeric fatty acids and growth*. The relationship between *trans*-fatty acids and pregnancy outcome has been investigated by several groups. Koletzko (35,49,57,58) reported that *trans*-fatty acids in the plasma of 29 preterm infants on day 4 of life correlated negatively with birth weight. This relationship was found between birth weight and 18:1*t* in sterol esters ($r = \pm 0.50$; $P < 0.01$), total *trans*-fatty acids in sterol esters ($r = \pm 0.40$; $P < 0.05$), and total *trans*-fatty acids in phospholipids ($r = \pm 0.42$; $P < 0.01$). Similar results were found by von Houwelingen and Hornstra (59), who reported that 18:1*t* in umbilical arterial vessel walls correlated negatively with birth weight ($r = \pm 0.35$; $P = 0.033$). In addition, these investigators found a negative correlation with head circumference ($r = \pm 0.37$; $P = 0.028$). This negative relationship between head circumference and *trans*-fatty acids is consistent with recent preliminary results reported by Lammi-Keefe and Makhoul (48; personal communication). A negative correlation was observed at 32–35 wk gestation between *trans*-isomers of 18:2 in maternal plasma and head circumference of infants ($r = \pm 0.52$; $P = 0.07$). Similarly, Elias and Innis (45) reported that the concentration of *trans*-fatty acids in the cholesterol ester fraction of infant plasma was inversely related to gestational length ($r = \pm 0.33$; $P = 0.006$). Thus, a number of investigators have reported inverse correlations between *trans*-fatty acids and various measures of pregnancy outcome.

The data on the relationship between *trans*-fatty acids and pregnancy outcome must be interpreted with some caution. Koletzko (49) did not find significant negative correlations between birth weight and some fractions of plasma, such as 18:1*t* in triacylglycerols or phospholipids. Moreover, Elias and Innis (45) did not find the relationship between *trans*-fatty acids in various fractions of the umbilical cord plasma lipids and length of gestation, birth weight, and birth length to be significant, except for *trans*-fatty acids in the cholesteryl ester fraction and gestational length. Lammi-Keefe and Makhoul (48; personal communication) did not report a significant negative relationship between *trans*-fatty acids and either birth weight or length at birth. Thus, definitive conclusions on the relationship between *trans*-fatty acids and fetal growth await future investigation.

*Isomeric fatty acids and EFA status*. EFA of both the n-6 and n-3 families play important roles in the perinatal period (51). AA is important as a component of membrane phospholipids, as a precursor for eicosanoids, and as a stimulant of growth and cell division. It also plays a role in second messenger and cell signaling pathways. During perinatal development, DHA accumulates in retinal and neural membrane phospholipids; as such, it is involved in visual and central nervous system function. Both AA and DHA are synthesized from their respective 18-carbon precursors, linoleic acid (18:2n-6) and linolenic acid (18:3n-3) by a series of desaturation and elongation enzymes.

The hypothesis that dietary *trans*-fatty acids could inhibit biosynthesis of long-chain PUFA with 20 and 22 carbon atoms and thus affect infant development is based on studies indicating that *trans*-fatty acids inhibit Δ6-desaturase, and perhaps

Δ5-desaturase activity in animals (60), in *in vitro* systems (52,53,61,62), or in cultured cells (54,63,64). This hypothesis is supported by the work of Koletzko (35,49,57,58) who demonstrated an inverse correlation of plasma *trans*-octadecaenoic acid and total *trans*-fatty acids with n-6 and n-3 long-chain PUFA in 29 preterm German infants at day 4 of life. Specifically, total *trans*-fatty acids in the sterol ester fraction of plasma were negatively correlated with AA and in all three lipid fractions (triglycerides, sterol esters, and phospholipid) with total n-6 long-chain PUFA. In addition, 18:1*t* demonstrated a negative relationship with AA in the sterol ester fraction and with total n-6 long-chain PUFA in triacylglycerols and sterol esters. With respect to n-3 fatty acids, negative correlations were observed for DHA with 18:1*t* and total *trans*-fatty acids in plasma triacylglycerols and sterol esters; total n-3 long-chain PUFA were negatively correlated with 18:1*t* in the triacylglycerol fraction. In addition, total long-chain PUFA (n-6 + n-3) and product/substrate ratios for both n-6 and n-3 long-chain PUFA were negatively correlated with total *trans*-fatty acids and/or 18:1*t* in plasma triacylglycerols and sterol esters. These data were interpreted as indicating a potential impairment of EFA metabolism by *trans*-fatty acids. The safety of high dietary *trans*-fatty acid intake during pregnancy and in the perinatal period was thus questioned.

A similar relationship was demonstrated in 53 German children, aged 1–15 yr, in that plasma phospholipid *trans*-fatty acids were significantly inversely related to AA, total n-6 PUFA, and the AA/linoleic acid ratio (65). Comparable significant relationships for the n-3 fatty acids were not observed in these children.

The recent data of Elias and Innis (45) support, in part, the earlier work of Koletzko (49). Inverse correlations between infant plasma *trans*-fatty acids and DHA concentrations in triacylglycerols and between *trans*-fatty acids and AA concentrations in cholesterol esters were observed in the plasma lipid fractions of 70 North American infants (45).

Similarly, in 50 mother/infant pairs living in Memphis, TN, Carlson and co-workers (66) reported inverse correlations between *trans*-fatty acids in cord venous plasma triacylglycerol and DHA in cord venous phospholipids. Also, positional *cis*-isomers of 18:1 (n-5 to n-7) in cord venous plasma triacylglycerols were inversely correlated with DHA and AA in cord venous phospholipids. In more recent work with 50 mother/infant pairs living in Kansas City, MO, Carlson's group (47) supported the hypothesis that *trans*-fatty acids could compromise the EFA status of infants. These investigators reported that the artery wall was the most robust indicator of an inverse relationship of *trans*-fatty acids with DHA and AA. Significant inverse correlations were found in the umbilical artery wall phospholipids for 16:1*t*, positional *cis*-isomers of 18:1, and 18:2*t*,*c* with both DHA and AA. Additionally, the phospholipids of umbilical vein walls, maternal plasma, cord venous plasma, and cord arterial plasma had significant inverse relationships between AA or DHA and one or more of the isomeric fatty acids mentioned above (16:1*t*, positional *cis*-isomers of 18:1, and 18:2*t*,*c*).

Lammi-Keefe and Makhoul in a preliminary report (48; personal communication) presented further evidence that *trans*-fatty acids may lead to compromised long-chain PUFA status during pregnancy. In a study of 16 Connecticut pregnant women, significant inverse associations between maternal plasma *trans*-fatty acids and n-3 and n-6 long-chain PUFA were demonstrated. For example, *trans*-isomers of 18:2 were inversely correlated with AA and total n-6 long-chain PUFA. Moreover, these investigators found that *trans*-fatty acids were significant covariates for long-chain PUFA during the course of pregnancy.

Further support for inhibition of EFA metabolism during pregnancy by dietary *trans*-fatty acids has been provided by a well-controlled animal study (67) in which rats were fed three levels of *trans*-fatty acids (0, 15, and 30% of total fatty acids) with linoleic acid and α-linolenic acid held constant. These investigators concluded that high intakes of *trans*-fatty acids partially inhibited Δ-6 desaturase in pregnant rats, which may explain, in part, the low concentrations of DHA that they found in tissues of pregnant and fetal rats. They reported, however, that the fatty acid composition of both fetal and pregnant rat brain remained mainly unaffected by dietary *trans*-fatty acids.

Although there is some evidence of a negative relationship between *trans*-fatty acids and long-chain PUFA in plasma, similar negative correlations have not been identified for human milk. Studies by Innis and King (11) did not support the hypothesis that long-chain PUFA in human milk would be suppressed by *trans*-fatty acids in the diet of lactating women. These investigators found that milk *trans*-fatty acids were not inversely related to AA or DHA in milk or infant plasma. In fact, these investigators reported that milk *trans*-fatty acids were inversely related to milk linoleic acid and linolenic acid, but not to their elongated products, AA and DHA. The earlier work of Chen *et al*. (10) was in agreement with that of Innis and King (11) in that they also observed a negative correlation between milk *trans*-fatty acids and 18:2n-6 and 18:3n-3, but not with n-6 or n-3 elongation products. As a possible explanation for these results, Innis and King (11) hypothesized that diets high in *trans*-fatty acids would also be low in all-*cis* n-6 and n-3 fatty acids because hydrogenation reduces the content of the naturally occurring all-*cis* isomers. Moreover, a recent study in rats (68) did not show a relationship between high intakes of *trans*-fatty acids in the maternal diet and long-chain PUFA in rat milk.

In summary, considerable evidence based on relationships among plasma fatty acids exists supporting the hypothesis that *trans*-fatty acids can inhibit EFA metabolism, thereby compromising the AA and DHA status of the developing infant. It must be emphasized that this evidence is correlational in nature and that the physiologic implications of this inhibition are largely unknown.

Only a few investigators have studied the physiologic implications of *trans*-fatty acids on development in humans consuming *trans*-fatty acids within the "normal" range. In a crossover experiment in which lactating women consumed a low *trans*-fatty acid diet and a high *trans*-fatty acid diet made

from commercially available fats, Craig-Schmidt *et al*. (8) found no effect of *trans*-fatty acids on human milk prostaglandins. Although *trans*-isomers of 18:3 can be elongated into *trans*-isomers of long-chain n-3 PUFA and incorporated into rat brain and retina (69), the physiologic implications of the incorporation of such *trans*-isomers into human tissues is not known. Similarly, elongated and further desaturated products of isomeric forms of oleic and linoleic acids have been identified in rats fed partially hydrogenated canola oil (70). Again, the importance of these compounds during perinatal development is unknown. If *trans*-fatty acids in the North American food supply remain greater than that in other parts of the world, the physiologic effects of increased dietary *trans*-fatty acids on the developing infant should be addressed in tightly controlled human studies conducted within the framework of currently available commercial sources of dietary isomeric fats.

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# Polyunsaturated Fatty Acids, Inflammation, and Immunity

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**ABSTRACT:** The fatty acid composition of inflammatory and immune cells is sensitive to change according to the fatty acid composition of the diet. In particular, the proportion of different types of polyunsaturated fatty acids (PUFA) in these cells is readily changed, and this provides a link between dietary PUFA intake, inflammation, and immunity. The n-6 PUFA arachidonic acid (AA) is the precursor of prostaglandins, leukotrienes, and related compounds, which have important roles in inflammation and in the regulation of immunity. Fish oil contains the n-3 PUFA eicosapentaenoic acid (EPA). Feeding fish oil results in partial replacement of AA in cell membranes by EPA. This leads to decreased production of AA-derived mediators. In addition, EPA is a substrate for cyclooxygenase and lipoxygenase and gives rise to mediators that often have different biological actions or potencies than those formed from AA. Animal studies have shown that dietary fish oil results in altered lymphocyte function and in suppressed production of proinflammatory cytokines by macrophages. Supplementation of the diet of healthy human volunteers with fish oil-derived n-3 PUFA results in decreased monocyte and neutrophil chemotaxis and decreased production of proinflammatory cytokines. Fish oil feeding has been shown to ameliorate the symptoms of some animal models of autoimmune disease. Clinical studies have reported that fish oil supplementation has beneficial effects in rheumatoid arthritis, inflammatory bowel disease, and among some asthmatics, supporting the idea that the n-3 PUFA in fish oil are anti-inflammatory and immunomodulatory.

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## THE IMMUNE SYSTEM

The immune system acts to protect the host from infectious agents that exist in the environment (bacteria, viruses, fungi, parasites) and from other noxious insults. The immune system has two functional divisions, i.e., the innate (or natural) immune system and the acquired (also termed specific or adaptive) immune system. Both components of immunity involve various blood-borne factors and cells (Table 1). These cells are generally termed leukocytes (or white blood cells). Leukocytes fall into two broad categories, i.e., phagocytes, including granulocytes (neutrophils, basophils, eosinophils), monocytes and macrophages, and lymphocytes. Lymphocytes are classified as T lymphocytes, B lymphocytes and natural killer (NK) cells. T lymphocytes are further divided into helper T (Th) cells (these are distinguished by the presence of the molecule CD4 on their surface) and cytotoxic T cells (these are distinguished by the presence of CD8 on their surface). All cells of the immune system originate in bone marrow. They are found circulating in the bloodstream, organized into lymphoid organs such as the thymus, spleen, and lymph nodes, or dispersed in other locations around the body.

### Innate and Acquired Immunity

Innate immunity is the first line of defense against infectious agents. It is present before exposure to pathogens and its activity is not enhanced by such exposures. Innate immunity is concerned with preventing entry of infectious agents into the body and, if they do enter, with their rapid elimination. Elimination can occur as follows: (i) by direct destruction of pathogens by complement, by toxic chemicals (e.g., superoxide radicals and hydrogen peroxide) released by phagocytes, or by toxic proteins released by NK cells; and (ii) by engulfment of pathogens by the process of phagocytosis, which is made more efficient by coating the invading pathogen with host proteins such as complement or antibodies, and their subsequent destruction.

Acquired immunity involves the specific recognition of molecules (antigens) on an invading pathogen, which distinguish it as being foreign to the host. The recognition of antigens is by antibodies (produced by B lymphocytes) and by T lymphocytes. However, in contrast to B lymphocytes, T lymphocytes are able to recognize only antigens displayed on cell surfaces. Therefore, infection of a cell by an intracellular pathogen is signaled to T lymphocytes by cell surface expression of peptide fragments derived from the pathogen. These fragments are transported to the surface of the infected cell and expressed there in conjunction with proteins termed major histocompatibility complex (MHC). It is the combination of the pathogen-derived peptide fragment bound to MHC that is recognized by T lymphocytes. There are two classes of MHC, MHC I and MHC II, and the source of the peptide bound to each differs. MHC I binds peptides that originate from pathogen proteins synthesized within the host cell cytosol; typically, these are from viruses or certain bacteria. The peptides bound to MHC II are derived from pathogens that have been phagocytosed by macrophages or endocytosed by antigen-presenting cells (macrophages, dendritic cells, B lymphocytes).

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Abbreviations: AA, arachidonic acid; ALNA, α-linolenic acid; COX, cyclooxygenase; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DTH, delayed-type hypersensitivity; EPA, eicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HLA, human leukocyte antigen; HPETE, hydroperoxyeicosatetraenoic acid; IFN, interferon; Ig, immunoglobulin; IL, interleukin; KLH, keyhole limpet hemocyanin; 5-LOX, 5-lipoxygenase; LT, leukotriene; MHC, major histocompatibility complex; NK, natural killer; PG, prostaglandin; Th, helper T cells; TNF, tumor necrosis factor.

**TABLE 1**
**Components of Innate and Acquired Immunity**

| | Innate immunity | Acquired immunity |
|---|---|---|
| Physicochemical barriers | Skin | Cutaneous and mucosal immune systems |
| | Mucosal membranes | Antibodies in mucosal secretions |
| | Lysozyme | |
| | Stomach acid | |
| | Commensal bacteria | |
| Circulating molecules | Complement | Antibodies |
| Cells | Granulocytes | B lymphocytes |
| | Monocytes/macrophages | T lymphocytes |
| | Natural killer cells | |
| Soluble mediators | Macrophage-derived cytokines | Lymphocyte-derived cytokines |

The MHC-peptide complex is recognized by the T-cell receptor on T lymphocytes. T lymphocytes expressing CD8 recognize MHC I, whereas T lymphocytes expressing CD4 recognize MHC II. Thus, intracellular pathogens stimulate cytotoxic T lymphocytes to destroy the infected cell, whereas extracellular pathogens stimulate a helper T cell–mediated response.

The acquired immune system includes a component of memory, such that if the antigen is encountered again (i.e., there is reinfection), the response is faster and stronger than the initial response. Although the immune system as a whole can recognize tens of thousands of antigens, each lymphocyte can recognize only one antigen; thus the number of lymphocytes specific for a particular antigen must be very low. However, when an antigen is encountered, it binds to the small number of lymphocytes that recognize it and causes them to divide so as to increase the number of cells that are capable of mounting a response to the antigen; this is the process termed lymphocyte expansion or proliferation. B lymphocytes proliferate and mature into antibody-producing cells (plasma cells), and T lymphocytes proliferate and are able to directly destroy virally infected cells (cytotoxic T lymphocytes) or control the activity of other cells involved in the response (helper T cells). The B lymphocyte response to antigen is termed humoral immunity, and the T cell response is termed cell-mediated immunity.

## Communication Within the Immune System

Communication within the acquired immune system and between the innate and acquired systems is brought about by direct cell-to-cell contact involving adhesion molecules and by the production of chemical messengers. Chief among these chemical messengers are proteins called cytokines, which can act to regulate the activity of the cell that produced the cytokine and/or of other cells. Each cytokine can have multiple activities on different cell types. Cytokines act by binding to specific receptors on the cell surface and thereby induce changes in growth, development, or activity of the target cell.

Tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 are among the most important cytokines produced by monocytes and macrophages. These cytokines activate neutrophils, monocytes, and macrophages to initiate bacterial and tumor cell killing, increase adhesion molecule expression on the surface of neutrophils and endothelial cells, stimulate T and B lymphocyte proliferation, upregulate MHC, and initiate the production of other cytokines (Fig. 1). Thus, TNF, IL-1, and IL-6 are mediators of both natural and acquired immunity and are an important link between them. In addition, these cytokines mediate the systemic effects of inflammation such as fever, loss of appetite, mobilization of protein and fat, and acute phase protein synthesis (Fig. 1). Production of appropriate amounts of TNF, IL-1, and IL-6 is clearly beneficial in response to infection, but inappropriate amounts or overproduction can be dangerous; these cytokines, especially TNF, are implicated in causing some of the pathological responses that occur in inflammatory conditions (1). The actions of proinflammatory cytokines are antagonized by anti-inflammatory cytokines such as IL-4, IL-10, transforming growth factor-β, and by cytokine inhibitors such as IL-1 receptor antagonist and soluble TNF receptors.

Helper T lymphocytes are subdivided functionally according to the pattern of cytokines they produce (Fig. 2). It is believed that helper T cells that have not previously encountered antigen produce mainly IL-2 upon initial encounter with antigen. These cells may differentiate into a population some-

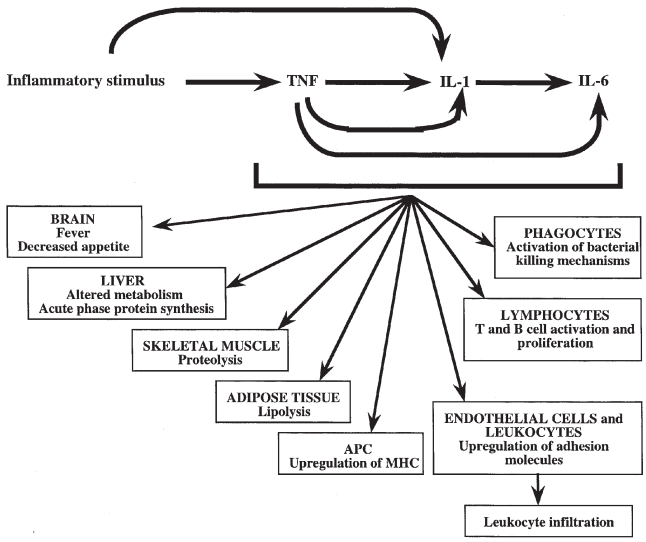


**FIG. 1.** Key roles of proinflammatory cytokines in mediating the host innate immune response and in integrating the innate and acquired immune responses. TNF, tumor necrosis factor; IL, interleukin; APC, antigen-presenting cells; MHC, major histocompatibility complex.

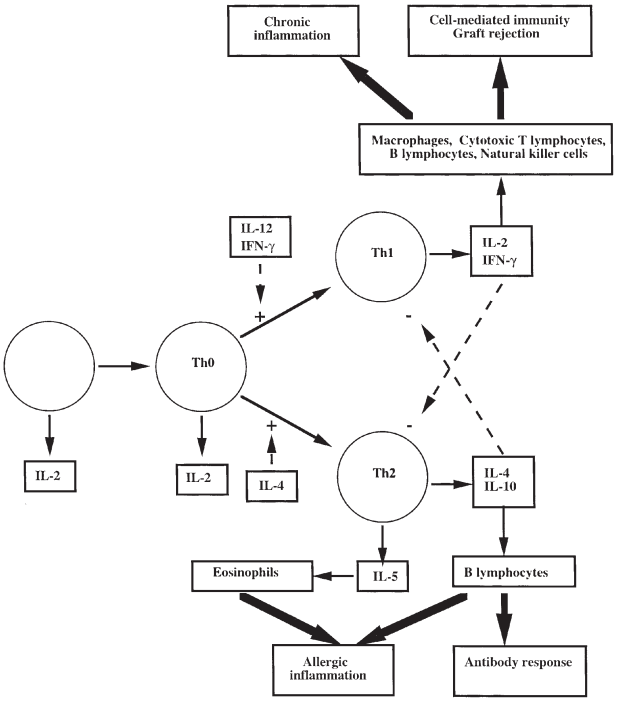


**FIG. 2.** Development and roles of helper T lymphocyte responses. IL, interleukin; IFN, interferon; Th, helper T cells.

times referred to as Th0 cells, which differentiate further into either Th1 or Th2 cells (Fig. 2). This differentiation is regulated by cytokines as follows: IL-12 and interferon-γ (IFN-γ) promote the development of Th1 cells, whereas IL-4 promotes the development of Th2 cells (Fig. 2). Th1 and Th2 themselves have relatively restricted profiles of cytokine production, i.e., Th1 cells produce IL-2 and IFN-γ, which activate macrophages, NK cells, and cytotoxic T lymphocytes and are the principal effectors of cell-mediated immunity (Fig. 2). Interactions with bacteria, viruses, and fungi tend to induce Th1 activity. Because Th1 cytokines activate monocytes and macrophages, these cytokines may be regarded as proinflammatory. Th2 cells produce IL-4, which stimulates immunoglobulin (Ig) E production by B lymphocytes, IL-5, an eosinophil-activating factor, and IL-10, and which together with IL-4 suppresses cell-mediated immunity (Fig. 2). Th2 cells are responsible for defense against helminthic parasites, which is due to IgE-mediated activation of mast cells and basophils. Because Th2 cytokines suppress Th1 responses, these cytokines may be regarded as anti-inflammatory. An imbalance or dysregulation between the Th1- and Th2-type responses is a characteristic of many human diseases (2).

## Inflammation

Inflammation is the body's immediate response to infection or injury. It is typified by redness, swelling, heat, and pain. These occur as a result of increased blood flow, increased permeability across blood capillaries, which permits large molecules (e.g., complement, antibodies, cytokines) to leave the bloodstream and cross the endothelial wall, and increased movement of leukocytes from the bloodstream into the surrounding tissue. Thus, inflammation is part of the normal, innate immune response.

## Integration of the Immune Response

The innate and acquired immune responses are integrated according to the direct cell-to-cell and cytokine interactions that result from the presence of a particular stimulus. The innate response, including its inflammatory component, reacts initially to the stimulus, acting directly to eliminate it by the activities of complement or phagocytosis, for example. Cytokines produced by the cells involved in the innate response, especially monocytes and macrophages, will regulate this response and also act systemically on the liver to promote acute phase protein synthesis, on skeletal muscle and adipose tissue to promote proteolysis and lipolysis, respectively (this is believed to be the body's way of providing fuels to the immune system), and on the brain to reduce appetite and induce fever (Fig. 3). These cytokines will also interact with T lymphocytes. Antigen-presenting cells, which include activated monocytes and macrophages, will present antigen to T lymphocytes and thus the acquired immune response will be triggered (Fig. 3). Now there will be a cell-mediated response to the antigen. T lymphocytes will produce cytokines, which will regulate the activity of the cells involved in the innate response (monocytes, macrophages, NK cells), promote the proliferation of B and T lymphocytes, and promote antibody production by B lymphocytes. By virtue of the integrated innate and acquired responses, the source of the antigen should be eliminated and a component of immunological memory will remain (Fig. 3).

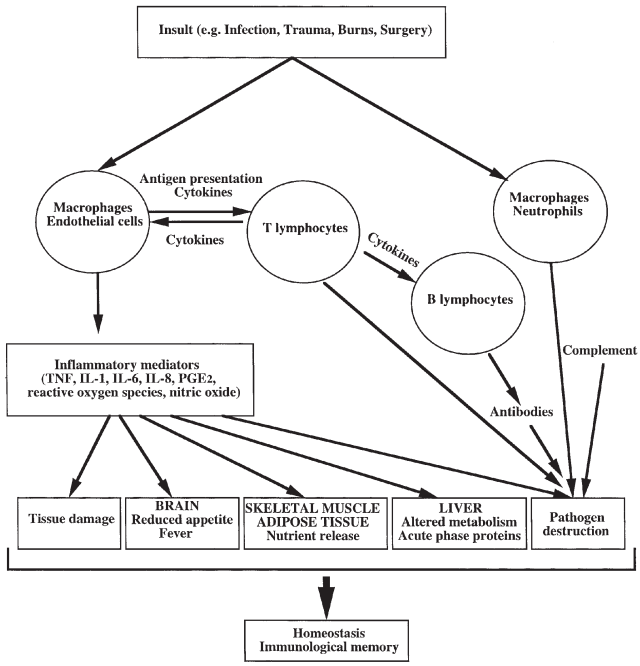


**FIG. 3.** The interrelationship between the innate and aquired immune responses. PG, prostaglandin; for other abbreviations see Figure 1.

### The Immune System in Health and Disease

Clearly, a well-functioning immune system is essential to health. It serves to protect the host from the effects of ever-present pathogenic organisms. Cells of the immune system also have a role in identifying and eliminating cancer cells. There are, however, some detrimental effects of the immune system, including the following:

(i) In the course of its activity to recognize and eliminate foreign antigens, the immune system is responsible for the rejection of transplanted tissues.

(ii) In some individuals, the immune system appears to recognize host antigens as "non-self" rather than as "self." As a result, an immune response to host tissues is generated and this leads to tissue damage. This is the characteristic of so-called chronic inflammatory or autoimmune diseases. Such diseases are linked to genes coding for proteins involved in antigen presentation or recognition such as the MHC II proteins and the T-cell receptor; thus there is a genetic predisposition to these diseases. These diseases are typified by an ongoing chronic inflammation involving the proinflammatory cytokines produced by monocytes and macrophages and by a dysregulated Th1 lymphocyte response. Examples of this type of disease include rheumatoid arthritis, type-1 diabetes, Crohn's disease, psoriasis, and multiple sclerosis.

(iii) The immune system of some individuals can become sensitized to usually benign antigens from the environment and can respond inappropriately to them. Such antigens can include components of foods or of so-called allergens (e.g., cat or dog fur, house dust mite, some pollens), such that this response can lead to allergies, asthma, and related atopic diseases. Although these diseases are often termed chronic inflammatory diseases, they have a different immune basis from the diseases described above, although again they are typified by inappropriate recognition of and/or responses to antigens. However, atopic diseases are characterized by a dysregulated Th2 lymphocyte response such that excessive amounts of IL-4, IL-5, and IL-10 are found. IL-10 suppresses the Th1 response, IL-4 stimulates IgE production by B lymphocytes (IgE promotes histamine release from mast cells), and IL-4 and IL-5 activate eosinophils, which are involved in the persistent inflammation that is a component of these diseases.

(iv) The innate immune system becomes activated as a result of trauma and surgery. This response is characterized by excess production of the proinflammatory cytokines and, if it persists, it can damage organs, causing their failure and leading to complications and sometimes death.

## EICOSANOIDS: A LINK AMONG POLYUNSATURATED FATTY ACIDS, INFLAMMATION, AND IMMUNITY

### Eicosanoid Precursors and Synthesis

Eicosanoids are a second group of chemical messengers that act within the immune system. These compounds provide a link among polyunsaturated fatty acids (PUFA), inflammation, and immune function. Eicosanoids are synthesized from PUFA, in particular dihomo-γ-linolenic acid (DGLA; 20:3n-6), arachidonic acid (AA; 20:4n-6), and eicosapentaenoic acid (EPA; 20:5n-3). Eicosanoids include prostaglandins (PG), thromboxanes, leukotrienes (LT), lipoxins, hydroperoxyeicosatetraenoic acids (HPETE), and hydroxyeicosatetraenoic acids (HETE). The fatty acid precursor for eicosanoid synthesis is released from cell membrane phospholipids, usually by the action of phospholipase $A_2$ activated in response to a cellular stimulus. Because the membranes of most immune cells contain large amounts of AA, compared with DGLA and EPA, AA is usually the principal precursor for eicosanoid synthesis.

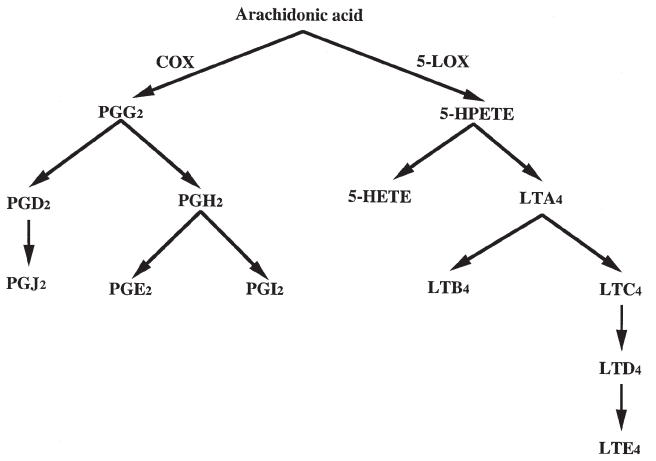


**FIG. 4.** Formation of eicosanoids from arachidonic acid. COX, cyclooxygenase; 5-LOX, 5-lipoxygenase; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene. For other abbreviation see Figure 3.

Metabolism of AA by cyclooxygenase (COX) gives rise to the 2-series PG and thromboxanes (Fig. 4). There are two isoforms of COX; COX-1 is a constitutive enzyme and COX-2, which is induced in immune cells as a result of stimulation, is responsible for the markedly elevated production of PG that occurs upon cellular activation. There are at least 16 different 2-series PG, and these are formed in a cell-specific manner. For example, monocytes and macrophages produce large amounts of $PGE_2$ and $PGF_2$, neutrophils produce moderate amounts of $PGE_2$, and mast cells produce $PGD_2$.

Metabolism of AA by the 5-lipoxygenase (5-LOX) pathway gives rise to hydroxy and hydroperoxy derivatives (5-HETE and 5-HPETE, respectively), and the 4-series leukotrienes (LT), $LTA_4$, $B_4$, $C_4$, $D_4$ and $E_4$ (Fig. 4). 5-LOX is found in mast cells, monocytes, macrophages, and granulocytes. The 12-LOX enzyme is found in platelets and some epithelial cells, and the 15-LOX is found in some epithelial cells.

### Roles for Eicosanoids in Inflammation and Immunity

$PGE_2$ has a number of proinflammatory effects, including induction of fever and erythema, increasing vascular permeability and vasodilation, and enhancing pain and edema caused by other agents such as histamine. $PGE_2$ suppresses lympho-

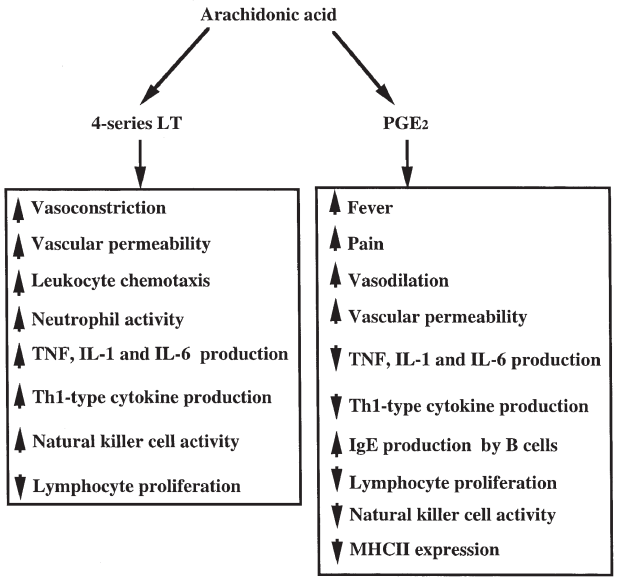


**FIG. 5.** Roles of some arachidonic acid–derived eicosanoids in regulating inflammation and immunity. Ig, immunoglobulin; Th, T helper cell; MHC, major histocompatibility complex; for other abbreviations see Figures 1, 3, and 4.

cyte proliferation and NK cell activity and inhibits production of TNF-α, IL-1, IL-6, IL-2, and IFN-γ; thus, in these respects, $PGE_2$ is immunosuppressive and anti-inflammatory (Fig. 5). $PGE_2$ does not appear to affect the production of the Th2 cytokines IL-4 and IL-10 directly, but it does promote IgE production by B lymphocytes (Fig. 5). $LTB_4$ increases vascular permeability, enhances local blood flow, is a potent chemotactic agent for leukocytes, induces release of lysosomal enzymes, enhances generation of reactive oxygen species, inhibits lymphocyte proliferation, and promotes NK cell activity. LT (4-series) also regulate production of proinflammatory cytokines; for example, $LTB_4$ enhances production of TNF, IL-1, IL- 6, IL-2, and IFN-γ (Fig. 5). 5-HETE enhances whereas 15-HETE inhibits lymphocyte proliferation. Thus, AA gives rise to a range of mediators that have opposing effects to one another so that the overall physiological effect will be governed by the concentration of those mediators, the timing of their production, and the sensitivities of target cells to their effects.

Feeding animals or humans increased amounts of fish oil, which contains EPA and its derivative docosahexaenoic acid (DHA; 22:6n-3), results in a decrease in the amount of AA in the membrane phospholipids of cells involved in inflammation and immunity (Fig. 6). In addition, long-chain n-3 PUFA appear to inhibit the release of AA from membrane phospholipids perhaps by inhibition of phospholipases (4). EPA also competes with AA for the active sites of COX and 5-LOX. Thus, fish oil feeding results in a decreased capacity of immune cells to synthesize eicosanoids from AA (Figs. 7, 8). In addition, EPA is able to act as a substrate for both COX and 5-LOX, giving rise to derivatives that have a different structure from those produced from AA (i.e., 3-series PG and 5-series LT) (Figs. 7, 8). Thus, the EPA-induced suppression in the production of AA-derived eicosanoids is mirrored by an elevation in the production of EPA-derived eicosanoids (Fig. 7). The eicosanoids produced from EPA are considered to be less biologically potent than the analogs synthesized from AA, although the full range of biological activities of these compounds has not been investigated. The best example of differential immunological potencies of eicosanoids produced from AA and EPA is that of $LTB_4$ vs. $LTB_5$. $LTB_5$ is at least 10-fold less potent as a neutrophil chemoattractant than $LTB_4$ and, on this basis, can be considered to be considerably less proinflammatory. One other aspect of the formation of alternative eicosanoids to those produced from AA is that they will share the same receptor on target cells and therefore will act to antagonize the AA-derived mediators (Fig. 8).

## PUFA AND IMMUNE FUNCTION

### Linoleic Acid (18:2n-6) and Immune Function

In vitro *studies*. Linoleic acid enhanced superoxide release from neutrophils and macrophages (7–10) and promoted neutrophil adhesion to endothelial cells (11), suggesting that it possesses proinflammatory effects. In contrast, linoleic acid inhibited the proliferation of rodent and human lymphocytes (12–16) and decreased the production of IL-2 by mitogen-stimulated rat and human lymphocytes (13,14), suggesting that it is potentially immunosuppressive.

*Animal feeding studies*. Essential fatty acid deficiency impaired the ability of mice to produce IgG and IgM in response to sheep red blood cells (17); this response was restored by feeding diets containing 130, 500, or 700 g corn oil/kg (17). In contrast to this apparent enhancing effect of linoleic acid on antibody production, dietary linoleic acid was found to impair the production of antibodies, including IgG and IgM, after antigenic challenges, compared with feeding diets containing low fat or high saturated fat (beef tallow, coconut oil) (18,19). Compared with diets high in saturated fatty acids, feeding rodents high-fat diets rich in linoleic acid decreased mitogen-stimulated lymphocyte proliferation and NK cell activity in some studies (see Ref. 20 for references). These studies suggest that very high levels of linoleic acid in the rodent diet impair cell-mediated and antibody responses. However, modest changes in the amount of linoleic acid in the rat diet did not markedly affect lymphocyte proliferation or NK cell activity (21).

*Human studies*. Surprisingly, few human studies have investigated the immunological effect of linoleic acid. The most detailed of the studies that have been performed are those of Kelley *et al*. (22,23), which involved providing volunteers with low-fat diets (25% energy as fat) that were rich (12.9% of energy) or poor in linoleic acid (3.5% of energy). No differences were observed in the responses of lymphocytes to various T-cell mitogens, in circulating IgM, IgG, IgE, or IgA levels, or in the delayed-type hypersensitivity (DTH) response to seven recall antigens. Yaqoob *et al*. (24) included a group consuming 9 g encapsulated sunflower oil/d for 12 wk in their study. This had no effect on lymphocyte proliferation,

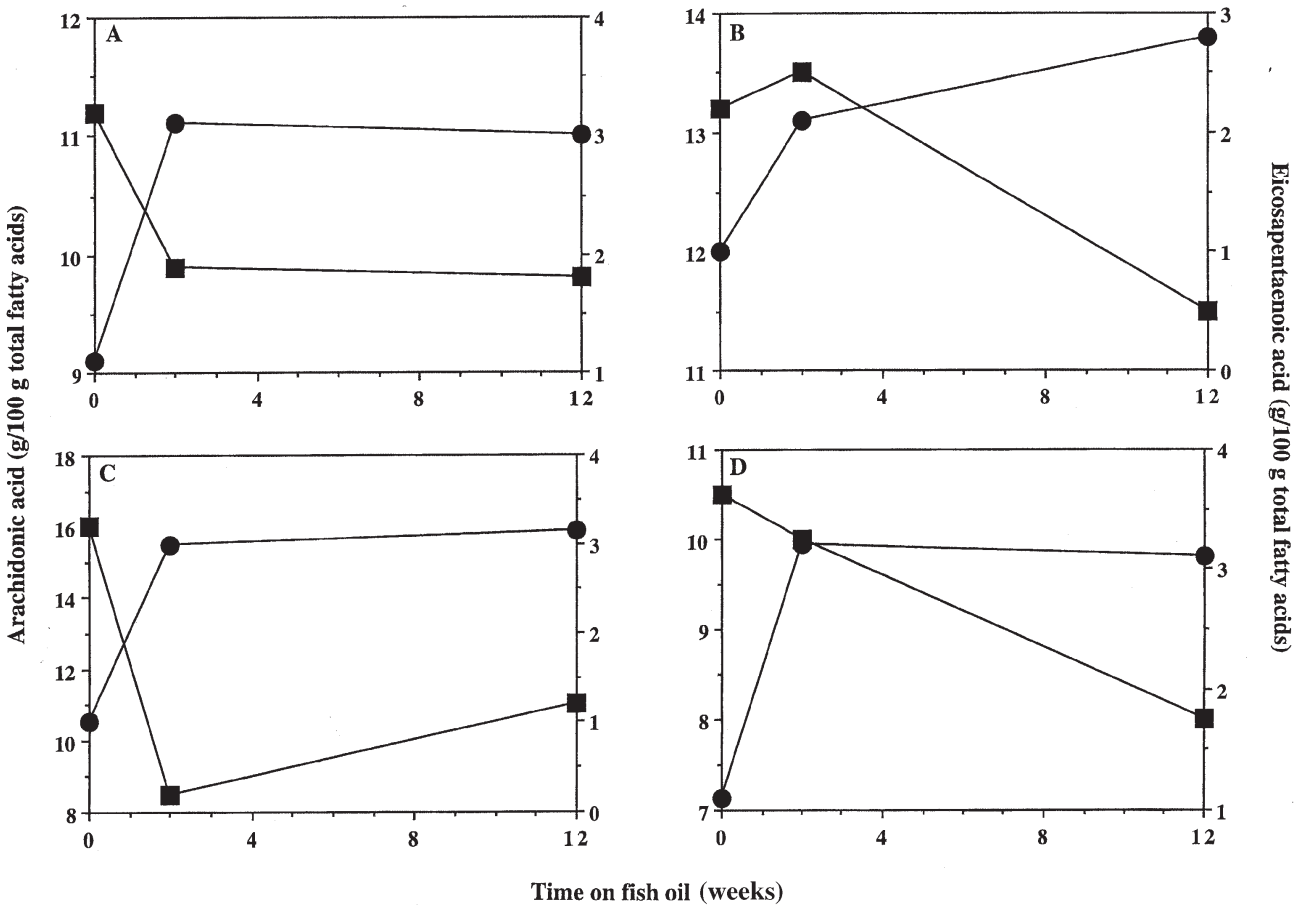


**FIG. 6.** The effect of dietary fish oil on the arachidonic acid (■) and eicosapentaenoic acid (EPA) (●) contents of human immune cell phospholipids. Healthy men aged 22–41 yr supplemented their diets with 10–15 g fish oil/d, providing 2.3–5.6 g EPA plus docosahexaenoic acid/d. After 2 and 12 wk, blood neutrophils (A), monocytes (B), T lymphocytes (C), and B lymphocytes (D) were prepared and the fatty acid composition of their phospholipids determined. Data are from Reference 3.

NK cell activity, or production of TNF-α, IL-1α, IL-1β, IL-2, and IFN-γ by mononuclear cells. These studies suggest a limited effect of linoleic acid (at a level ≥3.5% of dietary energy) on human immune function. However, in another study, the low-fat diet–induced increase in human NK cell activity (25) was reversed by adding 15 g safflower oil/d to the diet for 2 mo (26). Furthermore, the NK cell activity of blood lymphocytes from elderly Danish subjects correlated negatively with linoleic acid intake and with serum levels of linoleic acid (27).

## AA and Immune Function

In vitro *studies*. AA enhanced superoxide release from neutrophils and macrophages (7–10), promoted neutrophil adhesion to endothelial cells (11), and increased IL-1β production by a monocytic cell line (28) and by human monocytes (29). Thus, AA exhibits proinflammatory effects *in vitro*. AA inhibited the proliferation of rodent, pig, and human lymphocytes (12–16,30–32) and decreased the production of IL-2 by mitogen-stimulated rat and human lymphocytes (13,14), suggesting that it is potentially immunosuppressive.

*Animal feeding studies*. Feeding mice a diet containing 20 g safflower oil plus 10 g AA/kg did not affect spleen lymphocyte proliferation or IL-2 production compared with feeding a diet containing safflower oil (30 g/kg) (33). Inclusion of 4.4 g AA/100 g fatty acids in the rat diet did not significantly affect spleen lymphocyte proliferation, NK cell activity, or the graft vs. host response, despite the increased capacity of immune cells from rats fed AA to produce $PGE_2$ (34). These studies suggest that even significant amounts of AA in the rodent diet do not influence cell-mediated immunity.

*Human studies*. Two studies of the influence of dietary AA on human immune function have been performed. In the first, AA (1.5 g/d) was included in a low-fat diet (27% energy as fat) consumed for 8 wk by healthy men aged 20–38 yr (35,36). This level of AA did not alter the proliferation of lymphocytes in response to mitogens, NK cell activity, or the DTH response to seven recall antigens (35) and did not alter TNF-α, IL-1β, IL-6, or IL-2 production by mononuclear cells or the *in vivo* antibody responses to immunization with three strains of influenza virus (36). However, AA did increase production of $PGE_2$ and $LTB_4$ by endotoxin-stimulated mononuclear cells (36). The second study involved supplementing the diet of healthy subjects (men and women) aged 55–75 yr with

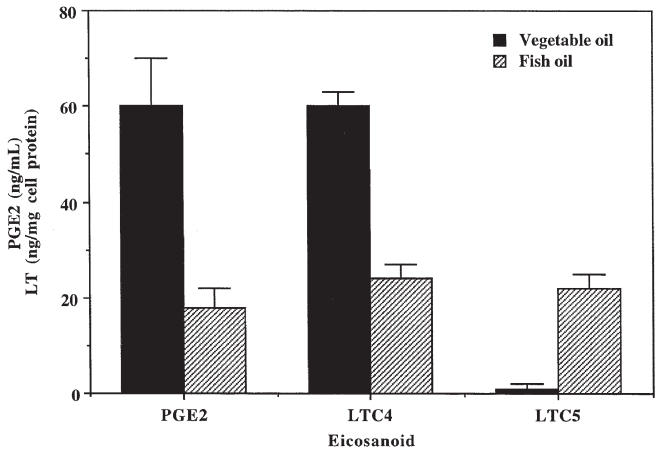


**FIG. 7.** The effect of dietary fish oil on the production of arachidonic acid and eicosapentaenoic acid–derived eicosanoids by murine macrophages. Mice were fed diets containing linoleic acid-vegetable oil or fish oil and thioglycolate-elicited peritoneal macrophages prepared. *Ex vivo* production of prostaglandin (PG)$E_2$, leukotriene (LT)$C_4$, and $LTC_5$ by stimulated macrophages was determined. $PGE_2$ data (ng/mL) are from Reference 5 and LT data (ng/mg cell protein) are from Reference 6.

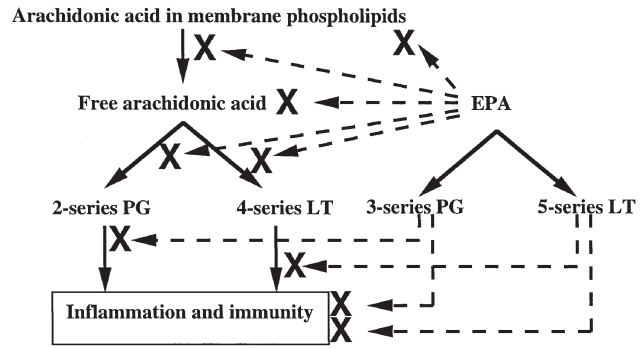


**FIG. 8.** The basis of the anti-inflammatory and immunomodulatory effects of fish oil. Eicosapentaenoic acid (EPA) acts at a number of sites (X) to antagonize the effects of arachidonic acid (AA). EPA replaces AA in immune cell membrane phospholipids, inhibits hydrolysis of AA from membrane phospholipids, and competes with AA for cyclooxygenase (COX) and 5-lipoxygenase (5-LOX). EPA-derived eicosanoids can oppose the effects of those derived from AA by competing for receptor binding. Finally, EPA-derived eicosanoids can have differing effects from those derived from AA. For abbreviations see Figures 3 and 4.

encapsulated AA (~700 mg/d) for 12 wk; there was no effect on NK cell activity (37), mitogen-stimulated lymphocyte proliferation (38), or production of TNF-α, IL-1β, IL-6, IL-2, or IFN-γ by mononuclear cells (38; Thies, F., Newsholme, E.A., and Calder, P.C., unpublished observations). Given that the habitual consumption of AA in free-living Western adults is <300 mg/d, these studies suggest that increasing AA consumption in healthy adults does not have adverse immunological effects. It should be noted, however, that the length of AA administration in these studies was ≤12 wk, and the immunological effects of AA over a longer term are not known.

## α-Linolenic Acid (ALNA; 18:3n-3) and Immune Function

In vitro *studies*. ALNA promoted neutrophil adhesion to endothelial cells (11). ALNA inhibited the proliferation of rodent and human lymphocytes *in vitro* (11–16,30–32), decreased the production of IL-2 by mitogen-stimulated rat and human cells (13,14), and inhibited degranulation of cytotoxic T lymphocytes (39). Thus, ALNA has potentially immunosuppressive properties.

*Animal feeding studies*. Compared with feeding a linoleic acid-rich diet, feeding rats linseed oil (100 g/kg diet) for 8 wk decreased superoxide production by peritoneal macrophages in response to phorbol ester but did not affect superoxide production in response to *Listeria monocytogenes* or phagocytosis of *L. monocytogenes* (40). Studies in rodents indicated that linseed oil increased production of TNF by resident macrophages, but had no effect on TNF production by inflammatory macrophages (41–43). High levels of linseed oil in the rodent diet led to decreased lymphocyte proliferation (44,45), NK cell activity (45), and graft vs. host response (45). The precise effect of ALNA on lymphocyte functions appears to depend on the level of linoleic acid and the total PUFA content of the diet (21).

*Human studies*. A high dose of ALNA (15 g/d for 4 wk) decreased IL-1 and TNF production by lipopolysaccharide-stimulated human monocytes (46). Adding linseed oil (providing ~15 g ALNA/d) to a low-fat diet (total fat provided 29% energy) resulted in a significant decrease in human blood lymphocyte proliferation and in the DTH response to seven recall antigens after 6 wk, but circulating antibody levels were unaffected (47). Supplementing the diet of healthy subjects aged 55–75 yr with linseed oil providing 2 g ALNA/d did not significantly affect NK cell activity (37), mitogen-stimulated lymphocyte proliferation (38), or production of TNF-α, IL-1β, IL-6, IL-2, or IFN-γ by mononuclear cells (38; Thies, F., Newsholme, E.A., and Calder, P.C., unpublished observations). Furthermore, supplementing the diet of healthy young men (aged 20–40 yr) with 4.2 g ALNA/d did not alter superoxide production by neutrophils (48). These studies suggest that a moderate increase in ALNA intake by healthy adults does not affect immunity, but that a marked increase in ALNA intake (e.g. 7- to 15-fold) can induce anti-inflammatory and immunosuppressive effects. It is not clear whether these are exerted by ALNA itself or by EPA, a product of ALNA metabolism.

## Long-Chain n-3 PUFA and Immune Function

Because dietary fish oil leads to decreased $PGE_2$ production (see earlier), it is often stated that it should reverse the effects of $PGE_2$, simply acting as a $PGE_2$ antagonist. If this were so, then fish oil would exert some anti-inflammatory actions (e.g., decreasing fever and vascular permeability) but it would also enhance production of the classic proinflammatory cytokines (TNF, IL-1, and IL-6), enhance production of Th1-type cytokines, increase MHC II expression, lymphocyte proliferation, and NK cell activity, and decrease IgE production

by B lymphocytes. As described below, many studies, especially those conducted in laboratory animals, have demonstrated that although in some situations fish oil does act as a "$PGE_2$ antagonist," it often induces effects that are the opposite to those expected on this basis. Thus, the situation is more complex than fish oil simply being a $PGE_2$ antagonist. $PGE_2$ is not the sole mediator produced from AA, and the range of mediators produced have varying, sometimes opposite, actions (see earlier). Thus, if fish oil was to act as a "4-series LT antagonist," it would be expected to decrease vascular permeability, leukocyte chemotaxis, neutrophil reactivity, production of proinflammatory and Th1-type cytokines, and NK cell activity. EPA itself will give rise to eicosanoids with varying actions, some augmenting the actions of AA-derived mediators and others antagonizing those actions. In addition, long-chain n-3 PUFA may exert a range of eicosanoid-independent effects, especially upon intracellular signaling mechanisms; these are discussed elsewhere (49,50). Thus, the overall effect of fish oil feeding cannot be predicted solely on the basis of an abrogation of $PGE_2$-mediated effects.

In vitro *studies*. Culture with EPA or DHA inhibited superoxide production (51) and phagocytosis (52) by human neutrophils. Incubation with EPA or DHA inhibited cytokine-induced cell surface expression of MHC II (in the mouse these antigens are termed Ia) on mouse peritoneal macrophages (53); DHA was more inhibitory than EPA and other 20-carbon fatty acids and acted by inhibiting the increase in Ia mRNA that occurs after stimulation of macrophages with cytokines (53). Hughes *et al.* (54) examined the effect of incubation of purified human monocytes with either EPA or DHA upon expression of MHC II, which is termed human leukocyte antigen (HLA); both EPA and DHA decreased the proportion of HLA-DR or -DP positive monocytes after incubation with IFN-γ and decreased the level of expression of these molecules on the monocyte surface (54). In accordance with this, the ability of monocytes cultured with EPA or DHA to present antigen (tetanus toxoid) to autologous lymphocytes was diminished (55). EPA and DHA inhibited production of IL-1β and of TNF-α by human monocytes (28,29) and IL-6 production by rat peritoneal macrophages (56). EPA and DHA inhibited mitogen-stimulation proliferation of rodent and human lymphocyte in culture (12–16,31,32,57–60), decreased the production of IL-2 by rat and human lymphocytes (13,14,59), and decreased human NK cell activity *in vitro* (59,61,62). Thus, in cell culture, both EPA and DHA exhibit potent anti-inflammatory and immunosuppressive effects.

*Animal feeding studies*. Feeding fish oil to laboratory animals decreased superoxide and hydrogen peroxide production by macrophages (63–66). Fish oil feeding decreased *ex vivo* production of TNF-α, IL-1β and IL-6 by rodent macrophages (5,67–69) and monocytes (70). Compared with feeding safflower oil, fish oil feeding resulted in lower peak plasma TNF-α, IL-1β, and IL-6 concentrations after intraperitoneal injection of endotoxin in mice (71). Furthermore, parenteral nutrition supplemented with fish oil decreased serum TNF-α, IL-6, and IL-8 concentrations in burned rats compared with n-6 PUFA–rich parenteral nutrition (72,73). Thus, animal studies reveal significant anti-inflammatory effects of dietary fish oil.

One study reported diminished phagocytosis of *Salmonella typhimurium* by murine Kupffer cells after feeding fish oil, although this was not associated with a reduced capacity of the cells to kill the bacteria (65). Another study showed that fish oil administered by gastric tube significantly diminished the ability of neonatal rabbits to clear a challenge of *Staphylococcus aureus* (63). However, there are reports that dietary fish oil does not affect phagocytosis of sheep erythrocytes or yeast particles by murine peritoneal macrophages (66), or of latex beads by porcine alveolar macrophages (74). Feeding fish oil decreased the level of MHC II expression on murine peritoneal macrophages (75) and on rat dendritic cells obtained by cannulation of the thoracic duct (76). Feeding mice an EPA-rich diet for a period of 4–5 wk resulted in diminished *ex vivo* presentation of antigen (keyhole limpet hemocyanin; KLH) by spleen cells (77). Compared with feeding a low-fat diet or a diet containing 200 g safflower oil/kg, feeding rats a diet containing 200 g fish oil/kg diminished *ex vivo* KLH presentation by dendritic cells obtained by cannulation of the thoracic duct to KLH-sensitized spleen lymphocytes (76). A recent study reported that dietary fish oil decreased expression of the IFN-γ-receptor on murine peritoneal macrophages (78). These studies suggest that dietary fish oil might impair the cell-mediated immune response by decreasing the activity of antigen-presenting cells and by decreasing the sensitivity of macrophages to T lymphocyte–derived cytokines. The effect of dietary fish oil on phagocytosis is unclear.

Animal feeding studies indicate that high levels of fish oil decrease NK cell activity (79–82), cytotoxic T lymphocyte activity (80), expression of the IL-2 receptor on activated lymphocytes (82–84), lymphocyte proliferation (82,83,85–90), and the production of IL-2 (74,90) and IFN-γ (90). Recently, Byleveld *et al*. (91) showed that feeding mice fish oil significantly impaired the elevation in lung IFN-γ that follows infection with the influenza virus. Dietary fish oil also decreased expression of the IFN-γ receptor on murine splenocytes (78). Dietary fish oil reduced the DTH response in mice compared with n-6 PUFA–rich or olive oil–rich diets (92), whereas addition of either EPA or DHA to the diet of mice consuming a safflower oil diet decreased the DTH response (93); both n-3 PUFA were equally effective. The DTH response to sheep red blood cells in mice was also diminished after tail-vein injections of emulsions of triacylglycerols rich in EPA or DHA (94). Feeding beagle dogs a diet with an n-6/n-3 PUFA ratio of 1.4 resulted in a reduced DTH response to intradermal KLH compared with diets with n-6/n-3 PUFA ratios of 31 or 5.4 (95); the increased n-3 PUFA content was brought about by replacing linoleic acid with EPA plus DHA. A suppressed host vs. graft response was observed in mice fed a diet containing 160 g fish oil/kg compared with those fed a standard chow diet (96); lower levels of fish oil (25, 50,

100 g/kg) did not significantly affect the response. Significantly diminished graft vs. host and host vs. graft responses were observed in rats fed a diet containing 200 g fish oil/kg compared with those fed a low-fat diet or diets containing 200 g coconut, olive, safflower, or evening primrose oil/kg (97). Taken together, these studies suggest that fish oil impairs cell-mediated immunity and induces a shift in T-lymphocyte response away from the Th1-type response, which is involved in both cell-mediated immunity and chronic inflammation. In accordance with this, fish oil enhanced production of IgE to ovalbumin in rats (98).

Animal studies have often used very large amounts of fish oil in the diet, i.e., a diet in which fish oil contributes 20% by weight will mean that EPA plus DHA comprise up to 30% of dietary fatty acids and up to 12% of dietary energy. Recent studies in rats and mice have indicated that relatively low levels of the long-chain n-3 fatty acids (EPA or DHA at a level of 4.4% of total fatty acids or 1.7% of dietary energy) are sufficient to bring about some of the effects of fish oil (99), that dietary EPA and DHA both inhibit lymphocyte proliferation (33,34) and IL-2 production (33), and that dietary EPA, but not DHA, inhibits NK cell activity (34).

*Human studies*. Fish oil, providing >2.3 g EPA plus DHA/d (and in some studies up to 14.5 g/d), decreased chemotaxis of neutrophils (100–104), decreased neutrophil superoxide production (105–107), and decreased neutrophil binding to endothelial cells (100). A recent study providing up to 2.4 g EPA plus DHA/d to healthy subjects for 12 wk did not detect effects on neutrophil chemotaxis or superoxide production (48).

Fish oil, providing 4.5–5.3 g EPA plus DHA/d, decreased chemotaxis of monocytes (101,102,108). Fish oil supplementation decreased zymosan-induced superoxide production by monocytes (109). A more recent study reported no effect of a low dose of n-3 PUFA (0.55 g EPA plus DHA/d for 12 wk) on monocyte chemotaxis (110).

Supplementation of the diet of human volunteers with 1.6 g EPA plus DHA/d for 3 wk resulted in decreased expression of MHC II (HLA-DP, -DQ and -DR) on the surface of blood monocytes (111). Fish oil providing >2.4 g EPA plus DHA/d has been shown to decrease production of TNF (46,108,112, 113), IL-1 (46,108,112,113), and IL-6 (112) by mononuclear cells. One other study in which subjects consumed a low-fat diet including oily fish daily (providing 1.2 g EPA plus DHA/d) showed decreased production of TNF, IL-1, and IL-6 (114). In addition, parenteral nutrition supplemented with fish oil decreased serum TNF-α and IL-6 concentrations in patients after major abdominal surgery compared with n-6 PUFA–rich parenteral nutrition (115). In contrast to these observations, a number of studies that provided from 0.55 to 3.4 g EPA plus DHA/d failed to demonstrate an effect of fish oil on production of TNF (24,110,116–118), IL-1 (24,110,116–119), and IL-6 (110,117).

Data from studies investigating the influence of fish oil on human lymphocyte functions are also conflicting. Supplementation of the diet of healthy human volunteers with fish oil providing 2.4 g EPA plus DHA/d resulted in decreased proliferation of lymphocytes from older (aged 51–68 yr) but not young (aged 21–33 yr) women and decreased IL-2 production (112). Molvig *et al*. (116) reported decreased lymphocyte proliferation after providing 1.7 or 3.4 g EPA plus DHA/d to men, and Gallai *et al*. (113) reported that 5.2 g EPA plus DHA/d decreased IL-2 and IFN-γ production. Providing 1.2 g EPA plus DHA/d to healthy subjects aged 55–75 yr resulted in decreased NK cell activity (37) and lymphocyte proliferation (38), but did not affect IL-2 or IFN-γ production (38). Finally, inclusion of oily fish providing 1.2 g EPA plus DHA/d in the diet of volunteers consuming a low-fat, low-cholesterol diet decreased lymphocyte proliferation, IL-2 production, and the DTH response to seven recall antigens (114). In contrast to these observations, there are reports of no effect of 3.2 g EPA plus DHA/d on NK cell activity, lymphocyte proliferation, and IL-2 and IFN-γ production (24) and of no effect of 4.6 g EPA plus DHA/d on lymphocyte proliferation and IL-2 production (120).

Taken together these studies indicate that addition of high levels of fish oil to the human diet exerts potent anti-inflammatory effects, particularly decreasing neutrophil and monocyte chemotaxis, superoxide production, and production of proinflammatory cytokines. A high level of dietary fish oil also impairs lymphocyte responses, at least in some studies. Other studies indicated that more modest addition of fish oil to the diet does not affect inflammatory or immune activities. However, there are a large number of studies that fall between the extremes of "modest addition" and "high levels," and these studies provide conflicting results. It is unclear what the reasons for these discrepancies are, but they might be related to different experimental protocols used, particularly those involving cell preparation, cell culture and cytokine assays, and/or to different subject characteristics (e.g., gender, age, habitual diet) (see Ref. 121 for a discussion).

Some recent studies have examined whether the effects of fish oil are due to EPA or DHA. There was no effect of 3.8 g of either EPA or DHA/d for 7 wk on phagocytosis of opsonized or unopsonized *Escherichia coli* by human monocytes (122). Kelley *et al*. (123,124) reported the effects in men aged 20–40 yr of including 6 g DHA/d in a low-fat diet (30% energy as fat) for 90 d. There was no effect of DHA on lymphocyte proliferation, serum IgG concentration, or the DTH response to seven recall antigens (123), or on the serum antibody response to immunization with three strains of influenza virus (124). NK cell activity was unaffected at d 55 but was decreased at d 90 (124). Similarly, the production of TNF-α and IL-1β tended to decrease at day 55 but was significantly decreased at day 80 (124). More recently, 750 mg DHA/d was shown not to affect NK cell activity (37), lymphocyte proliferation (38), or the production of TNF-α, IL-1β, IL-6, IL-2, or IFN-γ (38; Thies, F., Newsholme, E.A., and Calder, P.C., unpublished observations) in healthy subjects aged 55–75 yr. Taken together, these data indicate that high levels of DHA (e.g., 6 g/d) can mimic some of the effects of fish oil but that lower levels (e.g., <1 g/d) do not exert any immunological effects in healthy adults.

## EFFECTS OF n-3 PUFA ON INFLAMMATION AND IMMUNITY: IMPLICATIONS AND APPLICATIONS

As outlined above, a number of animal feeding and human supplementation studies indicate that fish oil can have potent effects on immune function and inflammatory cell responses. Of the two long-chain n-3 PUFA characteristic of fish oil, EPA appears to be more potent than DHA, although high levels of DHA can mimic some of the effects of fish oil. Moderate increases in the intakes of ALNA and AA appear to have little effect on immune function, although high intakes of ALNA have similar effects to fish oil and high intakes of AA have not been studied in humans. The immunological effects of long chain n-3 PUFA are generally termed as anti-inflammatory, and the applications of these effects have been described in terms of chronic inflammatory diseases, allergic inflammation, and acute systemic inflammation in response to trauma. In each of these situations, the benefits of decreased production of 2-series PG (especially $PGE_2$), 4-series LT, and the proinflammatory cytokines, especially TNF-α and IL-1, are evident. The potential detrimental effect on cell-mediated immune responses is often overlooked, although this effect has been demonstrated more easily in animals fed large amounts of fish oil than in humans consuming more moderate amounts. In the following sections, the contrasting effects of fish oil on host responses to live pathogens vs. purified endotoxin and the applications to chronic inflammatory diseases and allergic inflammation are described.

### Fish Oil, Infection, and Endotoxemia

*Animal studies*. The diminished cell-mediated immune responses observed after feeding diets rich in long-chain n-3 PUFA suggest that these fatty acids could impair the host response to infection. Some animal studies support this suggestion. Mice fed a diet containing 200 g fish oil/kg showed lower survival over 15 d (48%) to orally administered *S. typhimurium* than those fed corn oil (62.5%), coconut oil (87.5%), or a low-fat diet (88%) (125); spleens from the fish oil–fed animals contained a greater number of bacteria than those from animals fed the other diets. Similarly, a study of experimental tuberculosis in guinea pigs reported an increased number of bacteria (*Mycobacterium tuberculosis*) in the spleens of fish oil–fed animals, and it was concluded that this represented persistence of the experimental infection (126). Compared with safflower oil, fish oil decreased the clearance of bacteria (inspired *S. aureus*) in neonatal rabbits (63). A diet containing 170 g fish oil/kg decreased survival of mice after an intraperitoneal injection of *L. monocytogenes* compared with feeding 200 g/kg lard, but not compared with feeding 200 g soybean oil/kg, which also resulted in lower survival (127). The spleens from the fish oil–fed mice contained significantly more bacteria than those from the other two groups (127). Recently, fish oil feeding was shown to delay the clearance of influenza virus from the lungs of mice; this was associated with impaired IFN-γ appearance in lung lavage fluid (91). Because the response to microbial and viral infections is predominantly a Th1-mediated response [or at least requires Th1-type cytokines such as IFN-γ (2)], the reduced survival of rodents fed large amounts of fish oil after bacterial challenges confirms that large amounts of fish oil in the diet suppress the Th1 response *in vivo*. In contrast to these observations, some studies show that fish oil feeding does not affect resistance of laboratory rodents to some bacterial (*Pseudomonas aeruginosa*) and viral (murine cytomegalovirus) challenges (128,129). Furthermore, some studies have shown that dietary fish oil enhances survival during some infections. For example, Blok *et al*. (130) reported increased survival of fish oil–fed mice challenged by intramuscular injection with *Klebsiella pneumoniae*; 90% of fish oil–fed mice survived compared with 30, 40, and 0% in groups fed corn oil, palm oil, or chow, respectively. Cerebral malaria induced by intraperitoneal injection of erythrocytes infected with *Plasmodium berghei* occurred in only 23% of fish oil–fed mice compared with 61, 81, and 78% of mice fed corn oil, palm oil, or chow, respectively (130). The latter observation is interesting because in human cerebral malaria, an unrestrained Th1 response is detrimental and a Th2 response is helpful (2). The apparent shift away from a Th1- toward a Th2-type response after fish oil feeding fits with these observations.

In contrast to the detrimental effects of fish oil feeding after challenge with live intact bacteria reported in some studies (see above), intravenous infusion of a 10% (vol/vol) lipid emulsion rich in fish oil into guinea pigs significantly enhanced survival to intraperitoneally injected bacterial endotoxin compared with infusion of a 10% (vol/vol) safflower oil emulsion (131). Furthermore, feeding a diet containing 145 g fish oil/kg to guinea pigs for 6 wk significantly increased survival after an intraperitoneal injection of endotoxin compared with animals fed a diet containing 150 g safflower oil/kg (132). The decreased sensitivity of fish oil–fed animals to endotoxin could be because fish oil decreases the production of the proinflammatory cytokines (see earlier), which are in part the cause of endotoxin-mediated morbidity and mortality. In addition, other studies suggest that fish oil feeding decreases sensitivity to the effects of proinflammatory cytokines. For example, feeding weanling rats a diet containing 100 g fish oil/kg for 8 wk significantly decreased a number of metabolic responses to intraperitoneal TNF-α, i.e., the rises in liver zinc and plasma C3 concentrations, the fall in plasma albumin concentration, and the increases in liver, kidney, and lung protein synthesis rates were all prevented by the fish oil diet (133). Furthermore, fish oil feeding to rats or guinea pigs diminished the pyrogenic (134,135) and anoretic effects (133,136) of IL-1 and TNF-α compared with feeding linoleic acid–containing oils.

It is not entirely clear why fish oil increases susceptibility to pathogens in some studies but not others; this most probably relates to the precise components of the immune response that are required for defense against a particular pathogen, the age of the animals studied, the level of n-3 PUFA in the diet, the mode of administration of the pathogen, and the species

and strain of animal used. What is apparent, however, is that large amounts of fish oil can impair host defense to live pathogens *in vivo* (at least in some experimental animal models), and that they protect against the damage induced by pure bacterial endotoxin. These observations agree with the *ex vivo* measures of immune function. Defense against live bacteria and viruses requires an efficient cell-mediated immune response and this can be impaired by fish oil. In contrast, the damaging effects of purified endotoxin are mediated by macrophage-derived proinflammatory cytokines, whose production is diminished by fish oil feeding.

*Human studies.* There have been no reports of compromised immunity in humans supplementing their diet with n-3 PUFA. However, most studies of PUFA and immune function have been too small and of too short a duration to identify effects on infection rates; they have also not been designed to investigate rates of infection. However, an epidemic of measles in Greenland triggered by its introduction into the naive population by an infected Danish sailor showed the same characteristics (e.g., expected numbers of cases, complications) as previous epidemics recorded in other naive populations (137). This suggests that the traditional, very n-3 PUFA–rich diet of Greenland Eskimos did not worsen their response to the virus.

### Fish Oil and Th-1 Skewed Immunological Diseases

Chronic inflammatory diseases are characterized by a dysregulated Th1-type response and often by an inappropriate production of AA-derived eicosanoids, especially $PGE_2$ and $LTB_4$. The effects of fish oil outlined above suggest that it might have a role in the prevention and therapy of these diseases. Dietary fish oil has been shown to have beneficial clinical, immunological, and biochemical effects in various animal models of human chronic inflammatory diseases. These effects include increased survival and decreased proteinuria and anti-DNA antibodies in mice with autoimmune glomerulonephritis (a model of lupus) (138–141), decreased joint inflammation in rodents with collagen-induced arthritis (142), and less inflammation in rat models of colitis (143,144). The improvements in the model of lupus are associated with abolition of proinflammatory cytokine production and the induction of anti-inflammatory cytokines and antioxidant enzymes (145,146). It was recently reported that both EPA and DHA suppress streptococcal cell wall–induced arthritis in rats, but that EPA was more effective (147); this fits with the more potent effects of EPA than DHA on inflammation and immunity.

There have been a number of clinical trials assessing the benefits of dietary supplementation with fish oil in several inflammatory diseases in humans, including rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis, lupus, and multiple sclerosis. Trials in some of these diseases are summarized in Table 2. Many of the placebo-controlled, double-blind trials of fish oil in chronic inflammatory diseases reveal significant benefit, including decreased disease activity and a lowered use of anti-inflammatory drugs; the evidence for a beneficial effect of fish oil is strongest in rheumatoid arthritis (Table 2). It was recently observed that n-3 PUFA cause a concentration-dependent decrease in expression and activity of the aggrecanase enzymes that degrade cartilage, in expression of COX 2, but not COX 1, and in TNF-$\alpha$ and IL-1$\beta$ expression in cultured articular cartilage chondrocytes (156). These observations may explain in part the benefits of fish oil in rheumatoid arthritis. Trials of fish oil supplementation in systemic lupus erythematosus and multiple sclerosis have failed to show significant clinical improvement.

### Fish Oil and Th-2 Skewed Immunological Diseases

$PGD_2$, $LTC_4$, $LTD_4$, and $LTE_4$ are produced by the cells that mediate pulmonary inflammation in asthma such as mast cells and are believed to be the major mediators of asthmatic bronchoconstriction (Fig. 9). Although its action as a precursor to LT has highlighted the significance of AA in the etiology of allergic inflammation (Fig. 9), a second link with this fatty acid has been made. This is because $PGE_2$ regulates the activities of macrophages and lymphocytes (see earlier). Of particular relevance in the context of asthma and allergic diseases is the ability of $PGE_2$ to inhibit the production of the Th-1 type cytokines IL-2 and IFN-$\gamma$ without affecting the production of the Th-2-type cytokines IL-4 and IL-5, and to stimulate B cells to produce IgE. These observations suggest that $PGE_2$ regulates the development of these diseases (Fig. 9). As a result, there has been speculation that the increased intake of linoleic acid, the precursor of AA, that has occurred since the mid-1960s is causally linked to the increased incidence of asthma and allergic diseases over this period (157,158). Thus, a case has been made for increasing the consumption of n-3 fatty acids by patients with allergic diseases (157,158).

There is some epidemiological evidence to support a protective role of long-chain n-3 PUFA in allergic disease (see Ref. 159 for references). These observations make a compelling argument for trials of fish oil in asthma and related diseases, and a number of such trials have been performed. A fish oil–induced reduction in *ex vivo* $LTB_4$ production by neutrophils from asthmatic patients has been demonstrated (160); most likely, this was accompanied by increased production of 5-series LT. The 5-series LT might be beneficial in asthma because they are unable to elicit an asthmatic response and/or because they block 4-series LT binding to their receptors. Several studies of fish oil supplementation in asthma revealed limited clinical effect, despite significant biochemical changes (e.g., reduced 4-series LT production); details of these studies are discussed elsewhere (see Refs. 159,161). In contrast, some studies have shown significant clinical improvements at least in some patient groups and suggest that this type of approach may be useful in conjunction with other drug- and diet-based therapies (see Ref. 159). A very careful study by Broughton *et al.* (162) found that low n-3 PUFA ingestion resulted in increased methacholine-induced respiratory distress in asthmatic patients. In contrast, high n-3 PUFA ingestion resulted in an improved response in >40% of subjects; all measures of respiratory function were markedly improved in this group of patients who also showed a markedly elevated appearance of the

**TABLE 2**
**Summary of Clinical Trials of Fish Oil in Chronic Inflammatory Diseases[a]**

| Disease | Number of double-blind, placebo-controlled studies | Doses of EPA + DHA used (g/d) | Duration (wk) | Key findings | Reviews |
|---|---|---|---|---|---|
| Rheumatoid arthritis | 14 | 1–7.1 | 4–52 | All studies reported improvements, including reduced duration of morning stiffness, tender or reduced number of swollen joints, reduced joint pain, reduced time to fatigue, and increased grip strength. Twelve studies reported improvement in at least two clinical measures, and five studies reported improvement in at least four clinical measures. Nine studies reported decreased joint tenderness. Three studies reported significant decrease in the use of nonsteroidal anti-inflammatory drugs. | 148–152 |
| Crohn's disease | 3 | 2.7–5.1 | 12–52 | Two studies reported no benefit. One study reported a significiant decrease in relapses. One other study which used oily fish (100–250 g/d for 2 yr) reported a significant decrease in relapses. | 153 |
| Ulcerative colitis | 4 | 1.8–5.4 | 12–52 | One study reported no benefit (this study used the lowest dose of EPA plus DHA). One study reported a nonsignificant decrease in disease activity and a significant decrease in use of corticosteroids. Two studies reported benefit including improved histologic appearance of the colon, decreased disease activity, weight gain and decreased use of prednisolone. Two other "open" studies reported improved symptoms, improved histologic appearance of the rectal mucosa, and decreased use of prednisolone. | 154 |
| Psoriasis | 2 | 1.8 | 8–12 | One study reported significant improvement in itching, scaling and erythema. One study reported no benefit. Three open studies (providing 10–18 g EPA + DHA/d for 6–8 wk) reported mild-to-moderate (two studies) or moderate-to-excellent (one study) improvements in scaling, itching, lesion thickness, and erythema in the majority of patients. One open study,which combined fish oil with a low-fat diet, reported improvements. | 155 |

[a]Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

EPA-derived 5-series LT in their urine. However, some patients did not respond to the high n-3 PUFA intake. This study suggests that there are patients who respond positively to fish oil intervention and patients who are nonresponders. This suggests that such therapies should be approached cautiously until more is understood about the interaction between fatty acid consumption and disease activity.

## DIETARY PUFA AND IMMUNE FUNCTION IN THE NEONATE

None of the studies described above investigated the influence of altering the fatty acid composition of the maternal diet during pregnancy and/or lactation on immune outcomes in the offspring at birth, weaning, or later in life. Only a limited number of such studies have been reported and these are all in animals (163–165).

In the study by Berger *et al*. (163), female mice were fed before and throughout pregnancy and during lactation diets containing 100 g olive oil, safflower oil, linseed oil, or fish oil/kg. Spleen and thymus weights were determined in the offspring at weaning (d 18). Immune cell functions were determined in the offspring at day 42 but the paper does not indicate the postweaning diet; it is unclear whether it was the same as the maternal diet or was standard chow. At weaning, the offspring of dams fed the olive oil or fish oil diets had smaller spleens than those of dams fed the safflower oil or linseed oil diets. The offspring of dams fed the linseed oil or fish oil diets had smaller thymuses than those of dams fed the olive oil or safflower oil diets. NK cell activity in the offspring at day 42 was decreased by maternal fish oil compared with olive oil and safflower oil. Maternal diet did not affect spleen lymphocyte proliferation or IL-2 production in the offspring.

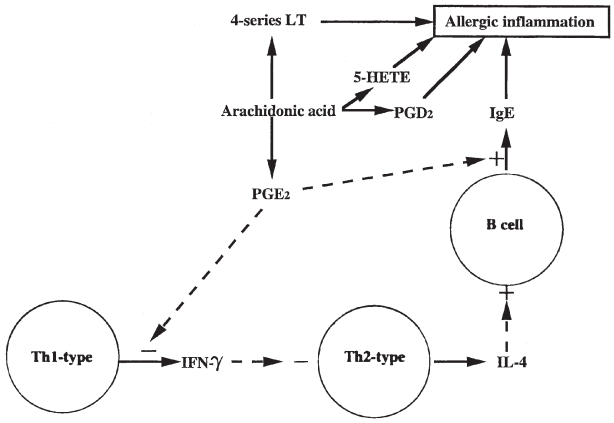


**FIG. 9.** Role of arachidonic acid–derived eicoanoids in allergic inflammation. For abbreviations see Figures 1–5.

Rayon *et al.* (164) fed rats diets containing either 100 g corn or fish oil/kg throughout pregnancy and lactation and then administered live *Streptococcus* to the 7-d-old pups. The offspring of dams fed corn oil were more susceptible to death (50% mortality over 2 d) than those of dams fed fish oil (25% mortality over 2 d).

The influence of feeding rats diets containing 100 g corn oil, 50 g corn oil plus 50 g coconut oil, or 10 g corn oil plus 90 g coconut oil/kg throughout pregnancy on lymphoid tissue weights and immune cell functions in the offspring was determined (165); the rats were transferred to standard laboratory chow once they had given birth. Spleen lymphocyte proliferation in the offspring at birth was higher if the maternal diet contained 90 g coconut oil. Spleen and thymus weight at weaning increased as the amount of corn oil in the maternal diet increased. Spleen and thymus lymphocyte proliferation at weaning were little affected by maternal diet, but tended to be lowest in the offspring of dams fed the 100 g corn oil/kg diet. Spleen NK cell activity at weaning was highest in the offspring of dams fed the 100 g corn oil/kg diet.

These studies indicate that the nature of the fatty acids in the maternal diet during pregnancy can influence lymphoid tissue development in the offspring, immune cell function in the offspring, and the ability of the offspring to withstand infectious challenges. It is not clear how long the influence of the fatty acid composition of the maternal diet affects the offspring. Nor is it clear how the fatty acid composition of the maternal diet affects the subsequent development of Th1- or Th2-type immunological diseases or subsequent resistance to infection. There is a clear need to explore this area further in appropriate animal models and in human epidemiological and intervention studies.

## GENERAL REMARKS

Among the fatty acids, it is the n-3 PUFA that possess the most potent immunomodulatory activities, and among the n-3 PUFA, those from fish oil (EPA and DHA) are more biologically potent than ALNA. Components of both natural and acquired immunity, including the production of key inflammatory mediators, can be affected by n-3 PUFA. Animal studies indicate that diets rich in EPA plus DHA are anti-inflammatory and immunosuppressive *in vivo*, although there have been relatively few good studies in humans. Although some of the effects of n-3 PUFA may be brought about by modulation of the amount and types of eicosanoids made, it is possible that these fatty acids might elicit some of their effects by eicosanoid-independent mechanisms, including actions upon intracellular signaling pathways and transcription factor activity (see Refs. 49,50). Such n-3 PUFA–induced effects may be of use as a therapy for acute and chronic inflammation, and for disorders that involve an inappropriately activated immune response. Moderate levels of AA and DHA do not appear to have any detrimental effects on human immune function, but the effects of these fatty acids have been studied only in healthy adults. The effect of fatty acids during pregnancy upon the maternal immune system and upon that of the infant are not known.

All studies of fatty acids and the human immune system have used adults as subjects, and most studies have used men only or a mixture of men and women. The only study that used women exclusively as subjects was that of Meydani *et al.* (112); in that study, it was found that the immune system of older women is more sensitive to fish oil than is that of young women. This age-related difference in sensitivity to dietary intervention may explain some of the contradictory observations in the literature [e.g., between (24) and (37, 38)]. It is clear that more needs to be understood about the effect of n-6 and n-3 PUFA on the human immune system and on how variations in age, gender, ethnicity, hormone status, antioxidant status, and genetics influence sensitivity to dietary PUFA. Long-chain n-3 PUFA have been used in a range of diseases characterized by dysregulated Th1- or Th2-type responses and, in a small number of studies, in trauma patients at risk of the systemic inflammatory response syndrome. In these situations, long-chain n-3 PUFA have been beneficial as therapeutic agents. It is not clear what the differential roles of n-6 and n-3 PUFA are in regulating the development of the immune system and how they might affect the likelihood of an individual developing a disease with an immunological component. Clearly, the interactions among PUFA intake, environmental factors, and genetics must be established if we are to fully understand the roles of n-6 and n-3 PUFA in the development and action of the human immune system.

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# Polyunsaturated Fatty Acids and T-Cell Function: Implications for the Neonate

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**ABSTRACT:** Infant survival depends on the ability to respond effectively and appropriately to environmental challenges. Infants are born with a degree of immunological immaturity that renders them susceptible to infection and abnormal dietary responses (allergies). T-lymphocyte function is poorly developed at birth. The reduced ability of infants to respond to mitogens may be the result of the low number of CD45RO+ (memory/antigen-primed) T cells in the infant or the limited ability to produce cytokines [particularly interferon-γ, interleukin (IL)-4, and IL-10]. There have been many important changes in optimizing breast milk substitutes for infants; however, few have been directed at replacing factors in breast milk that convey immune benefits. Recent research has been directed at the neurological, retinal, and membrane benefits of adding 20:4n-6 (arachidonic acid; AA) and 22:6n-3 (docosahexaenoic acid; DHA) to infant formula. In adults and animals, feeding DHA affects T-cell function. However, the effect of these lipids on the development and function of the infant's immune system is not known. We recently reported the effect of adding DHA + AA to a standard infant formula on several functional indices of immune development. Compared with standard formula, feeding a formula containing DHA + AA increased the proportion of antigen mature (CD45RO+) $CD4^+$ cells, improved IL-10 production, and reduced IL-2 production to levels not different from those of human milk-fed infants. This review will briefly describe T-cell development and the potential immune effect of feeding long-chain polyunsaturated fatty acids to the neonate.

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Infant survival depends in part on the ability to respond effectively and appropriately to environmental challenges. The required immune response to such a challenge is the result of the interaction among immune cells, signals, and other cells. Neonates are born with some degree of immunological immaturity, affecting both antibody- and cell-mediated function (1–8) and making them highly susceptible to infection (9). The developing neonatal immune system has functional, albeit limited, defensive, homeostatic, and surveillance capabilities; several excellent reviews of the development of the cellular components of the neonatal immune system and their physiologic interrelationships and limitations have been published (1,10). T-lymphocyte (T-cell) function is one of the most poorly developed systems at birth and is central to defense against infectious agents. This review will briefly describe T-cell lymphocyte function, review our current understanding of T-cell development in the newborn, and summarize the work on the effect of dietary long-chain polyunsaturated fatty acids (PUFA) on T-cell function (in the adult). The final section will highlight some work by our group and others that has examined the implications of feeding commercial infant formula with and without added long-chain PUFA on the development of the T-cell function in infants.

## T-CELL FUNCTION

The immune system is defined as part of the host's defense against destructive forces from outside the body (bacteria, viruses, and parasites) or those from within the body (malignant and autoreactive cells). The immune system can be divided loosely into two separate but interacting and interdependent arms, the innate and the acquired immune system (reviewed in Refs. 11,12).

Innate (natural) immune defenses are those components of the immune system (macrophages, monocytes, neutrophils) that do not depend on prior exposure to a particular antigen. This defense system provides the early phase of host defense, protecting the organism during the first 4–5 d before lymphocytes have been activated. Cytokines, growth factors, hormones, and lipid-derived molecules collectively control the proliferation, survival, differentiation, and function of immune cells (11,13,14).

The acquired or adaptive immune system is composed primarily of the cell-mediated and humoral responses (Fig. 1). Acquired immunity develops over the lifetime of individuals as they respond to their environment. T cells are an important part of this arm of the immune system and function by modulating the function of other immune cells and/or directly destroying cells infected with intracellular pathogens. The diverse host defense and immunoregulatory functions of T cells are performed by phenotypically heterogeneous subpopulations within two basic arms, CD4 (helper/inducer) and CD8 (suppressor/cytotoxic) cells. Maturation and expansion of T cells, a process essential to combating invading organisms, begins with the migration of mature T cells from the thymus,

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; NK, natural killer; PHA, phytohemagglutinin; PUFA, polyunsaturated fatty acids; sIL-2R, secretatory IL-2 receptor; Th, helper T cells.

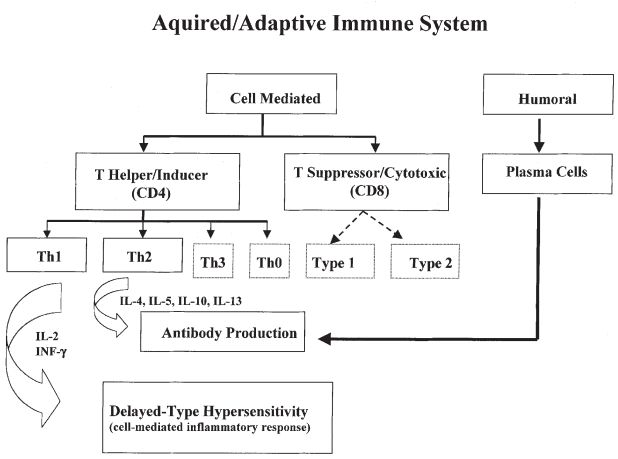


**FIG. 1.** The adaptive/acquired immune system. Th, helper T cells; IL, interleukin; INF, interferon.

and perhaps the gut, to the periphery. Here the cells encounter antigens by an interaction of their T-cell receptor with an antigenic peptide presented in association with major histocompatibility complex (MHC) class I or II molecules on the surface of a specialized antigen-presenting cell, i.e., a macrophage or B cell (15). Each T cell will generate a unique receptor through rearranging its receptor genes, which enables the cell to respond to a specific antigen.

The CD4 subset has been further subdivided on the basis of the type of cytokines produced and secreted (Fig. 1). The identification of helper T (Th) cell subsets has greatly improved the understanding of the regulation of immune effector functions (16). Th1 cells produce interleukin (IL)-2 and interferon (IFN)-γ, which generally promote a cell-mediated inflammatory response (17). Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which support a humoral response (17). Recently, a cytokine pattern defined as Th0 and Th3 has been described (18,19). Although Th1 and Th2 cytokine profiles are not so clearly defined in humans as they are in mice, it has been hypothesized that the balance between these two responses may explain how the immune system can respond appropriately to different challenges (15). Cytokine secretion is not confined to $CD4^+$ T cells; rather, Type 1 and Type 2 populations of $CD8^+$ T cells have been generated *in vitro* and have been isolated from *in vivo* situations (20).

T cells that reside in mucosal-associated lymphoid tissues are important in the protection against potentially infectious agents and other antigens that might gain entry through mucosal surfaces (21). This system includes immune cells that are found in the skin, the urinary tract, the respiratory tract, and the gastrointestinal tract (22–24). Most important for the neonate is the gastrointestinal mucosal immune system, a complex system with multiple interacting cells. This system is composed of T cells and other immune cells that reside in specialized lymph tissues known as Peyer's patches and in the intraepithelial and lamina propria regions of the gut (22,23). Immune cells work together to limit antigen passage from the gut lumen and thus promote normal antigenic response and ensure systemic tolerance (22,23). Because gastrointestinal infections are a major problem in infants (25), T cells in this region likely play an important role to the outcome of contact with infectious organisms (26). It has been hypothesized that the slow rate of maturation of this immune barrier might contribute to pathological inflammatory conditions that are frequently encountered by infants such as necrotizing enterocolitis, milk-protein enteropathy, and enteric bacterial infections (3,22).

## T-CELL DEVELOPMENT IN THE TERM NEONATE

Technical developments in immunophenotyping and function testing have greatly facilitated studies on the developing lymphocyte system in the infant (reviewed in Refs. 2,27). It is well established that newborn infants (term and preterm) have a higher absolute numbers of white blood cells compared with adults (28). As the infant ages, there is a progressive decline in the peripheral concentration of leukocytes, total lymphocytes and T cells, and natural killer (NK) cells (29–31). However, within the peripheral circulation, the proportion of T cells decreases without any changes in the relative proportion of $CD8^+$ and other cytotoxic cells (29,32,33), resulting in a decrease in the CD4/CD8 ratio (32,33). The following section will review what is known about T-cell maturation in the peripheral blood of term and preterm infants.

*T-cell maturation in the term infant*. The expression of a number of cell surface molecules is different on naïve and mature T cells. In the infant, the most frequently studied markers that delineate antigen naïve and mature T cells are the variant isoforms of CD45 (the common lymphocyte antigen), the CD45RA isoform (molecular weight 190,000–220,000), and the CD45RO isoform (molecular weight 180,000). $CD45RO^+$ T cells are believed to identify primed/memory (antigen-educated) cells and are derived from $CD45RA^+$ (naïve) cells after antigenic or mitogenic stimulation (34). The CD45RA antigen is first expressed late during intrathymic maturation and continues to be expressed by most circulating T cells in the infant (35–37). With increasing age and antigenic exposure, these phenotypically immature cells decline progressively in both absolute numbers and percentages. Recently it has been reported that they do not reach adult values until 2–4 y of age (38). With age, $CD4^+CD45RA^+$ cells have been shown to differentiate into $CD4^+CD45RA^-/CD45RO^+$ "memory" cells. These "primed/memory" cells are the predominant isoform of expressed T cells in the adult (30–40% of adult $CD4^+$ and $CD8^+$ cells) and convey most of the functions associated with T cells (2,34,35,38–40).

Clinical evidence has indicated that the neonatal immune response (cell-mediated) to primary infection is delayed compared with that of adults with the same primary infection (3). Additionally, the functional capacity of T cells (both *in vivo* and *in vitro*) to respond to stimulation in the neonate is reduced compared with adults (1,2,41). There are several possible mechanisms to explain this immaturity. The reduced ability to respond to mitogens may be the result of the low number

of $CD45RO^+$ (memory) cells in the infant (32,36,37). Analyses of the functional capabilities of $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ cells have shown that proliferative responses to "memory" recall antigens and the ability to provide help for antibody production are functions uniquely performed by $CD4^+CD45RA^-CD45RO^+$ cells (35,42). Extensive phenotypic analysis of naïve and memory T cells shows that the latter express greater amounts of adhesion molecules and low-level antigens indicative of activation (42). Alternatively, the major immunoregulatory functions described for $CD4^+CD45RA^+$ cells (those found in infants) involve suppression of immune responses, either directly or *via* the induction of suppressor activity by $CD8^+$ (35,43). Indeed, a high level of T-cell–mediated suppression has been reported in infant mononuclear cells (44).

It has been suggested that the immunological immaturity and susceptibility to infection of healthy neonates is the result of lower or altered expression of various cytokines (45). Neonatal peripheral T cells produce a limited repertoire of cytokines in response to activation (8,9,31,40,41,45–47), with steady improvement after birth (31). Specifically, the production of IFN-γ and IL-4, which participate in the maturation of cytotoxic cells, activation of macrophages, and maturation and modulation of B cell function and isotype expression, is reported to be reduced as much as 10-fold, compared with cells from adults (31,40,41,48). The higher susceptibility of newborns to fungal, viral, protozoan, and certain bacterial infections has been proposed to be due in part to a decreased IFN-γ production (9). Reports on IL-4 production in the neonate are a little less consistent. Transcriptions of IL-4, as determined by nuclear run-on assays, and IL-4 mRNA-containing cells, as determined by *in situ* hybridization, have been reported to be undetectable in neonatal T cells, whereas both are detectable in adult T cells (40). However, it was found recently that neonatal cells are able to produce IL-4 at levels comparable to that of adults (31). Human neonates exhibit broad immune deficits that parallel actions of IL-10. IL-10 suppresses phagocytic immune responses and accentuates humoral responses (49). In support of this hypothesis, the ability to produce IL-10 has been reported to be diminished in neonatal blood cells (31,50).

Neonatal cells (at least from full-term infants) appear to be able to make IL-2 with mitogen stimulation at levels equal to those of adults (31,40,51). Despite immature CD4 cells being able to make IL-2, only $CD45RO^+$ cells are found to be able to be stimulated by IL-2 (52). This may be due to a lower expression of IL-2 receptors on neonatal T cells (30,36). It has been suggested that the favoring of a type-1 response (increased IL-2 production) early in immune development may contribute to the ontogeny of the infant's immune system (31).

The mechanisms by which T lymphocytes acquire the capacity to produce cytokines during intrathymic and extrathymic development are poorly understood. The difference in cytokine expression in neonatal T cells has been suggested to be due to diminished transcription of these genes (possibly due to differences in essential promoter elements regulating transcription) in response to activation (41). Reduced IFN-γ production by neonatal T cells has been found to correlate with CD45RO expression, suggesting that extrathymic generation of memory T cells during postnatal life may result in an increased capacity for IFN-γ gene expression (40).

T cells do not function in isolation, and there are numerous reports of both humoral (4,7,47,53) and phagocytic (4,6,54) functions being impaired or immature in the neonate. These most certainly contribute to the increased tendency for the infant to develop infections. Owing to the constraints of sampling, little is known about T-cell development at other immune sites in the healthy infant. Additionally, the gastrointestinal immune system is reported to be poorly developed at birth, maturing with both age and antigenic stimulation (3).

*The contribution of human milk to T-cell function and development in the neonate*. Epidemiologic and clinical evidence indicates that there is a lower occurrence of infections (acute respiratory and diarrhea) in breast-fed infants >6 mon of age (25). In support of breast milk providing direct immune protection to the infant, there is considerable evidence that human milk contains a complex mixture of immunologically active components, such as immunoglobulins, immune cells, prostaglandins, cytokines (including IL-2, IL-10, IL-12), soluble cytokine receptors and other proteins (55–62). Many of these immune constituents have been shown in other models to provide both specific and nonspecific defenses against infectious agents in the gastrointestinal tract, but the physiological role of their consumption through breast milk has not been established. Additionally, the T cells found in breast milk may play a functional role in the infant's immunity. $CD4^+$ cells in human milk were found to express mainly the CD45RO isoform and two- to threefold more IL-2R than CD4 cells in adult blood (56). $CD8^+$ cells in human milk were also reported to express more activation markers (HML-1 and VLA-1) than the same population in adult blood (56).

Breast milk may also play a role in regulating the development of antigen-specific T-cell immunity in neonates. Breast-fed infants ingest a wide array of immunologically active ingredients present in maternal milk during a period of rapid maturation of gut-associated and peripheral lymphoid tissues. Although there have been few studies in this area, it is likely that human milk serves an immunodevelopmental role in the infant (reviewed in Ref. 58). At present, infant formula in North America does not contain the immune active substances found in human milk. This suggests that the infant who is not fed human milk is at an immunological disadvantage. As discussed above, there have been a number of papers over the past 20 yr describing the distribution of lymphocyte surface antigens in healthy neonates; more recently this has been extended to compare peripheral blood phenotypes between a large group of formula- and breast-fed infants (60). The concentration and percentage of T ($CD8^+$) and B lymphocytes in the peripheral blood of 6-mon-old infants were found to be the same, regardless of feeding regimen. However, breast-fed infants were found to have fewer $CD4^+$ T cells and more NK cells than the age-matched formula-fed

infants (60). Without maturation or functional data, the physiological consequences of these small changes are unknown; however, these descriptive data do support the hypothesis that diet/feeding regimen can affect the infant's immune system.

## T-CELL DEVELOPMENT IN THE PRETERM INFANT

Considerable maturation of peripheral T lymphocyte populations is believed to occur during the final trimester of gestation (41,63). This suggests that birth before the completion of this maturation process would result in an infant with a less mature immune system. This likely contributes to the increased susceptibility of preterm infants to illness and infection. Few studies have examined immune development in preterm infants.

In our recent study, unlike a number of reports on full-term infants (39,64), the percentage of $CD45RO^+$ T cells in peripheral blood obtained from preterm infants did not increase significantly during the first 42 d of life (33). Delayed expression of this isoform supports the hypothesis that T-cell maturation is delayed in preterm infants. The lower number of $CD45RO^+$ T cells, compared with the full-term infant, likely contributes to the higher susceptibility to infection in these high-risk infants (43). Consistent with the lower proportion of memory/antigen-mature T cells, we found that at 42 d of age, the proportion of $CD4^+CD45RA^+$ cells in preterm infants was higher (33) than that in the literature for full-term infants (2,34,39,64). Contrary to that reported in adults (41) and full-term infants, after 3 mon of age (39,64), ~10–20% of peripheral $CD4^+$ and $CD8^+$ cells in 42-d-old preterm infants did not express either of the CD45 isoforms (33). Lack of CD45 antigen expression (which appears during intrathymic development) further reflects the immaturity of the preterm infant's T-cell immune system (65). It has been suggested that the apparent lack of CD45 expression may be due to the maturation process, which involves a switch of isoform from $CD45RA^+$ to $CD45RO^+$ on peripheral cells (66). It is possible that the cells from the preterm infant may be expressing a very small amount of CD45 RA and RO that was not detectable by immunofluorescence during this process (66).

Compared with full-term infants, IFN-γ production (not due to a decrease in the relative proportion of T cells) in response to mitogens was reported to be even lower in lymphocytes from preterm infants (46). Unlike IFN-γ, IL-2 production by phytohemagglutinin (PHA)-stimulated peripheral mononuclear cells was reported in preterm infants to be higher than that in adults (46,51). The mechanism for these differences is not clear but may be due to alterations in the mRNA for the IL-2 receptor (51).

## PUFA AND T-CELL FUNCTION (ADULT AND ANIMALS)

There is considerable evidence that both the amount and type of dietary fat influence T-cell function (reviewed in Refs. 67,68). Animal studies have clearly demonstrated that the content of long-chain PUFA n-3 fatty acids and/or the ratio of n-6/n-3 fatty acids in the diet modulates many measures of T-cell function. These include the ability to proliferate in response to mitogen/antigen stimulation and to mount a delayed-type hypersensitivity response (68–73). The physiological interpretation of how T-cell function is altered by these lipids is far from clear. This is likely due in part to the wide variation in types of measures and the amounts of PUFA fed to both animals and humans.

Diets rich in n-3 PUFA are generally associated with suppression of cell-mediated immune responses {estimated by [$^3$H]thymidine uptake (71,73)}. Long-chain n-3 PUFA have been demonstrated both *in vivo* (in adult humans and animals) and *in vitro* to reduce IL-2 production (72,74,75). Recently, it was suggested that immunosuppressive and anti-inflammatory effects (delayed-type hypersensitivity response) of feeding fish oil to mice was due to the docosahexaenoic acid (DHA) rather than the eicosapentaenoic acid (EPA) in fish oil (75). Others have shown that the inhibitory effects of DHA on immune cell functions varied with the cell type, and these effects are not mediated through increased production of prostaglandin $E_2$ and leukotriene $B_4$ (76). Results like these have raised questions concerning the efficacy of feeding DHA to infants, particularly preterm infants.

Contrary to many of these studies, we have demonstrated in a healthy animal model that feeding long-chain n-3 PUFA can increase the response to mitogens when measures other than [$^3$H]thymidine incorporation are used (77). For example, we found an increased expression of activation markers (77) and a higher production of IL-2, IFN-γ, and nitric oxide by splenocytes from young rats fed a mixture of EPA and DHA (78). Recently, it was suggested that diets rich in DHA exert some of their immunomodulatory effects by a downregulation of surface expression of CD4 and CD8 and by an upregulation of a co-stimulatory signal [CD28 expression; (79)]. It was also reported that consumption of n-3 fatty acids may have slowed the inflammatory response compared with diets high in n-6 fatty acids, but that feeding n-3 lipids did not compromise overall immune potential (80). From animal and human work, it appears that feeding long-chain n-3 fatty acids is not clearly immunosuppressive. Because the T-cell response is the end point of a number of different components of the immune system and their interaction with other systems, one must examine more closely the type of response rather than simply the magnitude of a single estimate of T-cell function.

## PUFA AND T-CELL DEVELOPMENT/FUNCTION IN THE NEONATE

There are many considerations associated with the development of an optimal formula for infants. Preterm infants have been found to have an essential PUFA status significantly lower than that of term neonates (81). Most recently, emphasis has been placed on inclusion of 20:4n-6 (arachidonic acid; AA) and 22:6n-3 (DHA) fatty acids to promote optimal neurological and retinal function and membrane composition,

particularly in the preterm infant (82,83). As discussed above, a number of studies suggest that DHA added *in vitro* or fed to adults or animals may suppress some measures of T-cell function (67,72–74,84–86). Despite the amount of work done in healthy adults and human diseases and animal models of disease, little work has been done on the effect of PUFA on T-cell development in infants.

We recently attempted, within the limitations of tissue availability, to study the effect of adding long-chain PUFA to a standard infant formula on several functional indices of immune development (33). A large group of medically stable preterm infants were fed human milk, standard preterm infant formula, or a preterm infant formula containing DHA (0.4%) and AA (0.6%) for the first 42 d of life. With blood samples obtained at 14 and 42 d of age, the effect of diet on some parameters of immune development was studied. Compared with standard formula, feeding long-chain PUFA significantly increased the proportion of antigen mature ($CD45RO^+$) $CD4^+$ cells (by ~25%) compared with nonsupplemented formula-fed infants and lowered the proportion of immature ($CD45RA^+$) $CD4^+$ cells to levels not different from human milk-fed infants (33). These changes in the sialylation of the CD45 region (RA/RO) are believed to reflect the maturation of the immune system (87). Our data suggest that adding DHA and AA to preterm formula may have assisted in the maturation of peripheral $CD4^+$ cells.

Because $CD45RO^+$ (memory T cells) differ in their ability to synthesize and respond to a variety of cytokines *in vitro* (52), we also measured the effect of the three dietary treatments on the ability of lymphocytes to synthesize two key cytokines (IL-10 and IL-2) in response to stimulation with PHA. Between 14 and 42 d of age, the ability of peripheral mononuclear cells from unsupplemented formula-fed infants to produce IL-10 was lower than that of infants fed human milk. IL-10 production by cells from infants fed the formula containing DHA + AA did not differ from that of the human milk-fed infants. IL-10 production by stimulated peripheral lymphocytes has been reported by others to be lower for cells from preterm infants compared with adults and has been suggested to be due to differences in cellular maturation (50). IL-10 plays an important regulatory role in both cellular and humoral immunity; after activation, it is synthesized primarily by $CD45RO^+CD4^+$ cells (49,88). IL-10 is produced by T cells ($CD4^+$ Th2 cells), B cells, macrophages/monocytes, and keratinocytes. This cytokine can profoundly alter the expression of MHC class II antigens and the production of cytokines by monocytes, which in turn affect a variety of immunological responses including inhibition of Th1-type functions (anti-inflammatory), promotion of T-cell development, and increased proliferation, differentiation, and activation of B cells (16,49,88). Recently, it was suggested that an increase in IL-10 production, together with a decreased IL-12 production, is important in the induction of oral tolerance (89). To our knowledge, our study was the first to measure IL-10 production by lymphocytes from preterm infants fed different diets and suggests that the addition of DHA + AA to formula improves the ability of peripheral mononuclear cells from formula-fed infants to produce IL-10.

Unlike IL-10 (50) and most other cytokines (31,40, 41,45,48), neonatal cells stimulated with PHA have been reported to produce IL-2 at rates similar to those of adults (41). Feeding formula containing DHA + AA to preterm infants resulted in a significant decrease in the amount of secretory IL-2 receptor (sIL-2R) produced by stimulated peripheral mononuclear cells at 42 d of age compared with 14 d (33). Despite the lower level of sIL-2R compared with the unsupplemented formula, in the (DHA + AA)-supplemented formula group, the amount of sIL-2R produced by the DHA + AA group at both 14 and 42 d of age did not differ from the human milk-fed group. The activation and clonal expansion of T lymphocytes by IL-2 (produced by mitogen- or antigen-activated T cells) is mediated by IL-2 receptors (IL-2R) in such a manner that the level of activation usually corresponds to the level of expression of the IL-2R (90). During activation, sIL-2R is cleaved off and its concentration is used to estimate lymphocyte activation (91). *In vitro*, IL-2 is produced by activated Th1, and more recently, Th0-type CD4 cells [both $CD45RA^+$ and $CD45RO^+$ (40,92,93)]. The precise role of this cytokine *in vivo* is not known; however, a decreased production of IL-2 in response to an *in vitro* mitogen challenge has been interpreted to indicate a reduced capacity to recruit T cells (8,94). Thus, the physiological effect of reduced production between 14 and 42 d of age in the supplemented formula group requires the further study of Th1- and Th2-type cytokines. Despite immature CD4 cells being able to make IL-2, only $CD45RO^+$ cells are able to proliferate in response to mitogens (52). Proliferation was not directly measured in our study; however, the lower production of IL-10 by unsupplemented formula-fed infants, compared with human milk-fed infants, suggests a poorer proliferative response.

The mechanisms for the effects of adding AA + DHA to infant formula on immune maturation and cytokine production have not been established, but changes in fatty acid status (83) and lymphocyte membrane lipid essential fatty acid composition (33) are likely contributors. In lymphocytes, plasma membrane-associated events play an important role in immune functions such as the processing and recognition of antigens, expression of activation marker, and generation of membrane-mediated activation signals (reviewed in Ref. 74).

In conclusion, immunity is the end point of a number of different components of the immune system and their interaction with other systems. T lymphocytes are an important cellular component of this arm of the immune system that can both modulate the function of other immune cells and directly destroy cells infected with intracellular pathogens. At birth, T-cell function is not well developed and contributes to the immune immaturity of the infant, particularly the preterm infant. Interest and energy are currently being directed at identifying the role of specific nutrients in optimizing immune function. Our work suggests that the addition of small amounts of DHA and AA (at levels similar to that in human milk) to preterm infant formula can influence the concentration,

proportion, maturation, and cytokine production of peripheral blood lymphocytes (33). The goal of future studies is to determine whether the addition of long-chain essential fatty acids improves immune development and the ability of the neonate to respond to antigenic (bacterial, viral, parasitic, and dietary) challenges from the environment.

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# Polyunsaturated n-3 Fatty Acids and the Development of Atopic Disease

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**ABSTRACT:** The relationship between polyunsaturated long-chain fatty acids and atopy has been discussed for decades. Higher levels of the essential fatty acids linoleic acid and α-linolenic acid and lower levels of their longer metabolites in plasma phospholipids of atopic as compared to nonatopic individuals have been reported by several, but not all, studies. Largely similar findings have been reported in studies of cell membranes from immunological cells from atopics and nonatopics despite differences in methodology, study groups, and definitions of atopy. An imbalance in the metabolism of the n-6 fatty acids, particularly arachidonic acid and dihomo-γ-linolenic acid, leading to an inappropriate synthesis of prostaglandin (PG) $E_2$ and $PGE_1$ was hypothesized early on but has not been corroborated. The fatty acid composition of human milk is dependent on the time of lactation not only during a breast meal but also the time of the day and the period of lactation. This explains the discrepancies in reported findings regarding the relationship between milk fatty acids and atopic disease in the mother. Prospective studies show disturbances in both the n-6 and n-3 fatty acid composition between milk from atopic and nonatopic mothers. Only the composition of long-chain polyunsaturated n-3 fatty acids was related to atopic development in the children, however. A relationship between lower levels of n-3 fatty acids, particularly eicosapentaenoic acid (20:5 n-3), and early development of atopic disease is hypothesized.

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Linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) are essential fatty acids; that is, they cannot be synthesized by humans and thus they must be provided by the food. They compete for the same enzyme systems for desaturation–elongation of the carbon chain and are thus precursors to the long-chain polyunsaturated fatty acids (LC-PUFA) of the n-6 and the n-3 series, dihomo-γ-linoleic (DHGLA, 20:3n-6), arachidonic acid (AA, 20:4n-6), docosatetraenoic acid (DTA, 22:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) (1).

For many years, the accepted pathway, i.e., the classical pathway, has been one of alternating desaturation and elongation steps ending in the Δ4 desaturation of 22:4n-6 and DPA to 22:5n-6 and DHA, respectively (Fig. 1). In recent years, an alternative pathway has been suggested involving a Δ6-desaturation, elongation, and further β-oxidation of 22:4n-6 and DPA to 22:5n-6 and DHA, respectively. In fact, the latter has been suggested to be the primary pathway in adults (2). Newborn infants have an immature metabolic system, although desaturation and elongation of PUFA have been shown in formula-fed babies (3). It has been proposed that newborns use an alternative pathway involving an elongation of LA (and ALA) to 20:2n-6 (and 20:3n-3) and subsequent Δ8- desaturation to DHGLA (and 20:4n-3) (Fig. 1).

The desaturation–elongation products from the metabolism of polyunsaturated fatty acids (PUFA) are precursors to com-

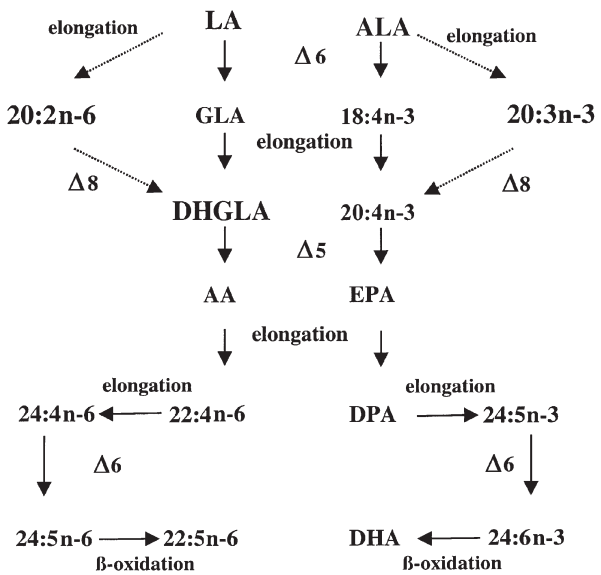


**FIG. 1.** The metabolic pathways of polyunsaturated fatty acids (PUFA). Linoleic acid (LA) and α-linolenic acid (ALA) are metabolized by the classical pathway in adults (arrows). The main metabolic step in the synthesis of 22:5n-6/22:6n-3 is through an elongation and Δ6 desaturation, rather than a Δ4 desaturation. An alternative pathway is used in infancy (dotted arrows) (2). GLA, γ-linolenic acid; AA, arachidonic acid; DHLGA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

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Abbreviations: AA, arachidonic acid, 20:4n-6; ALA, α-linolenic acid, 18:3n-3; APC, antigen-presenting cells; DHA, docosahexaenoic acid, 22:6n-3; DHGLA, dihomo-γ-linoleic acid, 20:3n-6; DPA, docosapentaenoic acid, 22:5n-3; DTA, docosatetraenoic acid, 22:4n-6; EFA, essential fatty acids; EPA, eicosapentaenoic acid, 20:5n-3; GLA, γ-linolenic acid, 18:3n-6; Ig, immunoglobulin; IL, interleukin; LA, linoleic acid, 18:2n-6; LC-PUFA, long-chain polyunsaturated fatty acids; $LTB_4$, leukotriene $B_4$; $LTB_5$, leukotriene $B_5$; MCSFA, medium-chain saturated fatty acids; $PGE_1$, prostaglandin $E_1$; $PGE_2$, prostaglandin $E_2$; PUFA, polyunsaturated fatty acids; Th, T helper.

pounds that are important for the structural function of cellular membranes. Thus, the proportion of unsaturated fatty acids in cellular membranes is the main factor regulating membrane fluidity (4). Furthermore, membrane LC-PUFA composition also modulates the activity and conformation of enzymes such as adenylate cyclase and $Na^+/K^+$ ATPase, and it may also influence activity and conformation of membrane-bound receptors (4).

DHGLA, AA, and EPA are the main precursors of prostaglandins and leukotrienes (5). These fatty acids are bound to cell membrane phospholipids. They are released by phospholipase $A_2$ for the generation of prostaglandins $E_1$ ($PGE_1$) and $E_2$ ($PGE_2$) after oxygenation of the n-6 fatty acids DHGLA and AA, respectively (6). Lipoxygenation of AA yields leukotriene $B_4$ ($LTB_4$) (6). Several cell types produce eicosanoids: neutrophils are activated by lipopolysaccharides to the synthesis of $LBT_4$ and induce leukocyte aggregation and adhesion to vascular endothelium, increased vascular permeability in postcapillary venules, and enhanced mucus secretion in inflammatory sites (7). Monocytes and macrophages are significant producers of DHGLA and AA metabolites, particularly $PGE_2$, which inhibits the production of interleukin (IL)-2 and interferon-γ (IFNγ) (8,9) and primes naive $CD4^+$ cells in cord blood to produce IL-5 and IL-4 (9). $PGE_2$ also enhances *in vitro* proliferation and differentiation of mast cells (10) as well as the IL-4-mediated immunoglobulin (Ig) E antibody class switch in uncommitted B-lymphocytes (11). Furthermore, enhanced $PGE_2$ production in antigen-presenting cells (APC) commits naive T helper (Th) cells toward a Th2-like cytokine pattern favoring IgE production (12,13). Furthermore, IL-4 induces CD23 membrane expression in monocytes, partly through the induction of $LTB_4$ synthesis (14). The IL-4-induced IgE production in humans can be further stimulated by $LTB_4$, through an increase of IL-4R positive cells and release of sCD23 from mononuclear cells.

The n-3 fatty acids, especially EPA and DHA, have also been shown to modulate cytokine responses such as IL-1β production by human mononuclear cells after stimulation with endotoxin and *in vitro* lymphocyte proliferation (16). Dietary treatment with n-3 fatty acids in inflammatory disorders such as ulcerative colitis and rheumatoid arthritis provides some clinical improvement of the disease (1), suggesting anti-inflammatory properties, especially of the n-3 LC-PUFA. This could be explained by the competitive inhibition of AA cyclooxygenation by the n-3 fatty acid EPA and production of less inflammatory compounds of the PG and the LT series, e.g., $PGE_3$ and $LTB_5$ (5).

## PUFA AND ATOPIC DISEASE

An abnormal metabolism of essential fatty acids (EFA) and LC-PUFA has been discussed in atopic disease. It has been suggested for decades that atopic disease in adults and children is associated with higher plasma levels of LA (18:2n-6) than in healthy controls (17) while the levels of γ-linolenic acid (GLA, 18:3n-6), DHGLA (20:3n-6), and AA (20:4n-6) are lower than those in healthy controls. Furthermore, disturbances in the n-6 and the n-3 series have been observed in several (18–24) but not all studies (25,26). Only one of these reports confirmed the results of the original study by Hansen (17), i.e., higher levels of EFA and lower levels of LC-PUFA in a small group of children with atopic eczema. In the other studies, there were higher EFA levels (21,23) or lower LC-PUFA levels (19,24) in serum phospholipids from atopic children and adults (Table 1).

The PUFA composition of the phospholipid fraction has also been studied in cord blood and early infancy (20,27–30) (Table 2). Disturbances in the n-6 PUFA composition were observed in cord blood of infants with a family history of allergy (20,27,29). In two prospective studies (24,30), however, there were no disturbances in the composition of n-6 or n-3 PUFA in cord serum phospholipids in children who developed atopic disease later in life. Furthermore, higher levels of n-6 and n-3 LC-PUFA have been found in cord blood phospholipids from babies of allergic mothers, as compared to babies of healthy mothers (31). This suggests that disturbances in the PUFA composition in cord blood phospholipids may merely reflect the atopic status of the mother rather than

**TABLE 1**
**Comparison Between Polyunsaturated Fatty Acid (PUFA) Composition of Serum Phospholipids in Atopic and Nonatopic Children and Adults[a]**

| | n-6 PUFA | | | | | | n-3 PUFA | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Study[b] | 18:2 | 18:3 | 20:3 | 20:4 | 22:4 | 22:5 | 18:3 | 20:5 | 22:5 | 22:6 |
| Manku *et al.* (18), 1984 (41A/50NA)§ | H | ND* | LL | LLL | LLL | LLL | — | L | LL | LLL |
| Rocklin *et al.* (19), 1986 (27A/21NA)§ | — | ND | — | — | ND | ND | — | — | ND | LLL |
| Sakai *et al.* (25), 1986 (6A/7NA)§§ | — | — | — | — | — | — | — | — | — | — |
| Strannegård *et al.* (20), 1987 (6A/107NA)§§ | HHH | | LLL | LLL | | | | | | LLL |
| Oliwiecki *et al.* (21), 1990 (48A/33NA) | H | ND | — | — | — | — | — | — | — | — |
| Griese *et al.* (22), 1990 (11A/10NA)§§ | — | — | — | — | — | — | — | H | — | — |
| Lindskov and Hølmer (26), 1992 (13A/12NA)§ | — | — | — | — | — | — | — | — | — | — |
| Leichsenring *et al.* (23), 1995 (17A/10NA)§§ | HHH | — | — | — | | | | | | |
| Yu and Bjorksten (24), 1998 (23A/22NA)§§ | — | — | — | — | — | — | — | — | L[c] | LL[c] |

[a]—, No difference between atopic and nonatopic individuals; H, HH, and HHH, higher levels in atopic individuals at $P < 0.05$ (H), at $P < 0.01$ (HH), and $P \ll 0.001$ (HHH) significance level; L, LL, and LLL, significance levels lower in atopic individuals at $P < 0.05$ (L), at $P < 0.01$ (LLL), and at $P < 0.001$ level; ND, not detected; ND*, not detected in atopic individuals.
[b]A, atopics; NA, nonatopics; §, adults; §§, children.
[c]Controls compared to atopic children with positive skin prick test.

**TABLE 2**
**PUFA Composition of Serum Phospholipids in Early Infancy and the Relation to Atopy or Atopic Heredity[a]**

| | n-6 PUFA | | | | | | n-3 PUFA | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Study[b] | 18:2 | 18:3 | 20:3 | 20:4 | 22:4 | 22:5 | 18:3 | 20:5 | 22:5 | 22:6 |
| Strannegård *et al.* (20),1987 (21/21)[a] | HH | | | | | | | | | |
| Galli *et al.* (27), 1994 | | — | — | LL | LL | — | — | — | — | — |
| Yu *et al.* (28), 1996 (25A/45NA)[b] | — | ND | — | — | — | — | ND | — | — | — |
| Beck *et al.* (29), 2000 (50/50)[c] | — | | — | L | LL | | ND | — | — | — |
| Duchén *et al.* (30), 2000 | | | | | | | | | | |
| (19A/40NA)[d] | — | ND | — | — | — | — | ND | — | — | — |
| (16A/35NA)[f] | — | ND | — | — | H | HH | ND | — | — | — |

[a]—, No difference between atopic and nonatopic individuals; H and HH, higher levels in atopic individuals at $P < 0.05$ (H) and at $P < 0.01$ (HH); L and LL, lower levels in atopic individuals at $P < 0.05$ (L) and at $P < 0.01$ (LL); ND, not detected. For other abbreviation see Table 1.
[b]A, atopics; NA, nonatopics.
[c]Comparision between cord sera with high and low total immunoglobulin E levels.
[d]Cord blood from children with/without atopy.
[e]Cord blood from children with and without atopic heredity.
[f]Serum phospholipids at 3 mon of age.

predict atopic development. It seems, though, that a disturbed metabolism of IgA and PUFA in atopic individuals can be detected early after birth, during the first months of life (30).

Abnormalities in PUFA composition have been reported consistently in cellular membranes from atopic individuals (21,22,26,32,33), even in studies in which the PUFA levels in plasma phospholids were similar in atopic and nonatopic individuals (26). Although the results vary in different cells, higher LA and lower n-6 LC-PUFA and n-3 LC-PUFA are consistently found in atopic individuals (Table 3).

Thus, despite differences in methodology, age of the study group, and type of atopic manifestations in the various studies, it seems reasonable to conclude that atopy is related to a disturbed metabolism of n-6 and n-3 LC-PUFA. The clinical significance of this relationship is still controversial, however. In a controlled study, clinical improvement of atopic eczema was reported after treatment with a GLA-rich oil from evening primrose (Epogam) (34), whereas in another study there was no effect of the treatment (35). Furthermore, the administration of GLA-enriched diet only partially corrects the differences between patients with atopic eczema and healthy individuals (18).

It has been suggested that a defect in the enzyme activity of Δ6-desaturase is present in atopic diseases (36). This would explain the higher levels of LA and lower levels of its longer metabolites in atopic children and adults (Tables 1 and 3, Fig. 1). It would also explain the high levels of 22:4n-6 and 22:5n-6 in atopics during early infancy when the metabolism is immature (Fig. 1). The disturbed correlations between

**TABLE 3**
**Comparison Between PUFA Composition of Cell Membranes in Different Cell Types in Atopic and Nonatopic Individuals[a]**

| | n-6 PUFA | | | | | | n-3 PUFA | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Study[b] | 18:2 | 18:3 | 20:3 | 20:4 | 22:4 | 22:5 | 18:3 | 20:5 | 22:5 | 22:6 |
| Red blood cells | | | | | | | | | | |
| Rocklin *et al.* (32)[b] | — | — | — | H | — | | H | — | — | — |
| Oliwiecki *et al.* (21)[c] | L | | L | L | — | | — | L | L | L |
| Lindskov and Hølmer (26)[d] | — | — | L | — | — | — | — | — | — | — |
| Biagi *et al.* (33)[e] | | HH | LLL | — | — | L | — | — | LL | — |
| Mononuclear cells | | | | | | | | | | |
| Griese *et al.* (22)[f] | — | — | — | — | — | — | — | H | — | — |
| Lindskov and Hølmer (26)[d] | HH | — | — | L | — | — | — | — | — | — |
| Monocytes | | | | | | | | | | |
| Rocklin *et al.* (32)[b] | H | — | — | L | — | — | — | — | — | — |
| Lymphocytes | | | | | | | | | | |
| Rocklin *et al.* (32)[b] | LLL | ND | — | H | — | — | — | — | — | — |

[a]—, No difference between atopic and nonatopic individuals; H and HH, higher levels in atopic individuals at $P < 0.05$ (H) and at $P < 0.01$ (HH) significance level; L, LL, and LLL, lower levels in atopic individuals at $P < 0.05$ (L) at $P < 0.01$ (LL), and at $P < 0.001$ (LLL) significance level; ND, not detected. See Table 1 for abbreviation.
[b]27 controls and 21 patients with allergic rhinitis and/or asthma bronchiale.
[c]32 controls and 26 patients with atopic eczema.
[d]12 controls and 13 patients with atopic eczema.
[e]15 controls and 24 children with atopic eczema and a positive skin prick test.
[f]10 controls and 11 children with atopic asthma bronchiale.

PUFA levels in cord serum phospholipids and atopy during childhood (24,28,31) raise the question of whether such a dysfunction is primary or secondary to a sustained allergic inflammation. The important role of AA in $PGE_2$ and $LTB_4$ metabolism and the relation to allergic inflammation (10–14) have led to the hypothesis that there is a dysregulation of AA metabolism in atopy (37). For example, a higher production of $PGE_2$ has been shown in monocytes from atopic individuals (38,39). Furthermore, reduced proliferative responses of peripheral blood mononuclear cells in adults with atopic dermatitis correlate with the IFNγ levels and are inversely related to T-cell production of IL-4 and the production of $PGE_2$ by monocytes (40). Thus, AA products seem to regulate allergic immune responses. Consequently, an abnormal PUFA metabolism could conceivably be associated with either enhanced or decreased susceptibility to allergic disease. An explanatory model has been suggested involving a disturbed n-6 fatty acid metabolism, particularly the balance between DHGLA and AA and between the PG metabolites $PGE_1$ and $PGE_2$, respectively (41).

## HUMAN MILK FATTY ACIDS AND ATOPY—A CLUE TO A FURTHER UNDERSTANDING?

The PUFA composition in human milk has been studied extensively (42). Human milk contains both medium-chain saturated fatty acids (MCSFA) and PUFA. The latter include the essential fatty acids LA and ALA and small amounts of LC-PUFA (42). The fatty acid composition in human milk differs in mothers of term and premature babies (43), and it is influenced by the maternal food habits, especially in mothers consuming fish or vegetarian diets (44). The composition of milk lipids varies over the day (45) and over the lactation period (46). MCSFA ($C_6$–$C_{14}$) are synthesized in the mammary glands and are probably β-oxidized and used as energy by the newborn (47).

Colostrum is characterized by a high percentage of monounsaturated fatty acids and a low proportion of saturated fatty acids, including medium-chain-length acids, low levels of LA and ALA, and high levels of their LC-PUFA (48). The composition of mature milk is the matter of some controversy, as there are only a few longitudinal studies of PUFA in milk from the same mothers through the lactation period (46,49,50). The levels of EFA (LA and ALA) in mature milk increase while the levels of LC-PUFA decrease during lactation in some (49,50), but not all (46), the studies. However, these findings were not confirmed in a cross-sectional study analysis of pooled milk samples (46).

The relationship between breast-feeding and the development of atopic manifestations early in life has been an issue of controversy for the last 50 yr (51). Differences in milk components that might influence infant immunity could possibly explain some of this controversy. The PUFA composition of human milk is one of several factors that have been studied in relation to the atopic status of the mother (50,52–54) and the development of atopic disease in the children (30,55,56).

The results are, at first view, contradictory. Lower levels of LA, ALA, and most LC-PUFA in both PUFA series have been reported in early transitional milk of atopic, as compared to nonatopic, mothers, but not at 3 mon of lactation (30,50,53) (Figs. 2 and 3). These observations partially agree with studies reporting similar PUFA composition (52) or lower levels of AA in milk from atopic mothers as compared to milk from nonatopic mothers (54) (Table 4). Differences in the composition of colostrum, transitional milk, and mature milk during the lactation period (49,50,53) clearly indicate that milk sampling should be standardized not only with regard to sampling procedure (45) but also with regard to period of lactation. The PUFA composition of transitional human milk, however, agrees well with the reported low levels of n-6 and n-3 LC-PUFA (i.e., DHGLA, AA, EPA, DPA, and DHA) in plasma (19,20,24,57) and membrane (19,21,26, 33) phospholipids in atopic children and adults.

It is known that extreme food habits influence the composition of human milk, particularly the levels of LA and ALA (58). Omnivorous diets influence the levels of saturated and monounsaturated fatty acids and LA but have little effect on the composition of 20- and 22-carbon LC-PUFA (44), with the exception of EPA and DHA levels in women with high intakes of fish (44,59). Maternal food habits did not explain the low levels of LA and ALA in transitional milk from atopic mothers (30) (Fig. 2). Although both the LA and ALA levels were lower at 1 mon of lactation in milk from atopic, as compared to nonatopic, mothers, the LA/ALA ratio was similar in the two groups [8.7 ± 2.8 wt% vs. 7.8 ± 1.5 wt% (mean ± SD), nonsignificant]. In contrast, the n-6 LC-PUFA/n-3 LC-PUFA ratio was higher in milk from the atopic mothers (2.1 ± 0.7 vs. 1.8 ± 0.42, $P < 0.01$), suggesting a relationship between maternal atopy and low n-3 LC-PUFA levels in transitional human milk.

The dual composition of colostrum and early milk may suggest a hormonally induced increasing Δ6-desaturase activity in mothers during pregnancy (60), which then later decreases after delivery. This would explain the increasing levels of LA and ALA and decreasing levels of n-6 and n-3 LC-PUFA (30,50,53) during the lactation period. A disturbed Δ6-desaturase activity in lactating atopic mothers might also explain the lower LA and ALA levels in their milk, as compared to the milk of nonatopic mothers, before reaching a stable activity in mature milk. A defect in the Δ6-desaturase activity previously has been suggested in atopic individuals (36,41) because this enzyme desaturates LA to GLA. The low levels of GLA in milk of atopic mothers (30) corroborate this hypothesis. Thus, a disturbance in the desaturation step may be reflected by low levels of most of the LC-PUFA, in both the n-6 and n-3 series, as has indeed been reported (53).

The Δ6-desaturase is also involved in an alternative final step in the PUFA desaturation and elongation (Fig. 1) (61). This could possibly explain the similar 22:4n-6 and 22:5n-6 levels in milk from atopic and nonatopic mothers (30). The limited capacity of the newborn baby to produce LC-PUFA (3) and the newborn's enhanced nutritional requirements stress the importance of breast-feeding for early human development (43,62,63).

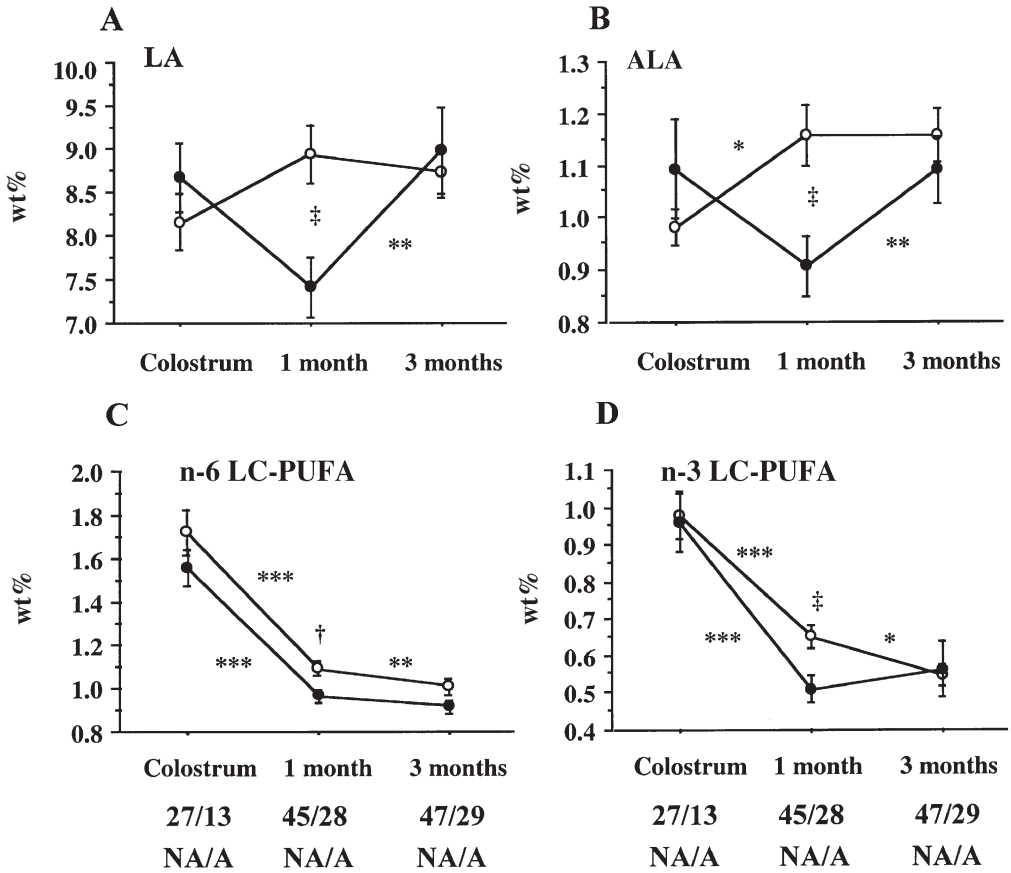


**FIG. 2.** The levels of LA (A), ALA (B), total n-6 long-chain polyunsaturated fatty acids (LC-PUFA) (C), and total n-3 LC-PUFA (D) in milk from atopic (closed circles) and nonatopic mothers (open circles) during 3 mon of lactation. The numbers of atopic (A) and nonatopic (NA) mothers are given for each sampling occasion. Mean levels ± SD are given. $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ for unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P \ll 0.001$ for paired *t*-test (adapted from Ref. 53). See Figure 1 for abbreviations.

## MILK PUFA IN RELATION TO ALLERGY DEVELOPMENT IN CHILDREN

Although there seem to be no nutrient abnormalities in the composition of PUFA in the cord blood of atopic infants, various disturbances seem to appear very early in infancy during the lactation period (Table 4). It is well known that the PUFA composition in maternal milk affects the PUFA status of the child (3,64,65). There are only a few studies of the relationship between the PUFA composition of material milk and development of atopy in the children (30,55,56), and only one of them was prospective (30). A different PUFA composition was demonstrated in milk from the mothers of atopic and nonatopic children in all the studies, despite considerable methodological differences (Table 4). In the prospective study (30), in which 160 allergic and nonallergic children were

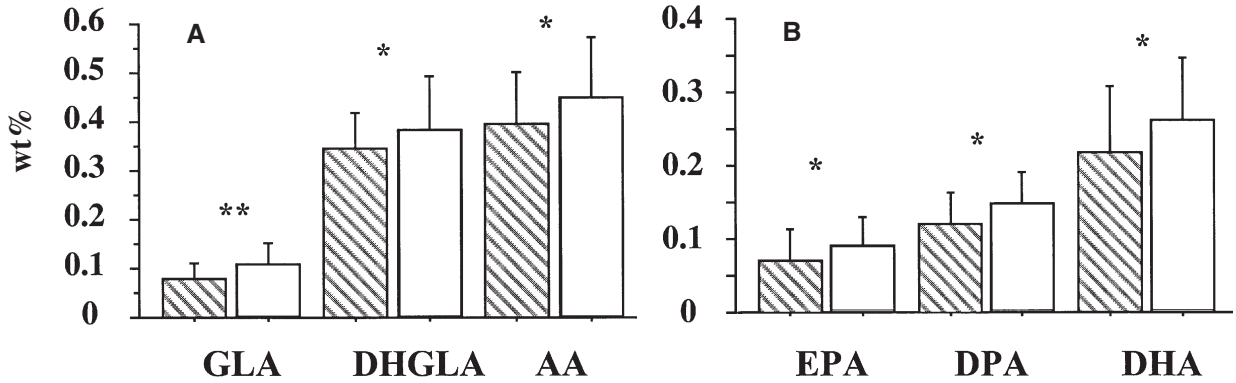


**FIG. 3.** Levels of individual n-6 (A) and n-3 (B) LC-PUFA contributing to the lower levels of total LC-PUFA in transitional milk from atopic (hatched bars) and nonatopic mothers (open bars). Mean levels ± SD are given. $^{*}P < 0.05$ and $^{**}P < 0.01$ for unpaired *t*-test (adapted from Ref. 53). See Figures 1 and 2 for abbreviations.

**TABLE 4**
**Comparison Between PUFA Composition of Total Lipids in Milk from Atopic and Nonatopic Mothers and the Relationship to Atopic Development in the Children[a]**

| | n-6 PUFA | | | | | | n-3 PUFA | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Study[b] | 18:2 | 18:3 | 20:3 | 20:4 | 22:4 | 22:5 | 18:3 | 20:5 | 22:5 | 22:6 |
| Milk from atopic and nonatopic mothers | | | | | | | | | | |
| Schroten *et al.* (52), 1992 (23A/29NA)[c] | — | — | — | — | — | | — | | — | — |
| Yu *et al.* (50), 1998 (17A/17NA)[d] | — | — | L | — | — | — | — | L | LL | L |
| Duchén *et al.* (30), 2000 (28A/45NA)[d] | LL | LL | L | L | — | — | LL | L | L | L |
| Thijs *et al.* (54), 2000 (20A/20NA)[e] | — | — | — | L | | | — | — | | — |
| Milk PUFA composition in relation to atopy in the children | | | | | | | | | | |
| Wright and Bolton (55), 1989 (25A/22NA)[f] | HHH | — | LLL | LLL | | | H | — | — | |
| Businco *et al.* (56), 1993 (23A/18NA)[f] | — | — | — | — | — | — | — | — | — | L |
| Duchén *et al.* (30), 2000 (38A/70)[f] | — | — | — | — | — | — | — | L | L | L |

[a]—, No difference between atopic and nonatopic individuals; H, HH, and HHH, higher levels in atopic individuals at $P < 0.05$ (H), at $P < 0.01$ (HH), and at $P \ll 0.001$ (HHH) significance levels; L, LL, and LLL, lower levels in atopic individuals at $P < 0.05$ (L), at $P < 0.01$ (LL), and at $P < 0.001$ (LLL) significance levels. See Table 1 for other abbreviation.
[b]A, atopics; NA, nonatopics.
[c]Milk samples at 2 wk of lactation.
[d]Differences at 1 mon of lactation; no differences found in colostrum or at 3 mon.
[e]Pooled sera from two occasions, between 2 and 12 wk of lactation.
[f]Milk samples from 2 to 8 mon of lactation.
[g]Milk samples at 3 mon of lactation.

included, no relationship was found between development of atopy and n-6 PUFA composition in maternal milk. The findings agree with those of one (56) of the cross-sectional studies, but not with those of the other (55). However, the levels of the n-3 LC-PUFA EPA, DPA, and DHA in mature maternal milk seemed to be related to allergic disease in early childhood (Table 4, Fig. 4). Possibly, the composition of milk, as analyzed in cross-sectional studies, merely reflects the milk PUFA composition of the atopic mothers (30,50), rather than being related to atopic disease in their children.

As both n-6 and n-3 PUFA compete for the same metabolic pathway (Fig. 1), the relationship between the two series can be expressed as ratios (Fig. 5). The total n-6/total n-3, LA/ALA, n-6 LC-PUFA/n-3 LC-PUFA, and AA/EPA ratios

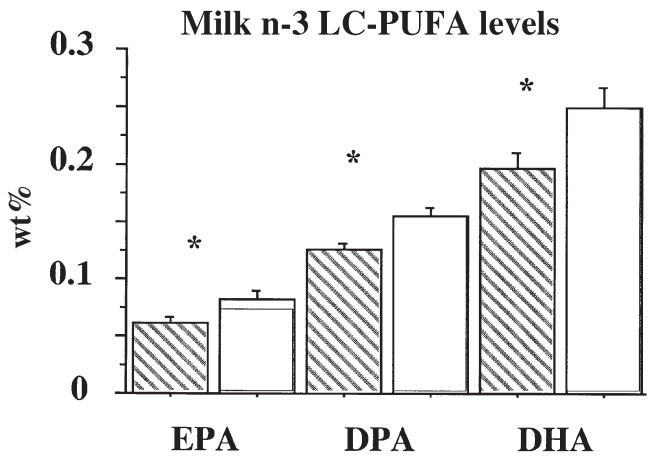


**FIG. 4.** Levels of individual n-3 LC-PUFA in mature milk at 3 mon of lactation, from mothers of 38 allergic (hatched bars) and 70 nonallergic children (open bars) at the age of 18 mon. Mean levels ± SD are given. *$P < 0.05$ for unpaired *t*-test (adapted from Ref. 53). See Figure 1 for abbreviations.

are similar in colostrum from mothers of atopic and nonatopic children. In transitional and mature milk, however, almost all ratios are significantly higher in milk from the mothers of atopic children (Fig. 5). This further strengthens the concept of a relationship between development of atopic disease in the infant and low n-3 LC-PUFA levels in maternal milk.

## MILK PUFA AND THE COMPOSITION OF SERUM PHOSPHOLIPIDS IN CHILDREN

As previously discussed, breast-feeding influences the PUFA composition in newborns (3,64,65). However, the relationship between maternal milk PUFA and serum PUFA composition in infants has been assessed in only one study (30). In this study, the relative fractions of 18:1n-9, LA, and n-6 LC-PUFA were similar in maternal milk and serum phospholipids of the infants, suggesting a relationship. Furthermore, the AA/EPA and AA/DHGLA levels correlated closely in milk and serum at 3 mon in the nonatopic, but not in the atopic infants. This again suggests that the milk PUFA content influences the composition of serum phospholipids in both atopic and nonatopic children and suggests a different ratio of AA and EPA, supporting a relationship between low n-3 PUFA and the development of atopy in childhood.

## IMBALANCE BETWEEN n-6 AND n-3 FATTY ACID METABOLISM IN ATOPY—A NOVEL HYPOTHESIS

The suggestion by Melnik and Plewig (41) that disturbed n-6 PUFA metabolism, particularly low AA and DHGLA levels, would lead to a low $PGE_1$ and $PGE_2$ production has not been corroborated. On the contrary, more recent research has assigned a major role to $PGE_2$ production by APC in the allergic

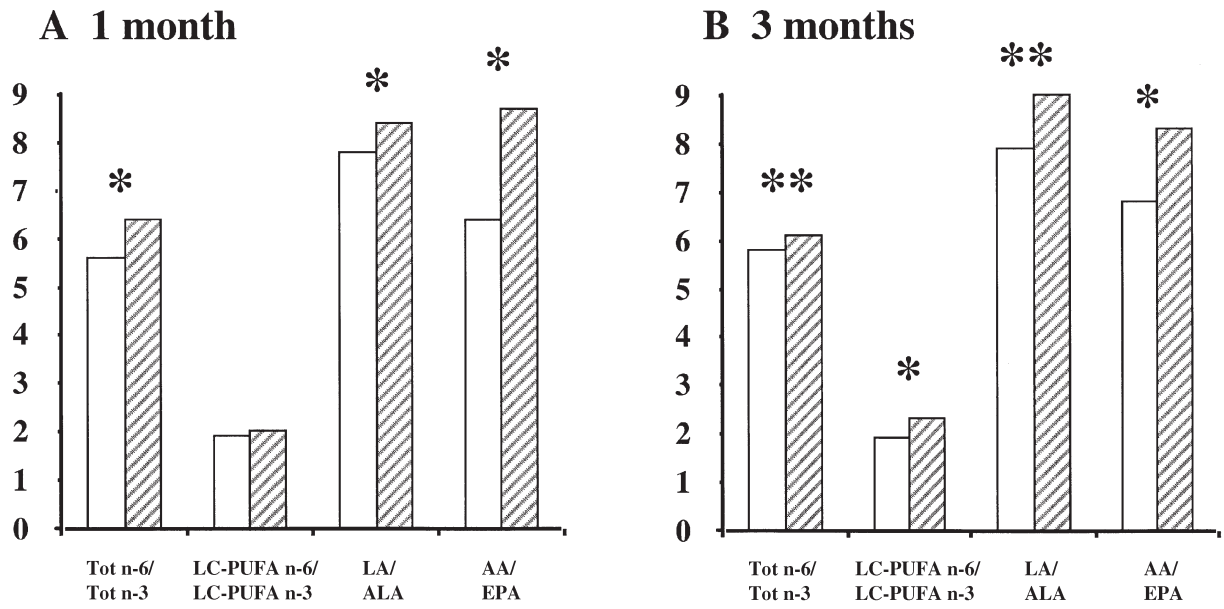


**FIG. 5.** The ratio between different n-6 and n-3 PUFA in milk from atopic (hatched bars) and nonatopic mothers (open bars), at 1 mon (A) and 3 mon (B) of lactation. LC-PUFA = the sum of all metabolites except LA and 20:2 for the n-6 and ALA for the n-3 PUFA. Mean levels ± SD are given. $^*P < 0.05$ and $^{**}P < 0.01$ for unpaired *t*-test (adapted from Ref. 53).

inflammation of $PGE_2$ by APC (8–13) (Fig. 6). Enhanced production of PGE2 by atopic monocytes after *in vitro* stimulation has repeatedly been reported (37,39, 66), corroborating a dysregulation in the synthesis of $PGE_2$ and possibly $LTB_4$ in atopic individuals. Thus, there seems to be a relationship between an abnormal AA metabolism and eicosanoid metabolism in atopic disease. As the clinical trials with PUFA substitution in allergic patients, which were limited to n-6 PUFA (Enfamil/Epogam), did not show any significant clinical efficacy (35), it seems reasonable to conclude that other components of fatty acid metabolism are important.

Recent studies suggest a more complex association between PUFA and atopic disease (30,50,53). The low levels of n-6 and n-3 EFA and LC-PUFA in milk from atopic mothers (30,50) could be explained by a relative Δ6-desaturase deficiency involving both series of PUFA in atopic individuals. Only low levels of n-3 LC-PUFA (EPA, DPA, and DHA) seem to be related to the development of atopic disease, however (30). The AA/EPA ratio, which is consistently higher in milk from mothers of atopic children and in serum phospholipids in atopics, could be a further clue to the relationship between n-3 LC-PUFA metabolism an atopy (30). A disturbed balance in the AA and EPA metabolism would lead to decreased synthesis of the less biologically active $LTB_5$ from EPA as well as an increased synthesis of $PGE_2$ and $LTB_4$ from AA by mononuclear cells. This would result in an overall "pro-allergenic" eicosanoid activity and a reduced synthesis of IL-12 in atopic individuals, leading to a polarization of the immune response toward a sustained Th2-like response (Fig. 6). A combination of an intrinsic dysmaturation of Δ6-desaturase in babies with atopic disease and composition of PUFA in the breast milk could explain both the sustained and transient IgE responses during early childhood.

Animal studies support this hypothesis, as a diet containing 1.3–3.3% of EPA and DHA given to mice significantly increases the proliferative responses of lymphocytes to T-cell mitogens, increases the production of IL-2, and suppresses

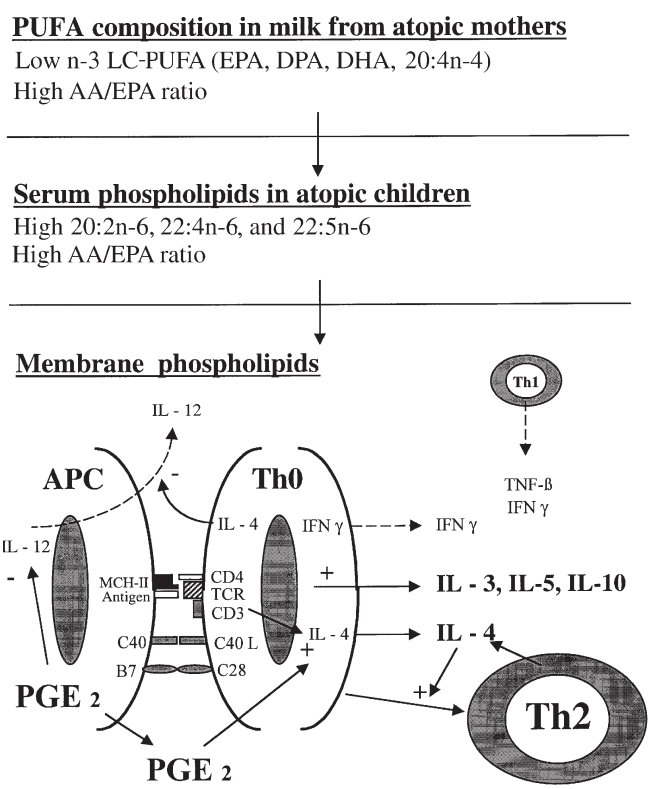


**FIG. 6.** The influence of milk PUFA on the composition of infant serum phospholipids early in life and the hypothesized effect on antigen-presenting cells (APC) and immune responses toward a T helper (Th)2-like response early in life. Abbreviations: TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; PGE, prostaglandin E; MCH, major histocompatibility complex.

the production of $PGE_2$ (67). Dietary n-3 fatty acids also have immunomodulatory properties (15,16), as particularly EPA competes for the same enzymes as AA and is the precursor to the less biologically active $LTB_5$ (5,15,16,67).

The Th2-like immune responses associated with atopic disease (68) are essential for a successful pregnancy (69). Furthermore, Th2-skewed cellular immune responses to common environmental antigens are present already *in utero* (70), suggesting the Th2-like response to be the primary response early in life (71). Prospective studies have shown that human infants synthesize IgE antibodies to both food and inhalant allergens early in life, but only children who develop an allergic disease maintain high IgE responses (72,73). Factors influencing early immune responses, for example, n-3 LC-PUFA in maternal milk, in combination with genetic factors, could then delay or prevent the Th1-skewing, thus promoting transient or sustained IgE synthesis. Breast-feeding is the natural source of PUFA in early infancy. If further research would confirm this hypothesis, dietary manipulation during early infancy might be a feasible way of primary allergy prevention. To corroborate this hypothesis, the composition of maternal diet and membrane phospholipids and their relation to eicosanoid synthesis should be studied in human cord blood monocytes. Such studies are lacking.

We conclude that altered PUFA composition in serum phospholipids and cell membranes is related to atopic disease and that the AA metabolism is disturbed in monocytes from atopics. These findings could be explained by a relating Δ6-desaturase deficiency in allergic individuals. Disturbances in the composition of the n-6 and n-3 fatty acids in milk from atopic mothers and serum phospholipids of their babies could at least be explained by a Δ6-desaturase dysfunction. Furthermore, mothers of atopic children provide their babies with low levels of n-3 fatty acids, and the balance between n-6 and n-3 fatty acids is disturbed. This imbalance, particularly a high AA/EPA ratio, is reflected in the serum phospholipid composition of those babies who develop atopic disease in infancy.

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# The Intestinal Mucosa as a Target for Dietary Polyunsaturated Fatty Acids

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**ABSTRACT:** Several studies have reported beneficial effects of dietary polyunsaturated fatty acids (PUFA) on various aspects of both human and animal health, and particular reference has been made to their effects on systemic immune responses. Both immune stimulation and immune suppression have been reported, with the outcome dependent on the type of PUFA, the target cell, as well as the immune competence of the cells before exposure. The systemic and the mucosal immune systems are discrete entities, which have evolved specific approaches in the defense of the host. The latter comprises several interconnected tissues, which communicate with one another through the action of soluble mediators and the trafficking of cellular components. After the oral mucosa, the intestinal epithelium and its associated gut-associated lymphoid tissue are the primary targets of dietary components. Absorption of dietary PUFA and its incorporation into intestinal tissues has been well studied, but the consequences of these events in relation to local immune responses have received little attention. This article describes some of the immune mechanisms operating at this barrier and, where possible, pinpoints areas for which a modulatory role for PUFA has already been demonstrated. Although not an exhaustive treatise of the subject, it is hoped that this review will foster research into the specific interaction between dietary PUFA and cell populations comprising the intestinal barrier.

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The intestinal mucosa is a dynamic barrier continually adapting to the ever-changing environment in the intestinal lumen and to the factors that are intrinsic to host defense. As such, it has a strategic role to play in the overall immune status of the host. Essentially, its overlying epithelium polices the luminal barrier and surveys which antigens cross the mucosa and gain access to the host's systemic immune system. As a consequence, the intestine has developed a highly organized immune system of its own, i.e., the gut-associated lymphoid tissue (GALT), which allows it to communicate with those in other mucosa. One of the most intriguing attributes of the GALT is its capacity to discriminate between pathogenic microorganisms to which it mounts a response, and the vast array of dietary antigens and commensal microbial flora to which it remains tolerant. This ambivalency is achieved through the precise regulation of pro- and anti-inflammatory mediators, which direct responses during homeostasis and pathological conditions (1). It is a potentially hazardous state, but, in general, the barrier assumes this challenge efficiently without any major detrimental effect to the host. Ordinarily, the chronic physiologic inflammation that ensues (2) is tolerated. However, inappropriate exposure to antigens and/or a failure of the system to restore the "injured" host to homeostasis, can culminate in aberrant immunological responses, which compromise the health of the host.

Dietary fatty acids such as linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) of the n-6 and n-3 series of polyunsaturated fatty acids (PUFA), respectively, are considered "essential" because they must be derived from the diet. Certain of these are precursors of the eicosanoids, which have well-documented effects on the induction and modulation of immune and inflammatory responses (3,4). The efficacy of dietary PUFA in modulation of such responses will depend on many factors, not least of all, the antioxidant status of the host's gastrointestinal tract. Both immune stimulation and immune suppression have been reported *in vitro* and *in vivo*, but it is clear that outcome is dependent on the admixing of different PUFA in specific proportions, the target tissue, and immune status of the host before exposure. Although a good appreciation of systemic immune effects has been achieved, studies on the modulation of intestinal immunity are lacking.

Because intestinal barrier integrity is a key element in the prevention of infection and clinical disease, this paper will introduce some of the nonspecific and specific immune mechanisms operating locally in the intestinal lumen. It will also briefly describe how the intestinal mucosa communicates with other mucosal tissues. Finally, it will address ways in which these specific mechanisms may be modulated by PUFA.

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; BALT, bronchus-associated lymphoid tissue; DHA, docosahexaenoic acid; EFA, essential fatty acids; EPA, eicosapentaenoic acid; FO, fish oil; GALT, gut-associated lymphoid tissue; GLA, γ-linolenic acid; HEL, hen egg-white lysozyme; IBD, inflammatory bowel disease; ICAM-1, intercellular adhesion molecule-1; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; KLH, keyhole limpet hemocyanin; LA, linoleic acid; LAB, lactic acid bacteria; MadCAM-1, mucosal addressin cell adhesion molecule-1; MHC, major histocompatibility complex; MLN, mesenteric lymph nodes; MMP, matrix metalloproteinase; NALT, nasal-associated lymphoid tissue; NEC, necrotizing enterocolitis; NK, natural killer; OA, oleic acid; $PGE_2$, prostaglandin $E_2$; PUFA, polyunsaturated fatty acids; TCR, T-cell receptor; TGF-β, transforming growth factor-β; Th, helper T cell; TNF-α, tumor necrosis factor-α.

## ORGANIZATION OF THE INTESTINAL MUCOSAL TISSUE

Host immune defense uses two arms in its battle against pathogens and harmful antigens, i.e., innate immunity and adaptive immunity. Nonspecific innate defenses are initiated the moment the host encounters these antigens, whereas the adaptive responses are specific for individual pathogens and require some days to be fully developed and operational. It is evident therefore that the integrity of the intestinal barrier and selective uptake of molecules are fundamental to the maintenance of homeostasis and the prevention of disease. A number of physical and immunological mechanisms have evolved to regulate antigen exposure at the epithelial surface. Although antigen uptake can be beneficial for sampling the intestinal milieu and providing essential trophic and regulatory factors, uncontrolled passage of antigen predisposes the host to disease (5). One strategy is to limit the antigenic load at the epithelial surface (Fig. 1). Nonimmune elements limiting uptake include gastric acidity, peristalsis, intestinal secretions, proteolytic digestion, the phospholipid layer, and mucus secretion (5). If these fail, the secretory immunoglobulin (Ig)A comes into action and limits the exposure by immune exclusion. For pathogens, an additional strategy is to limit the growth of the pathogen by bactericidal or bacteriostatic mechanisms such as antimicrobial peptides, defensins, and lactoferrin, antibody-dependent cellular cytotoxicity mechanisms or cellular effector mechanisms acting at the mucosal level (6). Yet another possibility is to limit the tissue damage that ensues through the action of antioxidants or complement inhibitors, or by the production of trophic factors.

If the antigen succeeds in overcoming each of these barriers, the mechanisms operating at the epithelium and its underlying lymphoid tissue enter into play. The epithelium itself represents a further physical barrier to antigen uptake, although it is not impenetrable. Unlike the systemic immune response, which is programmed to eliminate all foreign antigens, a differential response operates at the mucosa. Although the uptake of essential growth factors and nutrients is facilitated by specific receptor-mediated uptake (5), harmful antigens must be prevented from breaching the barrier. Some physical protection against large macromolecules is provided by the tight junctions between adjacent epithelial cells. Occludins (7) and claudins (8) are tight junction-specific integral membrane proteins, which are regulated by immune mediators (9) and whose expression restricts paracellular transport of macromolecules. During inflammatory processes, the junctions between the epithelial cells are disrupted, and the passage of luminal antigens across the epithelial layer augmented (10).

The Peyer's patches, lymphoid aggregates that occur along the length of the small bowel, are major inductive sites of the mucosal immune system (Fig. 1). They comprise all of the major lymphoid and accessory cells of the immune system and are covered by the follicular-associated epithelium, which

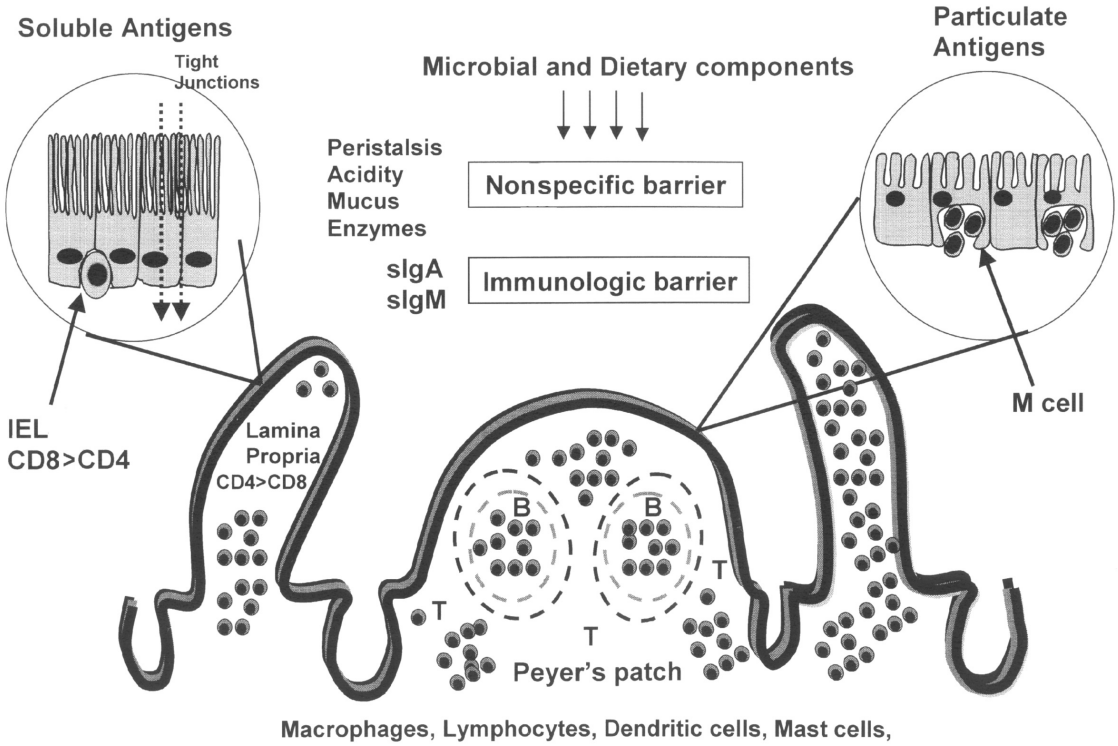


**FIG. 1.** Schematic representation of the intestinal barrier, depicting the physical and immunological mechanisms that regulate antigen exposure at the epithelial surface. Soluble antigens enter the mucosa *via* the villous epithelium, through either paracellular or transcellular routes. The Peyer's patches are the major sites for priming of intestinal lymphocytes. Microorganisms and other luminal antigens bind preferentially to the surface of M cells in the follicular-associated epithelium. These M cells are in intimate contact with immune cells in the underlying tissue. Ig, immunoglobulin; IEL, intraepithelial lymphocyte.

contains a specific epithelial cell type, the M cell (11). This cell facilitates the delivery of antigens, especially particulate antigens, from the lumen to immunocompetent cells, which are situated in its intraepithelial pocket (11). This intimate association favors further dissemination of signals to the macrophages, dendritic cells, and the many B and T lymphocytes underlying the epithelium. Unfortunately, the ease of uptake at this site is exploited by certain pathogens to cross the epithelial barrier and invade the mucosa.

## THE COMMON MUCOSAL IMMUNE SYSTEM

Dietary effects on the host are multifaceted. Specific components may act locally on gut tissues and elaborate immunomodulatory molecules, which then travel to other sites in the host. Alternatively, the dietary components themselves may be transported across the gut barrier to act in distal tissues. A final possibility is that the dietary components influence the phenotype of cells in the gut and that these cells themselves leave the gut environment to exert their effector functions in other tissues.

Lymphocytes continuously migrate through the body to increase the chance of encountering the cognate antigen (12). Labeling studies using Peyer's patch-derived cells injected into rodents demonstrated the phenomenon of the "common mucosal immune system" in which induction of a response in one mucosa-associated lymphoid tissue results in the departure of activated cells to effector sites in the same or other mucosal tissues (13). Once antigenic stimulation takes place in the Peyer's patches, antigen-specific precursor $IgA^+$ B cells and $CD4^+$ T helper cells leave these afferent sites and migrate through lymph to the mesenteric lymph nodes (MLN), then into the thoracic duct to enter into the bloodstream (Fig. 2). Some of the migrating cells express the $\alpha_4\beta_7$ mucosal and L-selectin homing receptors, which interact with the mucosal addressin cell adhesion molecule-1 (MadCAM-1) expressed on the microvascular endothelium and thereby direct the cells back into the lamina propria (14). Others express the integrin $\alpha_E\beta_7$, which interacts with the ligand E-caderin on the basolateral membrane of enterocytes (15) and is responsible for homing of lymphocytes into the intraepithelial compartment. Once lodged in these two sites, these activated cells finish their differentiation and participate in the effector mechanisms, the best known of which is the secretory IgA response. Other adhesion molecules are apparently used by immune cells primed in the bronchus-associated lymphoid tissue (BALT) and nasal-associated lymphoid tissue (NALT) but it is noteworthy that the lactating mammary glands receive immune cells primed in the inductive compartments of both the NALT and GALT (14).

Because activated T cells produce cytokines and express costimulatory molecules, they can induce the nonspecific proliferation of bystander lymphocytes (11). Selectively modifying the survival of these activated cells once they have

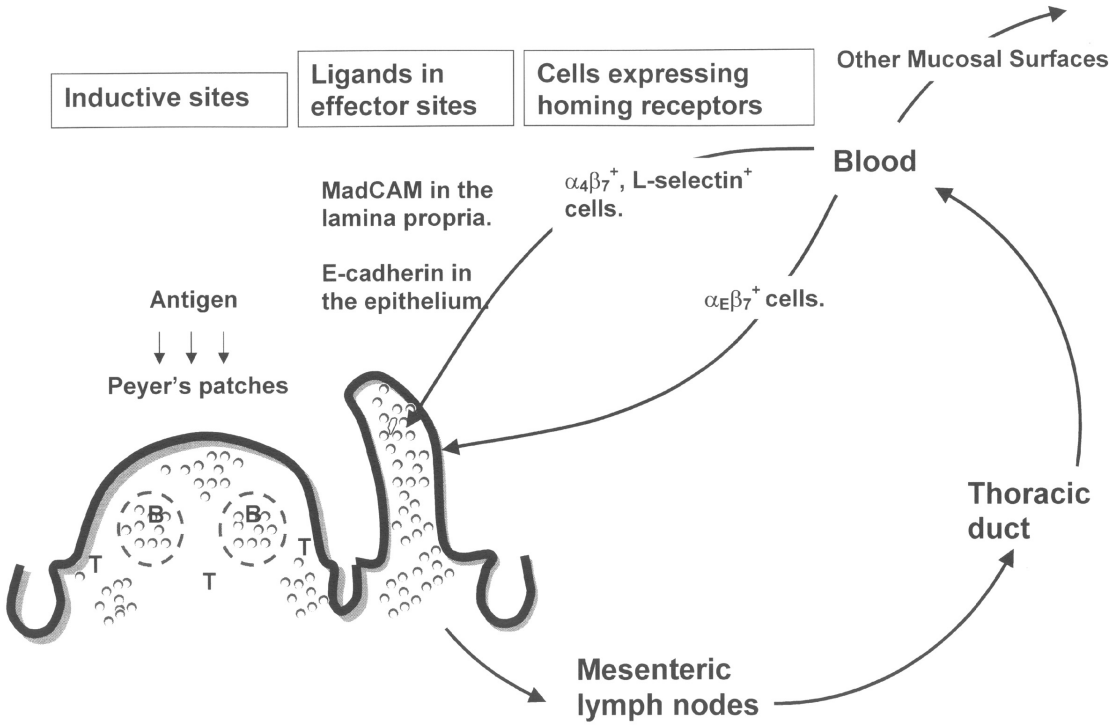


**FIG. 2.** Homing to intestinal tissues. T and B cells, primed in the Peyer's patches, migrate to the mesenteric lymph nodes *via* the draining lymphatics. They then enter into the bloodstream *via* the thoracic duct and finally home to different mucosal compartments depending on their expression of specific adhesion molecules. Cells expressing $\alpha_4\beta_7$ mucosal and L-selectin homing receptors interact with mucosal addressin cell adhesion molecule-1 (MadCAM-1) expressed on the microvascular endothelium and are thereby directed into the lamina propria. Cells that express the $\alpha_E\beta_7$ integrin interact with the ligand E-caderin on the basolateral membrane of enterocytes and are directed into the IEL compartment. For abbreviations see Figure 1.

randomly entered into the tissue or as they leave leads to a preferential accumulation of cells in sites where they are most needed. This might explain how immune responses can spread from the jejunum to the ileum and colon, while remaining confined to the small and large intestine (12).

## THE INTESTINAL EPITHELIUM

Another important component of the effector arm of intestinal immunity is the mucosal epithelium, which is composed of at least two types of immuncompetent cells, i.e., the intraepithelial lymphocyte (IEL) and the intestinal epithelial cell (IEC), which share an intimate association. The nonspecific protective mechanisms mentioned earlier are the first line of protection operating at the mucosa. They are naturally present and do not require prior contact with the antigen. The IEC response against pathogens has been considered part of this process.

IEC migrating from the crypt mature into discrete cell types (enterocytes, neuroendocrine cells, and goblet cells) upon entering into the villus, journey along the length of the villus over a period of 2–5 d, and terminate in apoptosis upon reaching the tip. Thereafter, the dead cells or their fragments are either extruded into the lumen or phagocytosed by macrophages in the lamina propria (16). Under normal conditions, the balance between the opposing forces of cell proliferation and cell death leads to a complete renewal of the epithelial cell population every few days and means that the number of cells within each villus remains constant. IEC produce many of the cytokines (17,18), which are known modulators of crypt cell proliferation. A variety of immune-mediated bowel disorders, including celiac disease and inflammatory bowel disease (IBD), are characterized by accelerated epithelial cell turnover and apoptosis, leading to altered crypt/villous morphology (19).

For many years, the intestinal epithelium was considered to represent a passive barrier and was not thought to participate in the initiation of immune responses until studies performed in the late 1970s revealed that the enterocyte expressed major histocompatibility complex (MHC) Class II (20,21). Later studies showed that this expression was dependent on both age and intestinal location (22), and that it endowed the epithelial cell with antigen-presenting capacity *in vitro* (23). It is now known that the epithelial cell expresses many important immune molecules and has the capacity to elaborate a wide range of cytokines, which modulate its interaction with T lymphocytes (17,18). Presentation can be to both $CD4^+$ helper cells and to $CD8^+$ cytotoxic/suppressor cells. Recent work suggests that presentation to the latter may be through both classical and nonclassical MHC elements such as the CD1 molecule. Although these molecules share similarities to Class I molecules, CD1 have a deeper and more hydrophobic antigen-binding groove (24), which enables them to present microbial glycolipids and synthetic lipids to natural killer (NK) T cells (25). Furthermore, they bind sphingolipid and phospholipid, but it is not known whether these molecules are self- or foreign antigens (26). It is interesting to speculate that dietary phospholipids may modulate immune function through an interaction with CD1.

Taken together, these observations confirm that the Peyer's patch is not the sole site for regulation of intestinal immune responses and that the IEC has a pivotal role to play in host responses to luminal antigen. Indeed, the enterocyte phenotype determines the development of specific subsets of T cells as well as the organization within the Peyer's patch (27).

## INTESTINAL T LYMPHOCYTES

Lymphocytes can be subdivided into various classes on the basis of their phenotype and functional attributes. The main properties distinguishing the various $CD4^+$ T helper cells and $CD8^+$ cytotoxic/suppressor cells are described in detail elsewhere in this volume (28). However, the lymphocytes that reside in the intraepithelial and lamina propria communities have unique characteristics.

The majority of IEL are T cells ($CD3^+$), which are predominantly $CD8^+$ $CD45RO^+$ (memory cells); although both γδ and αβ T-cell receptor (TCR)-bearing T cells are present, the latter are the most abundant (29). However, it has been suggested that IEL at the villous tip are quite distinct from those in the crypt region. Although there is no difference in αβ-$TCR^+$ IEL along the length of the villus, an increased frequency of γδ-TCR-bearing IEL is seen in villous tips (30). The γδ-$TCR^+$ IEL seem to be independent of bacterial challenge, but those with αβ-TCR increase on bacterial colonization of germ-free animals (6).

In contrast to the IEL population, the $CD4^+$ helper T-cell (Th) population predominates in the lamina propria compartment. These cells can be subdivided into functionally distinct phenotypes on the basis of the profile of cytokines they produce (Table 1). Th1 cell-driven responses are exemplified by the delayed-type hypersensitivity reaction for which regulation of phagocytes to fight microbial and viral infections is a major commitment. Th2 cells, on the other hand, promote IgE production and eosinophil activity, both of which contribute to the pathogenesis of allergic reactions. Classification of CD4 cells into these subsets is based on the cytokine profile of the cells. Once activated by allergen, Th2 cells secrete interleukin (IL)-4 and IL-5, which induce the production of IgE and IgG1 by B lymphocytes, and stimulate the recruitment of eosinophils from the bone marrow into inflamed tissues (31,32). Infections, on the other hand, induce Th1-type responses and the release of IL-2, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) (31,32). It has also been suggested that the environment promoted by Th1 cells may dampen the development of allergen-specific Th2 cells and the development of atopy. Th0 produce all of these cytokines and are considered the precursor of the Th1 and Th2 cells. However, immune responses driven by Th1 and Th2 cells are sometimes influenced by a third type of T helper cell that acts to downregulate the responses mediated by Th1 and Th2. Feeding low amounts of myelin basic protein to mice leads to

**TABLE 1**
**CD4$^+$ T Cell Subsets[a]**

| | Th1 | Th2 | Th3 | Tr1 |
|---|---|---|---|---|
| Cytokine profile | | | | |
| IL-2 | +++ | — | ± | ± |
| IFN-γ | +++ | — | ± | ++ |
| IL-4 | — | +++ | ± | — |
| TGF-β | ± | ± | +++ | + |
| IL-10 | — | ++ | ± | +++ |
| Growth/differentiation factors | IL-2, IL-12, IL-18 | IL-2, IL-4, IL-13 | IL-4 | IL-10, TGF-β |
| Suppressor effects | Th2 | Th1 | Th1/Th2 | Th1 |
| Antigens | Intracellular parasites | Allergens Helminths | Self antigens? Tolerogens? | Self antigens? Tolerogens? |

[a]Abbreviations: IL, interleukin; IFN, interferon; TGF, transforming growth factor; Th, helper T cell; Tr, regulatory T cell.

the induction of immunoregulatory cells, which prevent the development of experimental autoimmune encephalitis (33). These T cells, named Th3 cells, produce high levels of transforming growth factor (TGF)-β but little if any Th1- or Th2-type cytokines (34). Another type of regulatory T cell, named T regulatory cell 1 (Tr1), is distinct from the Th3 cell (35). Tr1 differentiates in the presence of IL-10, has a high production of IL-10, low levels of active TGF-β, and a moderate production of IL-5, IFN-γ, and TNF-α. A deficiency in this particular subset of cells has been suggested to be involved in the pathogenesis of IBD. However, studies on the role of Th3 and Tr1 cells in intestinal immune responses are in their infancy.

Virtually all lamina propria lymphocytes and IEL express chemokine receptors that have been associated with Th1 and Th0 lymphocytes, but chemokine receptors associated with Th2 cells are not expressed (36). Furthermore, these two lymphocyte populations express a specific and similar array of chemokine receptors whose ligands are constitutively expressed in the intestinal mucosa and whose expression is upregulated during intestinal inflammation.

IEC downregulate T cell proliferative and cytokine responses for both Th1 and Th2 within $\gamma\delta^+$ and $\alpha\beta^+$ IEL populations. Such interactions occur *via* cell surface molecules and may exist to prevent inflammatory responses at the intestinal mucosal surface (37). In addition, IEC-derived IL-6 enhances lipopolysaccharide-stimulated IgA secretion by mucosal B cells (38).

It is clear that lymphocyte trafficking, the interaction between IEC and lymphocytes, and the cytokine profile that results will determine the polarization of immune responses. Dysregulation of these processes may well explain the pathogenesis of intestinal inflammatory and allergic conditions.

## THE INTESTINAL BACTERIAL FLORA

The commensal microflora is an integral component of the intestinal milieu; as such, its composition and its contribution to the health of the host cannot be ignored. Specific flora populate each level of the intestine and are prevented from migrating to extraintestinal sites by the intestinal epithelial barrier. The low pH, peristalsis, and luminal secretions of the stomach and upper gut render these tissues sterile or sparsely populated by avirulent, aerobic, gram-positive organisms (6). However, descending toward the distal ileum and colon, gram-negative and anaerobic organisms predominate (39). It seems paradoxical that such a large population of indigenous bacteria establishes itself in this tissue without any adverse effect on the host. Indeed, the physiologic microflora affords protection to the host in two important ways. It functions as a physical barrier to prevent pathogens interacting with the mucosal surface; in addition, it interacts with the host and modulates its mechanisms of defense (6). It is clear that the members of this bacterial community are altered by components in both the diet and the intestinal milieu. Colonization is thought to occur at birth, is different between breast-fed and formula-fed infants, and is altered postweaning, in the elderly, and in clinical disease (6). Dietary components such as fiber and nondigestible oligosaccharides (40), and perhaps cytokines (41) elaborated into the intestinal lumen, may modulate its composition.

## ADAPTIVE CHANGES IN THE NEONATE

After birth, the mucosa undergoes a major adaptive change in the transition of the fetus to the extrauterine environment. This is a major physiologic event with the passage of the intestine from a sterile to a heavily colonized tissue. Clearly, effective barriers are required against the huge bacterial inoculum derived from the mother's genital tract and feces, and from the environment. Beneficial components in breast milk are certainly instrumental in ensuring that the adaptation of the intestine to this massive change occurs without compromising the health of the neonate. However, in the premature, the mucosal barrier is not fully functional and the infants are rendered particularly susceptible to clinical disease states such as necrotizing enterocolitis (NEC) or allergy.

Although immune homeostasis is quickly established in the postnatal period, and neonates are capable of mounting active immune responses, they are nevertheless relatively immunodeficient (42). Neonatal T cells produce mature levels of cytokines *in vitro* but only in response to powerful stimulation (43). Furthermore, both Th1 and cytotoxic T lymphocyte responses are poor or absent. As a consequence of these phenomena, the newborn is vulnerable to potentially hazardous antigens.

## PUFA INTERACTIONS WITH THE INTESTINAL MUCOSA

Uptake of dietary PUFA, past the nonspecific and immunologic barriers, to the intestinal epithelium is well documented. Indeed, there is an energy- and temperature-dependent carrier-mediated transport of ALA and possibly other long-chain fatty acids across intestinal cells (44). Once ingested, the essential fatty acids (EFA) are converted to longer-chain, more highly unsaturated fatty acids, including arachidonic acid (AA) from LA and eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) from ALA. This process probably already takes place at the level of the epithelium because the enterocyte has the ability to desaturate EFA and incorporate them into jejunal and ileal microsomes (45). Furthermore, the activity of these enzymes is regulated by both the dietary fat intake and cell maturation (45,46). If dietary PUFA are incorporated into IEC membranes, it is feasible that they may have an effect on IEC-intestinal lymphocyte crosstalk in both IEL and lamina propria compartments of the gut, as well as in distal immune responses taking place in the mammary gland.

Several studies have reported regulation of nonspecific barrier function by the products of PUFA, but more emphasis has been placed on the function in the gastric mucosa than in the small intestine. These reports suggest that eicosanoids, particularly those derived from AA, affect intestinal secretions, mucus secretion, phospholipid synthesis, and the density of surfactants in the mucus as well as providing cytoprotection of gastrointestinal mucosa; the majority of studies have provided evidence of upregulation of these phenomena (47–53). However, several studies have challenged the observations on mucous secretion and cytoprotection by finding no relationship between prostaglandin (PG) synthesis and these parameters (54–56). It has also been proposed that intestinal glycosyltransferases involved in cell differentiation and postnatal maturation of the rat small intestine are altered by the global unsaturation index of dietary fatty acids and that any alterations in intestinal glycosylation may result from a decrease in total PUFA (57).

Oleic acid (OA), an unsaturated fatty acid of the n-9 series, induces mucosal injury and increased intestinal permeability in developing piglet intestine that is dependent on both saturation and chain length. However, this can be avoided by using its ethyl ester (58). The authors suggest that ethyl ester forms of fatty acids, which are absorbed and metabolized similarly to free fatty acids, may provide a means of supplying long-chain fatty acids to the intestine without causing mucosal damage. However, a more natural way of providing ester forms of fatty acids is as triacylglycerols.

A diet deficient in EFA increases the incidence of bacterial translocation to MLN (59) and raises the possibility that these fatty acids help maintain a tight intestinal barrier. However, this may be dependent on the type and/or proportion of individual fatty acids. Treatment of endothelial cells *in vitro* with γ-linolenic acid (GLA) has been shown to increase transcellular electrical resistance and reduce paracellular permeability to large molecules. Occludin, which plays a major role in tight junctions, was upregulated by GLA and by EPA but was downregulated by AA and LA (60). The effects obtained may be related to the type of eicosanoids synthesized from these different fatty acids. Because IEC also express these tight junction molecules, it is possible that exposure to specific PUFA may tighten the intestinal barrier. However, more studies are required both *in vitro* with IEC and *in vivo*; in contradiction to the above observations, there is *in vivo* evidence that a safflower oil diet (rich in n-6 PUFA) but not a fish oil (FO; menhaden, rich in n-3 PUFA) diminishes *N*-formyl-methionyl-leucyl phenylalanine-induced permeability and neutrophil infiltration in rats (61).

Studies examining the effect of PUFA consumption on the phenotype and function of cells isolated from the MLN, lymph, or blood give a good indication of modifications to their counterparts in the intestine. Although immunostimulatory effects have been reported, the general consensus is that the n-3 PUFA, such as EPA and DHA, suppress immune responses compared with n-6 PUFA such as LA. However, the precise targets of these dietary PUFA and the mechanisms underlying the observed effects on immunity are not clear. Nevertheless, a modulatory effect of these PUFA on eicosanoid production and the expression of other genes, such as the fatty acid synthase, has been reported.

Consumption of n-3 PUFA, such as that found in FO, can influence the production and the biological effects of a number of cytokines both *in vitro* and *in vivo* (62). Their effects on peripheral blood cells include a decreased lymphocyte response to mitogens, reduced NK cytotoxic activity, and an inhibition of IL-1, IL-2, TNF-α, and IFN-γ production *ex vivo* (28,62,63). A reduction in cytokines such as IL-1, which are known to be co-stimulatory during antigen presentation, suggests that this activity may also be inhibited by n-3 PUFA. Indeed, consumption of FO or consumption of EPA and DHA in the same ratio (3:2) as that commonly found in FO suppresses the expression of MHC Class II and the accessory molecule, intercellular adhesion molecule-1 (ICAM-1) on blood monocytes and thereby inhibits the cells' capacity to present the antigen tetanus toxoid to autologous lymphocytes (64). Furthermore, oil consumption in rats decreases the expression of MHC Class II and CD11a and CD18 on lymph-borne dendritic cells and reduces the ability of the cells to present the antigen keyhole limpet hemocyanin (KLH) to KLH-sensitized responder spleen cells (65). Furthermore, both EPA and DHA inhibit IFN-γ-induced expression of MHC Class II on monocytes/macrophages (66,67). An inhibition of proinflammatory cytokines, MHC Class II expression, and antigen presentation suggest a beneficial role for FO in inflammatory conditions such as IBD, in which these parameters are increased; it also raises the interesting possibility that the absence of MHC Class II on intestinal epithelium (22) and lower immune reactivity during the suckling period may be due, at least in part, to the action of DHA in breast milk.

Although there is an abundance of studies reporting modulatory effects on lymphocytes of the blood or spleen, there have been few studies that have specifically addressed the effect of dietary PUFA on the immune phenotype or function

of intestinal lymphocytes. It is known, however, that these cells incorporate dietary PUFA into their cell membrane. Colonic lymphocytes of rats fed a diet high in corn oil have greater membrane concentrations of LA and AA and higher levels of $PGE_2$ production than those isolated from rats fed menhaden oil (68). As a consequence the cells are refractory to mitogen stimulation.

Miura *et al.* (69) reported that OA stimulates lymphocyte flux and blastogenesis in intestinal lymph and enhances T-cell migration to the Peyer's patches, perhaps through upregulation of homing receptors (70). Dietary fats have also been shown to influence adhesion of lymphocytes to extracellular matrix proteins (71) as well as to macrophage and endothelial cell monolayers (72). Together, these observations suggest that unsaturated fatty acids in the diet may regulate the extravasation of intestinal lymphocytes into the lymph and blood as well as their reentry into mucosal tissues.

Elemental diets reduce the CD4/CD8 ratio in MLN and ileal lamina propria, and the level of IgA positive cells in the lamina propria of rats, but administration of OA reverses all of these changes (73). Although similar studies have not been performed using PUFA, the group of Kleemann *et al.* (74) observed that in diabetes-prone BB rats, feeding a diet enriched with FO altered the IFN-γ and IL-10 levels in the GALT. The authors postulated that the FO had polarized the Th1/Th2 cytokine ratio toward Th2. However, it may be that administration of FO promoted functional development of the Th3 and/or Tr1 cell subsets, which produce high levels of TGF-β and IL-10, respectively (34,35). In support of this, Fernandes *et al.* (75) showed that feeding autoimmune-prone mice menhaden oil led to a higher production of TGF-β. Both IL-10 and TGF-β are well known for their role in the induction of oral tolerance and the prevention of autoimmune disease. Interestingly, dietary PUFA influence the fatty acid composition and secretory activity of lactating mammary epithelial cells (76). Furthermore, high levels of GLA and low levels of ALA and n-3 PUFA in human milk are associated with atopic sensitization during infancy irrespective of the atopic status of the mother (77).

Metabolites of AA have also been implicated in the development and maintenance of intestinal immune homeostasis. Feeding hen egg-white lysozyme (HEL) to mice expressing a HEL-specific transgenic TCR does not result in intestinal pathology. However, simultaneous administration of cyclooxygenase-2 inhibitors and HEL leads to increased proliferation of lamina propria lymphocytes and crypt epithelial cells with resultant crypt expansion and villus blunting (78). The suppression of T-cell proliferative responses could certainly be a result of increased production of IL-10 by Tr1 cells. However, the observations that both n-3 and n-6 fatty acid metabolites lead to increased production of IL-10 and TGF-β and have similar effects in *vivo* are difficult to reconcile, especially in light of the fact that consumption of FO reduces the extracellular release of AA by stimulated T lymphocytes (79). An alternative explanation is that yet another mechanism of control is acting during consumption of n-6 fatty acids. The recent findings that $PGE_2$ selectively inhibits activation and proliferation of human $CD4^+$ memory cells secreting low amounts of both IL-2 and IL-4 and that Th1, Th2, and Th0 clones become sensitive to the $PGE_2$ effects only when IL-2 and IL-4 are removed from the medium support this notion (80).

In inflammatory and pathological conditions, protective mucosal secretions may be limited or not active and production of proinflammatory mediators, including eicosanoids of the n-6 series, increased in inflamed intestine. Furthermore, dysregulation of cell turnover and apoptosis of IEC in the villus may lead to a reduction in the proportion of mature and differentiated cells occupying the upper villus. In this context, dietary n-3 PUFA suppress colonocyte proliferation (81) and increase the number of differentiating and apoptotic cells without modification of crypt morphology or the number of cells per villus (81).

In intestinal pathologies of the premature such as NEC, the increase in the number of goblet cells and enteric defensin expression has been suggested to reflect the pathologic process (82). The observation that the steady-state level of intracellular peptide did not increase in parallel with the increase in defensin mRNA suggested to the authors either that the defensin was actively secreted into the gut lumen or that there was translational regulation of the peptide. However, defensins are believed to be released in an inactive form in the gut and to require activation by particular matrix metalloproteinases (MMP) (83). The predisposition of the premature infant to bacterial overgrowth and infection could therefore be due to inadequate MMP activity in the premature gut. In addition, the increased levels of proinflammatory cytokines, IL-1 and TNF-α, seen in NEC and other intestinal pathologies, such as IBD, promote adherence of gram-negative organisms to intestinal mucosa both *in vitro* and *in vivo* (41). Certain dietary PUFA have been proposed as therapies for intestinal pathologies on the basis of their anti-inflammatory properties. However, the contribution of these dietary components to the colonization of the lumen with beneficial microorganisms and to the integrity of the intestinal barrier provides other target functions. Certainly, diets deficient in EFA increase the incidence of bacterial translocation to MLN (59). EPA significantly inhibits the growth of obligate anaerobes such as *Bacteroides* spp. but not *Escherichia coli* both *in vitro* and *in vivo* (84,85) perhaps through disruption of the outer membrane (86). Diets high in marine oils certainly reduce the anaerobe/aerobe ratio in the colon (87), and some dietary lipids have been demonstrated to increase the number of lactic acid bacteria (LAB) in mice (88) and fish (85). In addition, LAB are major unsaturated fatty acid hydrating bacteria in the rumen (89), whereas human fecal flora can hydrogenate LA and ALA but not AA (90). One interesting possibility that has been observed in fish (91,92) but that has not been tested in humans is that microorganisms colonizing the gut are a source of DHA. Taken together, it would appear that specific dietary PUFA may be used to antagonize potential pathogens and/or encourage health-promoting LAB.

In conclusion, there are numerous studies reporting the effects of the various PUFA and their metabolites on the immune response of peripheral blood cells or splenocytes *ex vivo*. These investigations have greatly increased our perception of how dietary lipids might influence the health of the host. However, to date, understanding the fundamentals of immune regulation in intestinal tissue remains a relatively virgin field of research. The latest work examining polarization of Th1/Th2 responses gave excellent clues as to the events that might be taking place in the intestinal compartment. However, the effect of dietary PUFA on intestinal T-cell subsets and phenotype, IEC phenotype, intestinal cytokine production, and IEC–T cell crosstalk must be known to reinforce the concept that dietary manipulation with specific PUFA not only reinforces immune homeostasis in the healthy individual but also reinstates such homeostasis in a number of immune-mediated disease states.

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# The Role of Polyunsaturated Fatty Acid Supplementation in Intestinal Inflammation and Neonatal Necrotizing Enterocolitis

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**ABSTRACT:** Dietary polyunsaturated fatty acid (PUFA) supplementation has been shown to reduce the incidence of necrotizing enterocolitis (NEC) in a recent randomized, controlled trial. These compounds are known to modulate the inflammatory cascade and to influence intestinal health in a variety of ways. Although the pathophysiology of NEC is not well understood, recent evidence suggests that platelet-activating factor (PAF) is a key endogenous mediator of intestinal necrosis in animals. Using a neonatal rat model of NEC that includes the key risk factors of asphyxia and formula feeding, we investigated the role of dietary PUFA supplementation on the incidence and pathophysiology of NEC. Our findings suggest that PUFA reduce the incidence of NEC by modulating PAF metabolism and endotoxin translocation.

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Long-chain polyunsaturated fatty acids (LC-PUFA) are a diverse group of compounds that have been purported to modulate inflammation and immunity (1,2). In high-risk premature neonates, dysregulation of inflammation and immunity may contribute to the pathophysiology of necrotizing enterocolitis (NEC), a serious gastrointestinal emergency that afflicts 10% of infants born weighing <1500 g (3,4). In a recent clinical trial, PUFA supplementation of formula for premature infants was shown to reduce the incidence of NEC in a high-risk population (5). Nonetheless, the specific effects of PUFA on intestinal inflammation and necrosis remain poorly characterized. Studies have shown that n-3 PUFA can reduce the inflammatory response *via* multiple potential pathways (6). Platelet-activating factor (PAF) is an important phospholipid mediator that has been shown to play a crucial role in the initiation and pathophysiology of NEC in several animal models (7). In this report, we will discuss the effect of PUFA on PAF metabolism and delineate the importance of these changes on intestinal inflammation and necrosis.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; IL, interleukin; iNOS, inducible nitric oxide synthase; LC-PUFA, long-chain polyunsaturated fatty acids; NEC, necrotizing enterocolitis; NF, nuclear factor; PAF, platelet-activating factor; PAF-AH, PAF-acetylhydrolase; PKC, protein kinase C; $PLA_2$-II, phospholipase $A_2$-II.

## EFFECT OF PUFA ON NEONATAL NEC: CLINICAL TRIAL

Although PUFA supplementation of neonatal formula has been studied extensively for the outcomes of central nervous system development and visual acuity (8), limited information is available regarding PUFA effects on neonatal intestinal inflammation and necrosis. As part of a risk-benefit trial for diet and infant development, Carlson *et al.* (5) performed a prospective, randomized, controlled trial that included neonatal NEC as one of the secondary outcome variables. Using an egg phospholipid preparation of PUFA in experimental formula, they found a reduction of NEC in supplemented patients compared with controls (1 of 34 vs. 15 of 85, $P < 0.05$ using Fisher's Exact Test). The authors discussed several potential mechanisms that could explain the results, but also suggested that due to low numbers of patients in the experimental group, a type I error may have erroneously produced the difference identified between groups. Thus, a larger randomized trial was suggested to confirm these interesting results. Nonetheless, the mechanisms responsible for this potential benefit on neonatal NEC remain speculative.

## PUFA EFFECTS ON INFLAMMATION AND INTESTINAL INJURY

Many recent studies have confirmed that PUFA modulate the inflammatory response. Supplementation in various systems with n-3 PUFA result in anti-inflammatory responses, with recent clinical trials suggesting improvement in rheumatoid arthritis, psoriasis, inflammatory bowel disease, and asthma (9–11). In other studies, n-6 PUFA seem to act in opposition to the n-3 compounds, and activate the inflammatory response *via* interleukin (IL)-1 production and increased tissue responsiveness to additional cytokines (12,13). Further analyses suggest that the balance between n-3 and n-6 PUFA is critical and contributes to the regulation of inflammation *via* cytokine activation and effects. Nonetheless, specific cellular mechanisms responsible for these events have been evaluated, and preliminary evidence suggests multiple effects, including the following: (i) modulation of gene expression, e.g. nuclear factor (NF)-κB (14); (ii) alteration of protein kinase C (PKC) activity (15); (iii) inhibition of apoptosis (16); (iv) inhibition of $Mg^{2+}$-ATPase (17); (v) action on antioxidant system (18); and (vi) modulation of cytokine and prostaglandin metabolite production and receptor activation (2,19).

Additional studies have considered the specific effects of PUFA on intestinal inflammation and necrosis. It is known that multiple cytokines (including IL-1, IL-6, IL-10, IL-11, IL-12, and tumor necrosis factor) and arachidonic acid (AA) metabolites (i.e., prostanoids, leukotrienes, and thromboxanes) influence intestinal health *via* their effects on intestinal blood flow, mucosal permeability, and activation of secondary inflammatory mediators at the mucosal environment (20,21). However, the link between PUFA supplementation and cytokine/prostaglandin modulation leading to alteration of intestinal inflammation and necrosis remains conjectural. One study evaluated the role of n-3 PUFA on hypoxia-induced intestinal injury in mice and found a significant reduction in necrosis with the dietary fish oil supplementation (22). In addition, the results indicated that n-3 PUFA protected against hypoxia-induced leukotriene $B_4$ and PAF production in intestinal homogenates. This study indicated that dietary PUFA may influence local, intestinal mediator production, which in turn could modulate intestinal inflammation and necrosis. The association of intestinal PAF with intestinal injury in this study was of interest because recent evidence has supported a primary role for this mediator in other animal models of neonatal NEC.

## ROLE OF PAF IN NEONATAL NEC

PAF is a potent phospholipid mediator with diverse biological actions (23). PAF is synthesized in most cells and tissues from phosphatidylcholine precursors under the influence of a PAF-specific phospholipase $A_2$-II ($PLA_2$-II) to form lysoPAF, and subsequent acetylation to PAF *via* an acetyltransferase enzyme (see Fig. 1). The half-life of PAF is remarkably short due to rapid degradation catalyzed by the important enzyme PAF-acetylhydrolase (PAF-AH) (24). Of interest, PAF-AH activity is present in breast milk, is deficient in the circulation of neonates, and is significantly reduced in NEC patients compared with age-matched controls (25–27). PAF exerts its biological effects after binding to a G protein-coupled PAF receptor that is present in most tissues, but is highly expressed in intestinal samples (28).

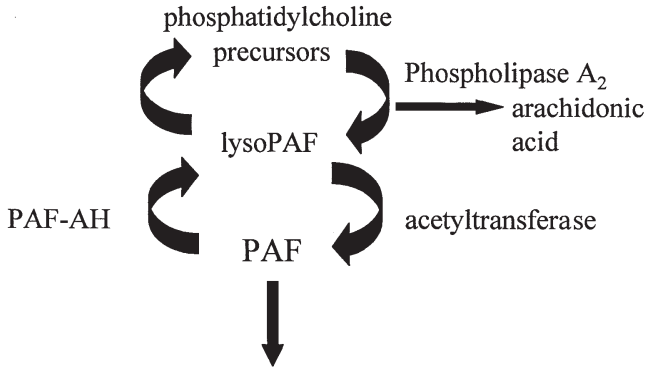


**FIG. 1.** The platelet-activating factor (PAF) cycle. PAF-AH, PAF-acetylhydrolase.

Although PAF is associated with systemic responses of capillary leak, vasoconstriction, platelet and neutrophil activation and degranulation, bronchoconstriction, and myocardial depression, studies suggest that PAF is intimately linked with intestinal necrosis (7,29). Hsueh and colleagues (30–32), using the adult rat model, have confirmed the importance of PAF in intestinal necrosis by showing the following: (i) intravenous PAF results in ischemic bowel necrosis (30); (ii) lipopolysaccharide-induced intestinal injury is blocked by PAF receptor antagonists; and (iii) PAF is increased in intestinal tissue after endotoxin stress (32). In addition, PAF activates several secondary mediators that contribute to the propagation of intestinal necrosis (33). We analyzed the importance of PAF in a neonatal rat model of NEC and showed that PAF receptor antagonists and enteral PAF-AH supplementation reduced the incidence of disease after asphyxia and formula feeding (34,35). Additional experiments have shown that PAF-AH is taken up in the intestinal epithelium, where it remains functionally active but does not translocate into the systemic circulation. Preliminary studies in our laboratory to delineate the cellular mechanisms of PAF-induced injury showed that PAF acts locally on the enterocyte PAF receptor to stimulate chloride transport, intracellular acidosis, and apoptosis, thereby leading to alterations in tight junctional integrity of the intestinal epithelium (Caplan, M., and Jilling, T., unpublished observations). Concurrent studies using the neonatal rat *in vivo* have shown that apoptosis is an important early event that precedes necrosis after asphyxia and formula feeding. Further study of these mechanisms may help clarify the pathophysiology of neonatal NEC, a disease whose etiology has so far remained elusive.

## ROLE OF PUFA SUPPLEMENTATION IN NEONATAL NEC

To investigate the specific role of PUFA supplementation in neonatal NEC, we utilized a well-described model that incorporates the typical risk factors for clinical NEC into the experimental protocol (36). Neonatal rats were fed formula for premature infants and challenged with asphyxia twice daily in a manner that has been shown to produce gross and microscopic NEC in 75% of animals by 96 h of life (37). To evaluate the effect of PUFA supplementation, experimental animals were fed formula supplemented with AA (34 mg/100 mL) and docosahexaenoic acid (DHA; 23 mg/100 mL) to provide an AA/DHA ratio of 1.5:1. Rats were followed up to 96 h for clinical signs of abdominal distention, abdominal wall discoloration, bloody stools, and respiratory distress; as symptoms developed, animals were killed and intestines recovered for gross and microscopic analysis of necrosis and apoptosis. In addition, separate groups of animals were studied at 24 and 48 h; before the development of intestinal injury, samples were frozen for subsequent analysis of mRNA expression of $PLA_2$-II, PAF receptor, and inducible nitric oxide synthase (iNOS), and for apoptosis of epithelial cells. Plasma was collected concurrently at these points for quantification of endotoxin, a crude estimation of bacterial translocation in this model.

We found that PUFA supplementation in this model markedly reduced the incidence of NEC and death compared with control formula (Table 1). Furthermore, PUFA significantly reduced plasma endotoxinemia that occurred by 48 h in animals treated with the protocol (Fig. 2). Analysis of apoptosis suggested no effect of PUFA on programmed cell death of intestinal epithelium. However, using reverse transcriptase-polymerase chain reaction normalized to β-actin, we found that PUFA reduced intestinal gene expression of $PLA_2$-II (at 24 h) and PAF receptor (at 48 h), but had no effect on iNOS at any time point (Figs. 3–5). The results suggest that PUFA reduced the risk for NEC in this model and that altered PAF metabolism might contribute to the beneficial effect of PUFA. The reduction in $PLA_2$-II mRNA lowered local PAF synthesis, and the suppressed PAF receptor expression prevented PAF-induced cellular effects, thereby together contributing to reduced likelihood of PAF-induced intestinal inflammation and necrosis after asphyxia and formula feeding. Although it is thought that bacterial translocation, activation of iNOS, and apoptosis participate in the pathogenesis of NEC (38–40), we found that PUFA had no effect on iNOS or apoptosis but did protect against bacterial translocation. The mechanisms responsible for the effect of PUFA on PAF metabolism and bacterial translocation may include many of those described above because NF-κB activation, PKC inhibition, and modulation of cytokines and prostaglandin metabolites affect PAF, mucosal integrity, and intestinal injury (20,41,42).

In summary, the effects of PUFA on intestinal inflammation and necrosis are complex, but recent studies suggest that these compounds reduce the incidence of NEC in premature infants and prevent NEC in neonatal rats with associated changes in PAF metabolism and bacterial translocation. Nonetheless, interpretation of human and animal studies with PUFA is difficult because differing preparations and ratios of n-3/n-6 fatty acids have been investigated. Additional

**TABLE 1**
**Effect of Polyunsaturated Fatty Acids (PUFA) on Necrotizing Enterocolitis (NEC) and Death**

| | NEC | Death |
|---|---|---|
| Control | 17/24 | 18/24 |
| PUFA | 8/24[a] | 8/24[a] |

[a]$P < 0.05$.

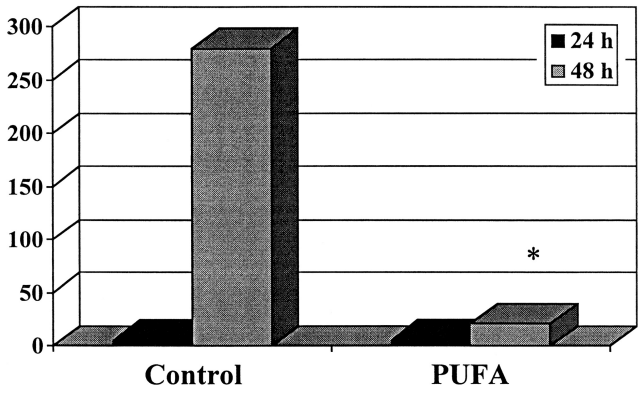


**FIG. 2.** Effect of polyunsaturated fatty acids (PUFA) on plasma endotoxinemia (endotoxin units/mL).

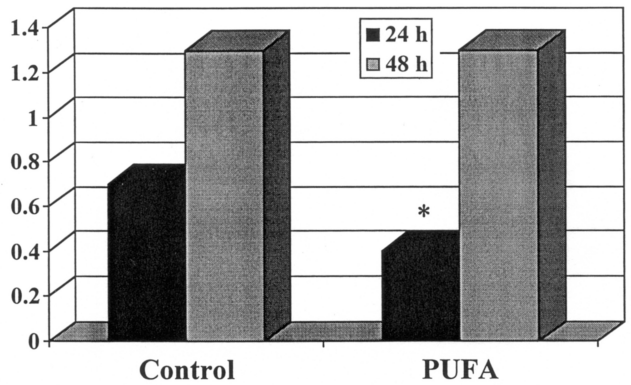


**FIG. 3.** Effect of polyunsaturated fatty acids (PUFA) on phospholipase $A_2$ ($PLA_2$) expression (ratio to β-actin).

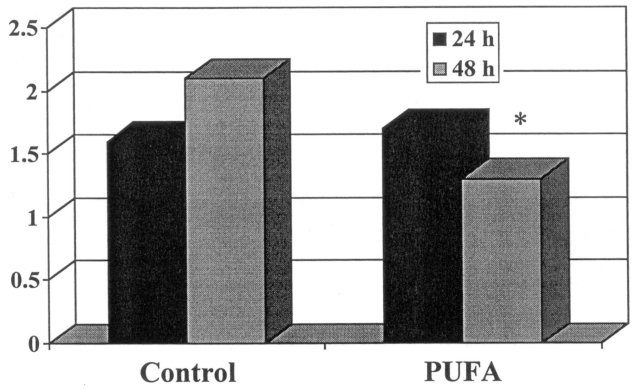


**FIG. 4.** Effect of polyunsaturated fatty acids (PUFA) on platelet-activating factor receptor expression (ratio to β-actin).

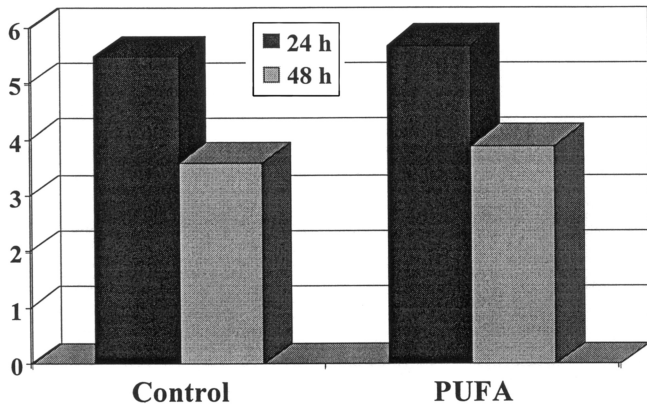


**FIG. 5.** Effect of polyunsaturated fatty acids (PUFA) on inducible nitric oxide synthase expression (ratio to β-actin).

research is warranted to delineate the specific cellular effects of PUFA on intestinal integrity, to clarify the specific components of PUFA responsible for improved intestinal health in animals, and to confirm the beneficial role of these dietary supplements for premature infants.

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# Abstracts

## A Symposium on PUFA in Maternal and Child Health

■ LCPUFA in Disorders of Protein and Carbohydrate Metabolism. C. Agostoni, E. Verduci, E. Racchi, and E. Riva, Department of Pediatrics, San Paolo Hospital.

In spite of the many trials on the role of LC-PUFA in fat metabolism, few studies are available on their functions in disorders of carbohydrate and protein metabolism. Breast-fed infants show higher percentage levels of DHA and total LC-PUFA in muscle phospholipids with lower plasma glucose levels when compared to formula-fed counterparts. These findings may suggest an early derangement of the insulin axis regulation in formula-fed infants. The relationship between the LC-PUFA synthesis rate and the insulin response seems to be quite complex, however. Obese children with elevated insulin levels and patients affected by type-1 glycogen storage disease, whose dietary regimen provides a high intake of slowly-absorbed carbohydrates, show increased LC-PUFA in plasma lipids. On the other hand, negative correlations between indices of delta-6 desaturase activity and blood insulin levels have been found in obese preadolescents. These data suggest that the insulin-associated stimulatory effects on desaturase activity, shown in earlier experiments, could depend on the individual degree of insulin sensitivity. In inborn errors of protein and amino acid metabolism, the dietary intervention provides semisynthetic diets without LC-PUFA. Dietary LC-PUFA on brain would be of potential utility for those conditions possibly affecting the functional development of the nervous tissues. We have recently investigated the effects of a 12-month supplementation with LC-PUFA in school-age children affected by type-I hyperphenylalaninemia (HPA). These patients follow a low phenylalanine diet, severely restricted in animal foods and LC-PUFA. At baseline HPA children showed a poorer DHA status and prolonged P100 wave latencies than reference children. After 12 months of LC-PUFA, we have found a significant increase in DHA levels of both plasma and erythrocyte lipids and a decrease of the P100 wave latency. In conclusion, LC-PUFA may 1) help regulate carbohydrate metabolism, 2) depend on the individual degree of insulin sensitivity as far as synthesis, and 3) aid brain development in inborn errors of amino acid metabolism that require unusual dietary intakes.

■ n-3 Fatty Acid Deficiency and Hippocampal Morphology. A. Ahmad, M. Murthy, R. Greiner, T. Moriguchi, and N. Salem, Jr., National Institute on Alcohol Abuse and Alcoholism, NIH.

Studies in our lab have previously shown that n-3 deficiency leads to deficits in learning and performance in rats on a number of behavioral tasks. To investigate the anatomical correlates of these deficits, we carried out morphological analysis of the hippocampus in these animals to ascertain the effects of n-3 deficiency. Nineteen adult female rats were raised for three generations on two n-3 deficient and two n-3 adequate diets. Deficient diets (Def and Def+LA) contained low linolenic acid (LNA) or low LNA and elevated linoleic acid (LA), and adequate diets (Adq and Adq+DHA) contained high LNA, or high LNA and docosahexaenoic acid (DHA). Unbiased stereological methods were used to investigate changes in the volume, density, and total number of neurons in the hippocampus of these animals. Cell size of neurons was also measured in CA1-3, granular, and hilar layers of the hippocampus at septal and temporal locations. To confirm the effect of diets on the total and phospholipid fatty acid compositions of the hippocampus, a separate group of 24 rats was used. We found that the cell body size of CA1 neurons decreased in the Def group compared to Adq and Adq+DHA groups at both septal and temporal locations, and the difference was statistically significant ($P<0.01$) between the Def and Adq+DHA groups at the septal location. No significant difference was detected in the size of neurons in other layers except that deficient diets led to a general decrease in the size of hippocampal neurons. No differences were detected in the volume of hippocampal layers, neuronal density, or total neurons across the dietary groups. However, magnitudes of these parameters were marginally higher in both deficient groups. The total fatty acid composition of the hippocampus was indeed dependent on the diets with profound changes in the n-6/n-3 ratios. DHA in the hippocampi of Def and Def+LA animals decreased by about 90% compared with animals on Adq and Adq+DHA diets with a reciprocal increase in docosapentaenoic acid (DPAn-6). The largest decrease in DHA was observed in phosphatidylserine (PS), followed by phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC), in Def and Def+LA groups with a similar and reciprocal increase in DPAn-6. Since the size of CA1 neurons decreased, and the density of neurons along with layer volume marginally increased in the deficient groups, it appears that the density of processes in these groups may be altered. Processes in oriens, radiatum, and lacunosum-molecular layers are being labeled with growth-associated protein 43 (GAP-43) to quantify arbor density in the deficient and adequate dietary groups in order to test this hypothesis.

Key Words: Hippocampus, n-3 Fatty Acids, Morphology

■ Docosahexaenoic Acid (DHA) Mediates Anti-Apoptotic Effects Through Phosphatidylinositol 3-Kinase Pathway: A Novel Observation in Neuronal Apotosis. M. Akbar and H.-Y. Kim, NIAAA.

Neuronal cells contain high levels of long chain polyunsaturated fatty acids, particularly docosahexaenoic acid (DHA). This fatty acid is considered to be essential for many physiological functions of the brain. Recently, we have demonstrated the antiapoptotic effect of DHA enrichment in serum-starvation-induced apoptosis, as pretreatment of Neuro 2A cells for 48 h with DHA (25 μM), but not with oleic acid (OA) and arachidonic acid (AA), significantly inhibited Caspase-3 activity and DNA fragmentation in comparison to non-enriched control cells. In the present study we found that DHA enrichment also prevented apoptotic cell death induced by staurosporine (ST). By using these two model systems, we further investigated the signaling mechanism involved in the antiapoptotic effect of DHA. We found that the protective effect of DHA was abolished by the treatment with a PI3-kinase inhibitor wortmanin (WM), but was not affected by inhibitors of protein kinase A, MEK or MAP kinase. Both serum starvation and ST-treatment significantly decreased phosphorylation of Akt, a well-known downstream kinase of PI3-kinase pathway involved in cell survival, and DHA enrichment restored the phosphorylation status of Akt. In the presence of WM, however, DHA enrichment did not recover the Akt phosphorylation which was suppressed by either serum starvation or by ST-treatment. Taken together, our findings strongly suggest that DHA may exert its antiapoptotic effect through the PI3-kinase/Akt pathway.

Key Words: Apoptosis, PI3-kinase, Akt Phosphorylation

■ The Relationship Between the Essential Fatty Acid Status at Birth and Neurophysiological Function of the Visual System at 8 Years of Age. E.C. Bakker[1,2], J. Reulen[2], F. Spaans[2], C.E. Blanco[2], and G. Hornstra[1], [1]Universiteit Maastricht, Maastricht, The Netherlands, [2]University Hospital Maastricht, Maastricht, The Netherlands.

Our aim was to investigate whether the concentrations of long chain polyunsaturated fatty acids (LCP) in phospholipids of umbilical plasma or umbilical vessel walls are associated with neurophysiological function of the visual system at 8 years of age. Several studies have found an influence of LCP status on the functioning of the visual system in young infants. This study is the first to investigate the long-term effects of LCP

NOTE: The first author was also the presenter; if otherwise, the presenting author has been indicated with an asterisk (*).

on the visual function. The study consisted of a follow-up of 60 children, born healthy and at term, who had been characterized with respect to their LCP status at birth. At 8 years of age, their visual function was assessed by electroretinography (ERG), visual-evoked potentials (VEP), and opthalmological measurements (including visual acuity, stereopsis, and contrast sensitivity). Results of these measurements have been related to the LCP status at birth by means of multiple regression analysis, correcting for potential confounding factors such as gestational age and age at measurement. Based on results of studies in young infants, a positive correlation between LCP status and the functioning of the visual system was expected. However, preliminary results of the 30 children evaluated so far give no evidence for such a correlation. None of the neurophysiological and opthalmological outcome measurements at 8 years was found to be significantly correlated to the LCP status at birth. The results from all 60 children including their essential fatty acid status at 7 years of age will be presented.

Key Words: LCP, Visual Function, Development

■ Distribution of Long Chain Polyunsaturated Fatty Acids in Canine Milk Parallels Changes in Plasma Total Phospholipid Fatty Acids During Lactation. J.E. Bauer[1], K.E. Bigley[1], G.E. Lees[1], and M.K. Waldron[2], [1]Texas A&M University, College Station, TX, USA, [2]Ralston-Purina Co., St. Louis, MO, USA.

Retina and central nervous system of mammals are rich in long chain polyunsaturated fatty acids (LC-PUFA) including arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3). Although human milk contains DHA and AA in amounts high enough for their rapid accumulation in developing nervous tissues, the need for LC-PUFA supplementation of infant formulas to reflect human milk remains controversial. Visual and neurologic development in dogs is not only important, but information obtained using canines may provide useful comparative data. The objective of this study was to investigate fatty acid (FA) profiles and LC-PUFA content of total milk lipid extracts from dogs fed a diet of known fatty acid composition and compare them with plasma phospholipid (PL). Milk and plasma samples were obtained from 11 nursing, adult dogs at <1 week lactation, between 1 and 2 weeks, and >2 weeks lactation. Diet was a mixture of canned and dry puppy growth foods with similar fatty acid profiles. Total lipids were extracted from all samples; lipid classes were fractionated from sera; and FA methyl esters were prepared and subjected to capillary gas chromatography. Total milk fat was determined gravimetrically. Milk FA profiles reflected the diet. Plasma PL fatty acids also reflected the diet although relative amounts of 18:0 and 20:4n-6 were higher in plasma PL, consistent with active *de novo* synthesis, desaturation, and chain elongation during PL synthesis. A decrease in relative amounts of 20:4n-6, 22:5n-3 and 22:6n-3 in milk paralleled a similar decrease in plasma PL from early to later lactation, and total fat dropped slightly. Milk contained approximately 11–12% total fat with moderate amounts of AA, DPA, and DHA. At 1 week, % AA, DPA, and DHA in dog milk were 1.8%, 0.2%, and 0.07%, respectively. Compared to 1 week, total milk LC-PUFA content decreased by 8% at 1–2 weeks lactation and by 12% at >2 weeks; similar to humans. By contrast, recommendations for human infant formula based on human milk are lower for AA (0.3–1.1%) and higher for DHA (0.25–0.76%). Amounts of AA in canine milk in this study are moderately higher, while those of DHA are markedly lower compared with humans. Why these differences exist is unknown. They may initially depend on dietary fatty acid intake but appear to reflect plasma phospholipid profiles. Amounts of n-6 and n-3 LC-PUFA necessary for optimal puppy neonatal development require further study.

Key Words: Canine Milk, Polyunsaturated Fatty Acids, Lactation

■ Essential Fatty Acids and the Development of Childhood Atopic Disease. B. Björkstén[1] and K. Duchen[2], [1]Centre for Allergy Research, Karolinska Institutet, [2]Department of Paediatrics, Linkoping University, Sweden.

The potential relationship between the intake of long chain fatty acids and childhood allergy has been discussed for many years. Conclusive evidence is still lacking, although several recent clinical studies suggest that atopic manifestations in infancy may be associated with a low dietary intake of essential fatty acids (FA), particularly alpha-linolenic acid (LNA). Most often, atopic dermatitis has been linked to an abnormal FA metabolism. Clinical studies of treatment of atopic dermatitis and asthma, by modifying the FA content of the diet have been largely disappointing, however. At least some atopic infants seem to have an imbalance in FA composition. The most consistent findings include lower levels of n-3 PUFA and high ratios of n-6/n-3 FAs in allergic individuals. Also, the relationships between individual FAs of the n-6 and n-3 series are abnormal, indicating disturbances in the FA metabolism. A delta-6 desaturase dysfunction has been suspected in several studies. As the atopic phenotype may be the consequence of any of several genotypes, particularly those related to the regulation of IgE antibody formation, it is not to be expected that allergic disease would be linked to an abnormal FA metabolism in all patients. It is likely, however, that some cases of allergic disease are a consequence of disturbed FA regulation. This would open the possibility for prevention and treatment of some patients by regulation of the diet. Similarly, the allergy preventive properties of human milk are controversial. Recent studies have demonstrated that there are individual variations in the FA composition of human milk, as well as in the content of components of the immune system. Thus, low levels of linoleic acid (LA), LNA, n-6 LCP and particularly LA/LNA and AA/EPA ratios have been reported in human milk of atopic mothers. Furthermore, low levels of n-3, including EPA, DPA, and DHA, as well as altered n-6/n-3 FA and AA/EPA ratios have been found in milk from mothers of allergic babies. It is reasonable to suggest that some of the controversy regarding the role of breast-feeding in allergy prevention is due to individual variations in the composition of human milk. If a relationship between an abnormal FA composition and the development of allergic disease would be confirmed, then this would open for primary prevention of allergies by giving lactating mothers a dietary supplement of n-3 FA.

Key Words: Human Milk, Allergy, Fatty Acids

■ Stable Isotope Studies of Maternal and Infant Polyunsaturated Fatty Acid Metabolism Using Primate Models. J.T. Brenna, Cornell University.

Recent and continuing advances in stable isotope availability and analytical techniques present increasingly powerful tools for investigation of a wide variety of metabolic issues. Specifically, high-precision compound-specific isotope analysis permits the investigation of *in vivo* metabolism at high sensitivity using highly enriched $^{13}C$-labeled tracers and/or by taking advantage of natural variability in $^{13}C/^{12}C$. Tracers are most appropriate for endogenous molecules when it is desirable to label specific parameters involving metabolism of a compound. An example is the labeling of two or more substrates for a single product, such as dietary 18:3 and 22:6 as substrates for brain 22:6. An alternative on this theme is labeling of two forms of the same fatty acid to evaluate utilization efficacy, for example investigating the relative accretion 20:4 consumed as phospholipid or as triglyceride. Other examples include labeling of a route of entry, such as oral and intravenous administration, or labeling of time points using multiple isotopes or isotopomers. Labeling can also be used to investigate the disposition of a single uniformly labeled compound, such as the relative amount of essential fatty acid carbon converted into saturates and monounsaturates, compared with that excreted as $CO_2$. These approaches all have analogues in radiotracer studies, but are often more conveniently accomplished with stable isotopes and do not present the hazards and increasing expense and regulatory burden of radiotracers.

In maternal/infant PUFA nutrition, the question of amount and form of dietary PUFA for long chain PUFA has been under active investigation for several years. Such studies have established that premature human infants can convert 18:3 to 22:6. However, human studies typically suffer from severely limited sampling, typically blood and little or no target tissue, particularly when infants are involved. Kinetic modeling based on blood sampling can provide only rough, tentative estimates of metabolism. In contrast, animal studies permit sampling from critical target tissues such as retina, brain, and liver and deliver direct measurements of tracer concentrations of interest. Quantitative estimates of 18:3 to 22:6 conversion are straightforward using animals. Examples of several of these principles will be presented.

Key Words: Stable Isotopes, Tracers, Primates

■ The Effect of Dietary Docosahexaenoic Acid (DHA) Supplementation on Human Milk Cytokine Production. D-L. Bryan[1], J.S. Hawkes[2], M. Makrides[3], M.A. Neumann[1], and R.A. Gibson[4], [1]Flinders University of South Australia, [2]Flinders Medical Centre, [3]Women's and Children's Hospital, [4]Child Health Research Institute, Adelaide, Australia.

An increased requirement for dietary DHA (22:6n-3) during pregnancy and lactation has resulted in some expert nutrition committees recommending supplementation for pregnant and lactating women. n-3 PUFA have also been recognized as regulatory agents for inflammatory cytokine production. We have previously reported on levels of the inflammatory cytokines tumor necrosis factor α (TNFα), interleukin (IL) 1β and IL6, in the aqueous fraction of human milk (Hawkes, J.S., *et al*., *Pediatr. Res. 46*, 194–199, 1999). The aim of this study was to examine the effects of dietary DHA supplementation on two of these potentially inflammatory human milk cytokines, TNFα and IL6, as well as the cytokines transforming growth factor (TGF) β1 and TGF β2. Mothers were randomized into three groups and asked to consume a dietary supplement from day 3 postpartum. Group 1 (placebo vegetable oil, *n*=28), group 2 (Lo DHA 300 mg of DHA per day, *n*=27) and group 3 (Hi DHA 600 mg of DHA per day, *n*=28). Milk samples (50mL) were collected by manual expression at week 4 and fractionated by centrifugation to provide fat for fatty acid analysis and the aqueous fraction for measurement of TNFα, IL6, TGFβ1, and TGFβ2. Levels of these aqueous phase cytokines were determined by ELISA, established using matched antibody pairs. Supplementation increased breast milk DHA (mean %±SD) from 0.26±0.08 (placebo) to 0.39±0.08 (Lo DHA) and 0.66±0.18 (Hi DHA) (*P*=0.00). The aqueous phase cytokines TNFα, IL6, TGFβ1, and TGFβ2 were detectable in 60–100% of samples. Preliminary analysis shows no difference between levels of these cytokines in each of the supplementation groups. Our results indicate that increased maternal consumption of n-3 PUFA during lactation does not cause perturbations in these immune mediators in human milk.

Key Words: Docosahexaenoic Acid, Lactation, Cytokines

■ Incorporation of α-Linolenic Acid (ALA) and Linoleic Acid (LA) into Human Epithelial Cell Lines. D.-L. Bryan[1], P. Hart[1], K.F. Forsyth[2], and R.A. Gibson[3], [1]Flinders University of South Australia, [2]Flinders Medical Centre, [3]Child Health Research Institute, Adelaide, Australia.

There have been numerous animal and human studies designed to examine the effects of α-linolenic acid (ALA) and linoleic acid (LA) supplementation on the fatty acid composition of plasma and tissues. A feature of all these studies is the marked difference in incorporation into plasma and tissue phospholipids of these 18-carbon precursors of the long chain polyunsaturates. While dietary LA and ALA are linearly related to tissue phospholipid levels, the levels of tissue LA can be 10-fold higher than tissue ALA even when dietary levels are equivalent. There is some dispute whether this disparity is due to ALA being more rapidly metabolized to its products by the liver or whether LA is preferentially incorporated into cellular phospholipids. We examined the level of incorporation of polyunsaturated fatty acids (PUFA) into human respiratory epithelial cell lines (A549, 16HBE) by determining the dose-dependent incorporation of ALA and LA free fatty acids (5–150 μg/mL FFA) over 24 hours. Cell membrane phospholipid ALA and LA were increased up to ~26–30% total fatty acids with a concomitant decrease in other membrane fatty acids before significant toxicity was observed (50 μg/mL). Analysis of culture media showed little to no conversion of FFA to other lipid classes or of PUFA to their metabolites. Our results indicate that both LA and ALA can be incorporated to similar levels into human cell lines *in vitro* without a concomitant rise in long chain metabolites such as AA, EPA, or DHA. The potential role for LA and ALA to regulate synthesis of mediators of immunity (cytokines, eicosanoids) and other processes per se can thus be explored using this model. Our data support the concept that rather than any inherent inability by human cells to incorporate ALA into membrane phospholipids, the lack of incorporation in human and animal models *in vivo* is due to the rapid metabolism of this fatty acid to other n-3 PUFA in the liver.

Key Words: Alpha-Linolenic Acid, Linoleic Acid, Human Epithelial Cell Lines

■ Relationship Between Spanish Lactating Mothers' Diets and the Antioxidant Vitamin Content in Breast Milk. C. Campoy[1], R.M. Baena[1], A. López-López[2], J. Garrido[1], C. López-Sabater[2], M. Rivero[3], R. Bayés[1], and J.A. Molina-Font[1], [1]Dept. of Paediatrics, School of Medicine, University of Granada, Spain, [2]Dept. of Nutrition and Bromatology, School of Pharmacy, University of Barcelona, Spain, [3]Scientific Dept. of Laboratories Ordesa, Barcelona.

Maternal nutrition during lactation may play an important role in breast milk composition, and thus in the nutrition of their infants. Breastfed newborns depend on maternal milk for their supply of vitamins A (A) and E (E). The aim of this study was to determine if there was a relationship between the lactating mother's food (especially fatty acid) intake and the antioxidant vitamin composition of breast milk. METHODS: 100 samples of breast milk from healthy lactating women between 17 and 37 years of age were collected: 34 colostrum (1–6 days) (C), 32 transition milk (7–14 days) (T), and 34 mature milk (15–41 days) (M). Women were asked to record their food intake for three days before they supplied milk. Evaluation of the questionnaires was accomplished with software developed by Suarez *et al.,* using Wander food composition tables. The milk from each breast was obtained at the beginning and end of each feed throughout the day to minimize any effects of diurnal rhythm on the composition of the milk. The milk samples of one mother were stored individually at -20°C without light and pooled at the end of the day. They were then stored under nitrogen at -80°C until analysis. E and A were measured in mg/dL using HPLC. ANOVA, Student/Welch *t* test and correlation analyses were performed. RESULTS: Significant correlations were found between the different components studied and the days of lactation. Breast milk: E: $r$=-0.49, A:$r$=-0.47. Mother's Intake: MUFAs: $r$=0.34, SFAs: $r$=0.48, CHOL: $r$=0.24, Total Fat: $r$=0.29; Fe: $r$=-0.30, Zn: $r$=-0.53, Carbohydrates: $r$=-0.47. A and E content in breast milk diminished significantly from colostrum to mature milk while the intake of these vitamins by the lactating mother was unaltered in the same period. This effect could be explained by the efficient mechanism of mammary gland E uptake around parturition and related to the higher fat content in colostrum milk. An increase in the mother's intake of MUFAs and SFAs during the periods of transition and mature milk compared to the colostrum was observed. As far as the nutrient content of womens' diets, the mean intake of protein was 109 g/day compared with the RDA recommendation of 65 g/day. The consumption of fats was higher than has been recommended (30%) as a percentage of total energy: 39.2% and 36.8% at the time of providing transition and mature milk, respectively. The profile of fatty acids was also different from that recommended, although possibly due to the high consumption of olive oil (90%). Ca, Fe, and vitamin A and D intake were less than RDA recommendations.

Key Words: Vitamins E and A, Breast Milk, Mother's Diet

■ Docosahexaenoic Acid (DHA) and Arachidonic Acid Content of Breast Milk in Spanish Women: Influence of Mothers' Diets. C. Campoy[1], A. López-López[2], E. Blanca[1], R.M. Baena[1], A. Jerez[1], C. López-Sabater[2], M. Rivero[3], and R. Bayés[1], [1]Dept. of Paediatrics, School of Medicine, University of Granada, Spain, [2]Dept. of Nutrition and Bromatology, School of Pharmacy, University of Barcelona, Spain, [3]Scientific Dept. of Laboratories, Ordesa, Barcelona.

Long chain polyunsaturated fatty acids (LC-PUFA), especially arachidonic (AA) and docosahexaenoic (DHA) acids, are important for normal visual and brain development. PUFAs can be provided to the infant by human milk (HM). HM composition can be affected by different factors such as gestational age, parity, diseases, individuality, and mother's diet. This study analyzed the influence of mother's diet on the HM PUFA composition. METHODS: A total of 100 samples of HM from healthy lactating women—17 to 37 years of age—were collected: 34 colostrum (1–6 days lactation) (C), 32 transition milk (7–14 days lactation) (T) and 34 mature milk (15–41 days lactation) (M). Women recorded dietary intake during the 3 days before C, T, and M milk were obtained. The questionnaires were evaluated using Wander food composition tables. The HM from each breast was obtained at the beginning and end of each feed throughout the day to minimize the effects of diurnal rhythm on the composition of the HM. The sample were kept at -20°C protected from light and pooled and preserved at -80°C under nitrogen at the end of the day. DHA and AA acids were measured in mg/dL by HPLC. ANOVA, Student/Welch *t* test for paired and unpaired data, and correlation analyses were performed. RESULTS: Linolenic acid content in HM increased from C to M milk (days of lactation-

linolenic acid: $r=0.44$, $P<0.0001$). DHA, AA concentrations, and linoleic/linolenic ratio in HM decreased with duration of lactation ($r=-0.45$, $r=-0.47$, and $r=-0.54$, $P<0.0001$, respectively). A correlation between the mother's PUFA intake by the diet and linolenic content in C ($r=0.36$, $P<0.03$) was demonstrated. DISCUSSION: There is a relationship between the maternal dietary DHA and higher concentrations of DHA in the blood of the newborn infant. Our data demonstrate that mother's PUFA intakes in the first days after birth influence the PUFA content in their milk, as well as the supply of these important nutrients to the breast-fed infant. With the progress of lactation and the increase of MUFAs and SFAs in milk, this relationship disappears, and there is a decrease in the DHA, AA, and linoleic acid content of milk. However, the progressive increase of the linolenic acid concentration in mothers' milk with longer lactation suggests a natural mechanism for the infant to obtain the substrate for endogenous production of DHA to meet its needs. CONCLUSION: The levels of PUFAs in the mother's diet can influence her PUFA status, her milk PUFA, and, therefore, the quality of the fat given to the infant.

Key Words: PUFAs, Human milk, Mother's diet

■ Isomeric Fatty Acids in Mothers and Their Infants: A Further Exploration of Relationships to DHA and AA in a New Population with Benefit of Umbilical Vessel Wall Fatty Acids. C.M. Smuts[1]*, M.I. Huang[2], D.C. Mundy[3], and S.E. Carlson[2], [1]MRC, Tygerberg, South Africa, [2]Univ. of Kansas Med. Center, Kansas City, KS, [3]Univ. of Missouri-Kansas City, Kansas City, MO.

Background: We have reported an inverse correlation between cord venous plasma (CVP) triglyceride (TG) *trans* fatty acids and CVP phospholipid (PL) DHA ($n$=50), but stronger inverse correlations between 18:1 *cis* isomers in CVP TG vs. CVP PL DHA and AA in a population studied in Memphis, Tennessee (*Ped. Res. 39,* 304A, 1996). Methods: We studied mothers and babies in Kansas City, Missouri ($n$=50) for whom we prepared PL and TG fatty acid methyl esters (FAMES) with $BF_3$ after TLC separation of CVP, cord arterial plasma (CAP), maternal plasma (MP), and cord umbilical artery and vein walls at delivery. FAMES were analyzed by GLC with an SP-2560 (Supelco) 100-M column. Results: Maternal plasma TG 18:1 *trans* and *cis* isomers were nearly equivalent (3.81 vs. 3.86% of total fatty acids). In comparison, CVP TG had much lower 18:1 *trans* (0.92%) but 4.4% 18:1 *cis*. CAP and CVP TG had similar relative 18:1 *trans* and 18:1 *cis* isomers. Correlations of 16:1 *trans*, 18:1 *cis,* and 18:2 *cis,trans* isomers with DHA and AA in tissues and plasma are shown below. All PL measured (artery, vein, MP, CVP, and CAP) had significant inverse relationships between AA and DHA and one or more of the following: 16:1 *trans,* 18:1 *cis,* and 18:2 *cis,trans* isomers. However, the artery wall was the most robust indicator of these inverse relationships. Conclusion: The artery wall (which carries deoxygenated blood from fetus to placenta) showed the most consistent inverse relationship between isomeric fatty acids and both AA and DHA in PL.

| | AA vs. 16:1*t* | AA vs. 18:1*c* | AA vs. 18:2*t,c* |
|---|---|---|---|
| Artery PL | –0.313* | –0.649** | –0.405** |
| CAP PL | –0.418** | N.S. | –0.410** |
| CAP TG | +0.395* | –0.452** | N.S. |
| Vein PL | N.S. | –0.357* | N.S. |
| CVP PL | N.S. | N.S. | –0.371* |
| CVP TG | –0.318* | –0.564** | N.S. |
| MP PL | –0.325* | N.S. | N.S. |
| MP TG | N.S. | N.S. | N.S. |
| | DHA vs. 16:1*t* | DHA vs. 18:1*c* | DHA vs. 18:2*t,c* |
| Artery PL | –0.317* | –0.776** | –0.513** |
| CAP PL | –0.473** | –0.495** | –0.307* |
| CAP TG | N.S. | –0.481** | N.S. |
| Vein PL | N.S. | –0.481** | N.S. |
| CVP PL | –0.384** | –0.353* | N.S. |
| CVP TG | N.S. | N.S. | N.S. |
| MP PL | –0.333* | N.S. | –0.400** |
| MP TG | N.S. | N.S. | N.S. |

Key Words: Isomeric Fatty Acids, n-3 and n-6 LC-PUFA, Umbilical Arteries and Veins

■ Polyunsaturated Fatty Acid Regulation of Gene Expression. S. Clarke, M. Nakamura, and C. Nelson, The University of Texas at Austin.

Dietary polyunsaturated fatty acids (PUFA) function as fuel partitioners in that they direct glucose toward glycogen storage, and direct fatty acids away from triglyceride synthesis and into fatty acid oxidation. 20-Carbon PUFA are more effective than 18-carbon PUFA. PUFA enhance fatty acid oxidation by inducing the transcription of genes encoding proteins involved in fatty acid oxidation (e.g., acyl-CoA oxidase), and PUFA reduce triglyceride synthesis by down-regulating genes encoding proteins of lipid synthesis (e.g., fatty acid synthase). The induction of oxidative genes involves the fatty acid activation of the transcription factor, peroxisome proliferator activated receptor (PPAR). Once activated, PPAR binds to a hexameric repeat (-AGGTCAnAGGTCA-) located within the 5′-flanking region of genes involved in fatty acid oxidation, ketogenesis, and thermogenesis (e.g., UCP-3). PUFA inhibition of lipogenic genes results from the ability of PUFA to reduce the nuclear content of the powerful lipogenic transcription factor, sterol regulatory element binding protein-1 (SREBP-1). PUFA accomplish this by suppressing the proteolytic release of SREBP-1 from its precursor form anchored in the endoplasmic reticulum and by accelerating the rate of SREBP-1 mRNA decay, thereby reducing the amount of precursor SREBP-1. In addition to their effects on lipid oxidation and lipogenesis, PUFA, particularly 20-carbon n-3 PUFA, are strong suppressors of delta-6 and -5 desaturase gene transcription, as well as inducers of skeletal muscle UCP-3 gene expression. The outcome of these coordinated gene events includes decreased blood triglycerides and increased lipid utilization.

Key Words: Transcription, Fatty Acids, SREBP-1

■ A Possible Role of Essential Fatty Acids in Pregnancy-Related Cognitive Impairment. R.H.M. de Groot, J.J. Adam, J. Jolles, A.C. van Houwelingen, and G. Hornstra, Maastricht University.

Background: Memory loss is a commonly observed phenomenon during pregnancy. Previous studies have shown that the overall essential fatty acid (EFA) status as well as the functional docosahexaenoic acid (DHA, 22:6n-3) status declines during pregnancy. Moreover, earlier studies also demonstrated that DHA plays an important role in brain functioning. Hence, it is tempting to hypothesize that the decline in DHA status during pregnancy might be functionally related to impaired cognitive functioning. Methods: In order to investigate a possible relationship between the decrease in EFA and/or DHA status during pregnancy and cognitive functioning, 58 pregnant women at week 14 of gestation were compared with different control groups. Two neurocognitive tests were used: the Stroop-Color-Word test as a measure of selective attention, and the spatial finger-precuing task as a measure of response preparation. Findings: Preliminary results at week 14 of gestation suggest a substantial neurocognitive impairment as indexed by performance on the spatial finger-precuing task and the Stroop test. Pregnant women demonstrated impaired reaction times comparable to those observed in elderly people. Nonpregnant controls the same age as the pregnant group were significantly faster (50 msec) on the finger-precuing task. The score on the Stroop test of the pregnant women was delayed, with an average of 3.6 sec compared with a control group of women matched for age and education, suggesting a lack in attention. Interpretation: These results could be taken as evidence to suggest that the decrease in EFA status, particularly DHA, during pregnancy might have a negative effect on neurocognitive functioning. However, randomized, double blind, placebo-controlled intervention studies are necessary to investigate whether a prevention of the pregnancy-associated DHA status decline also prevents neurocognitive impairment during pregnancy. Such studies are presently in progress in our laboratory. Results of an alpha-linolenic acid (18:3n-3) intervention study will be available later this year.

■ The Influence of Body Weight on the Metabolism of $^{13}$C-Linoleic Acid in Newborns. H. Demmelmair[1], P. Szitanyi[2], A. Mydlilova[2], and B. Koletzko[1], [1]Dr von Haunersches Kinderspital, Ludwig-Maximilians-University, Munich, Germany, [2]Thomayer Hospital, Charles University Prague, Czech Republic.

Birth weight is an indicator of endogenous lipid stores and is related to infant health and development. Several studies have shown that post-

natal growth is related to arachidonic acid availability. By comparing the disposal of stable isotope labeled linoleic acid during the first week of life in newborns with appropriate weight for gestational age (AGA) to small for their gestational age (SGA) neonates, we wanted to identify the relationship of birth weight to linoleic acid metabolism. Subjects and methods: Healthy, full-term, breast fed (AGA, $n$=10, birth weight: 3.4±0.2 kg (M±SD); SGA, $n$=9, 2.2±0.3 kg) neonates were enrolled in the study at day 2 of life. Each infant received 1 mg/kg body weight of uniformly $^{13}C$ labeled linoleic acid orally prior to feeding. Expired air (over 6 h) and blood (basal, 24 h, 48 h, and 72 h) samples were taken, and total $CO_2$ production was measured by indirect calorimetry. Fatty acids were quantified by gas chromatography and $^{13}C$ content of expired air and plasma phospholipid fatty acids were determined by isotope ratio mass spectrometry. Using a three-compartment model with assumed bolus injection of the tracer into the plasma phospholipid pool, fractional turnover of linoleic acid and its relative conversion to dihomo-γ-linolenic acid and arachidonic acid were estimated. Results: The concentration of phospholipid linoleic acid was significantly lower in AGA than in SGA infants (81.9±6.8 mg/L vs. 131.9±7.4 mg/L, M±SE), while no significant differences were found for dihomo-γ-linolenic (25.1±1.3 mg/L vs. 27.4±2.0 mg/L, SGA vs. AGA) and arachidonic (133.7±9.3 mg/L vs. 146.1±6.7 mg/L) acids. After 6 hours 7.4±0.6% of administered linoleic acid had been recovered in $CO_2$ in the AGA group and 5.1±1.1% in SGA ($P$<0.05). The fractional turnover of plasma phospholipid linoleic acid not directed towards dihomo-γ-linolenic acid or arachidonic acid was similar in both groups (3.1±0.3%/h vs. 3.3±0.3%/h, NS). Relative conversion of linoleic acid to dihomo-γ-linolenic acid (0.02±0.01%/h) and to arachidonic acid (0.01±0.01%/h) was significantly lower in AGA than in SGA, which showed 0.11±0.04%/h and 0.14±0.05%/h, respectively. Conclusions: Assuming an equal efficiency of tracer absorption in both groups, AGA infants oxidize a higher proportion of exogenous linoleic acid than SGA infants, which might be due to lower plasma concentrations or less exchange with body stores. Overall turnover of phospholipid linoleic acid is similar between groups, but there is a higher relative conversion of linoleic acid to dihomo-γ-linoleic acid and arachidonic acid in SGA infants. The reduced availability of long chain polyunsaturated fatty acids from body stores in SGA infants seems to be counterbalanced by increased endogenous production.

Key Words: Arachidonic Acid Synthesis, Birth Weight, Neonates

■ Investigation of Fatty Acid Metabolism Using Natural Abundance Variation of $^{13}C$. H. Demmelmair and B. Koletzko, Dr. von Haunersches Kinderspital, Ludwig-Maximilians-University, Munich, Germany.

Metabolic fluxes can be studied *in vivo* by the introduction of tracer molecules into the organism. Molecules enriched in a stable isotope, such as $^{13}C$, which naturally contributes only about 1.08% to total carbon in biomass, are very suitable for this purpose. While tracers with a $^{13}C$ content above 99% are available, highly precise gas isotope ratio mass spectrometry offers the possibility to exploit natural variations in $^{13}C$ content. There is a small enrichment difference between biomaterial originating from photosynthesis of $C_4$ plants (about 1.097% $^{13}C$) and carbon fixed by $C_3$ plants (about 1.081% $^{13}C$). In Europe the diet is largely based on $C_3$ plants (e.g., wheat, potato, sugar beet); therefore, lipids from $C_4$ plants (e.g., corn) can be used as tracer substances. In conventional infant formula and breast milk we have found a $^{13}C$ content of 1.077% for linoleic acid, while linoleic acid in corn oil showed 1.093% $^{13}C$. After switching the infant diet from breast milk or regular formula to a corn oil-containing formula, we observed a subsequent increase in the $^{13}C$ content in plasma linoleic acid (+0.012%) and its metabolites dihomo-γ-linolenic acid (+0.006%) and arachidonic acid (+0.002%) during the following 4 days when samples were taken. Since plant oils do not contain long chain polyunsaturated fatty acids, the increased $^{13}C$ contents in products demonstrate their endogenous synthesis from the dietary linoleic acid. The advantage of using naturally $^{13}C$ enriched corn oil is a relatively uncomplicated application as it is an established dietary component and low in cost. On the other hand, the signal to noise ratio is comparatively low with a precision of measurements around 0.0003% $^{13}C$, and dietary amounts rather than tracer amounts have to be fed. This excludes study diets deviating from the natural substrate composition. Furthermore, labeling of defined intramolecular positions is not possible with natural substrates.

While enrichment data obtained after the application of naturally or artificially enriched tracers can demonstrate the activity of a pathway by increased enrichment in the product, it is difficult to obtain quantitative data *in vivo* in humans. Direct measurement of tracer quantities is only possible in excretion products, such as breath $CO_2$, milk, or urine. From tracer concentrations in plasma, only approximate estimates of metabolic activities can be obtained. This usually requires the setup of models with inherent simplifications and assumptions, e.g., plasma concentrations are representative for corresponding pools in metabolically active tissue. Nevertheless, tracer studies elucidate metabolic fluxes, and estimators can be obtained for a comparison between different dietary or physiological states.

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Key Words: $^{13}C$, Natural Abundance, Quantification

■ Transport of Long Chain Fatty Acids Across the Human Placenta: Role of Fatty Acid-Binding Proteins. A.K. Dutta-Roy, Rowett Research Institute, Aberdeen, Scotland, UK.

Essential fatty acids (EFA) and their long chain polyunsaturated fatty acid (LC-PUFA) derivatives play a crucial role in fetal development and pregnancy outcome. The critical importance of maternal dietary intake or synthesis of LC-PUFA and their subsequent preferential transport by the placenta to the fetus is now well recognized. We have identified several membrane-associated (FAT, FATP, and p-FABPpm) and cytoplasmic fatty acid-binding porteins (H-FABP and L-FABP) in human placental trophoblasts. Although the exact roles of the membrane-associated and cytoplasmic fatty acid-binding proteins in complex FFA uptake and subsequent metabolism in the human placenta are yet to be understood, studies suggested that these proteins alone or in tandem may be involved in effective uptake and transfer of LC-PUFA by the placenta. The placental plasma membrane fatty acid-binding protein (p-FABPpm), with a molecular mass of ~40 kDa, preferentially binds LC-PUFA over nonessential fatty acids. The exclusive location of p-FABPpm on the maternal side of the placenta may favor the unidirectional flow of maternal LC-PUFA to the fetus by virtue of preference for these fatty acids, whereas location of FAT and FATP on the both sides of the bipolar placental cells may allow bidirectional flow of all FFA across the placenta. There are significant differences in the properties and regulation of these two FABP types. H-FABP only binds long chain fatty acids, whereas the L-FABP binds heterogeneous ligands such as bile salts, haem, peroxisome proliferators, selenium, lysophosphatidic acid, and eicosanoids. It has been suggested that FABPs may also interact with several fatty acid-mediated cellular processes, such as control of cell growth, cell signaling, and regulation of gene expression (such as PPAR). Studies on the distribution of fatty acids in the cellular lipids of trophoblast cells have shown that DHA is incorporated mainly into the triacylglycerol fraction, followed by the phospholipid fraction, whereas for arachidonic acid (AA) the reverse is true. The greater cellular uptake of DHA and its preferential incorporation into the triacylglycerol fraction suggest that both uptake and transport modes of DHA by the placenta to the fetus are different from that of AA. Certain LC-PUFA upregulate PPARg in human placenta. Since PPARg regulates terminal epithelial differentiation of the trophoblast and fetal cardiac membrane development, and is the only source for embryonic tissues, this indicates importance of placental LC-PUFA transport in feto-placental growth and development.

Key Words: Fatty Acid Binding Protein, Placenta, LC-PUFA

■ NMR Investigations of Docosahexaenoic Acid Structure and Flexibility. K. Gawrisch, L.L. Holte, B.W. Koenig, I.V. Polozov, A.M.K. Safley, and W.E. Teague, NIAAA, NIH, Rockville, MD.

We investigate conformation and flexibility of docosahexaenoic acid (DHA) chains in lipid membranes by magic angle spinning NMR approaches. Order parameters of all 22 carbon segments are measured. The excellent chemical shift resolution of DHA resonances enables us to partially assign order parameters to specific chain segments. We also measure cross-relaxation rates between the well-resolved DHA proton resonances using the NOESY experiment. In comparison to saturated chains,

DHA order parameters are very low, reflecting both a change in bond geometry and an increase in angular fluctuation amplitudes for some bond angles. DHA is often perceived as rigid due to the presence of six double bonds that eliminate a significant fraction of the internal degrees of freedom for structural transitions. Our NMR results support a very different model. Docosahexaenoic acid is surprisingly flexible with rapid structural transitions between a large number of conformations. The high flexibility of DHA is the consequence of lower potential barriers for rotations around the C-C bonds between methylene- and vinyl groups compared to C-C bonds in saturated chains. This enables these chains to adapt to looped conformations that have shorter length and larger area per molecule. Some neural receptors appear to have a specific requirement for high concentrations of lipids with DHA chains in the membrane. NMR experiments on reconstituted membranes containing the photoreceptor rhodopsin suggest that lipids with polyunsaturated DHA chains preferentially locate near the receptor. Lipid–protein interaction results in minor changes of chain order parameters only. Within the limits of experimental sensitivity, we found no evidence for existence of DHA that is tightly bound to protein. Perhaps low energetic barriers to membrane receptor conformational transitions are essential, making adaptability in structure of lipids with DHA chains a crucial biophysical property.

Key Words: Docosahexaenoic Acid, NMR, Conformation

■ DHA-Deficient Rats Exhibit Learning Deficits in Olfactory-Based Behavioral Tasks. R.S. Greiner[1], J.N. Catalan[1], T. Moriguchi[1], B.M. Slotnick[2], and N. Salem[1], [1]Lab. of Membrane Biochemistry and Biophysics, NIAAA, Rockville, MD, USA, [2]The American University, Washington, DC.

Rats are macrosmatic; this high olfactory acuity is necessary for rats to learn and adapt to their environments and ultimately for their survival. Rats actually demonstrate higher cognitive functions, previously only attributed to primates, when odors are used as the salient cues in behavioral tasks. Development of a learning set or errorless learning is an example of a higher cognitive function that has been demonstrated in rats using the olfactory modality.

We assessed the effects of a chronic n-3 fatty acid (DHA) deficiency on cognitive function in rats using a novel technique based on olfaction. DHA-deficient and DHA-adequate diets were designed and fed to rats over two generations. The dietary treatments were successful in depleting brain DHA as DHA-deficient rats had an average 83% loss of DHA in the total lipid extracts from whole brain or olfactory bulb compared to DHA-adequate rats. In the first behavioral task, rats fed an LNA-deficient diet made significantly more errors compared to LNA-fed rats in a series of seven, two-odor discrimination problems. Furthermore, when tested on a series of 20 two-odor discrimination problems, the number of errors in a DHA-fed group decreased over time. On problems 16–20 this group reached near errorless performance, demonstrating the acquisition of a learning set. DHA-deficient rats did not achieve this near-errorless learning and thus never demonstrated the acquisition of a learning set. These results indicate that DHA is critical for higher level performance associated with acquisition of a learning set when the olfactory modality is employed in the behavioral task.

Key Words: Docosahexaenoic Acid, Fatty Acid, Behavior

■ DHA and AA Supplementation of Infant Formulas Improves Motor Skills and Visual Orientation, Decrease CSF 5-HIAA in Infancy and Improve Heart Rate Variability in Adolescence. Joseph R. Hibbeln[1], Paolo DePetrillo[1], Maribeth Champoux[2], J. Dee Higley[1], Stephen Lindell[1], Courtney Shannon[2], Stephen Suomi[2], and Norman Salem, Jr.[1], [1]National Institute of Alcohol Abuse and Alcoholism, [2]National Institute of Child Health and Human Development.

Cognitive and visual changes result from suboptimal intake of docosahexaenoic acid (DHA) arachidonic acid (AA) during human infancy, but long term neuro-psychiatric consequences have not previously been described. The omega-3 essential fatty acid DHA and AA, are essential nutrients for optimal brain development and are found in both human and rhesus *(Macaca mulatta)* breast milk. Standard infant formulas are virtually devoid of these nutrients. In a well-established animal model, rhesus monkeys fed standard infant formulas, after separation from their mothers at birth, are at increased lifetime risk of lifetime aggressive and depressive behaviors and decreased CSF 5-HIAA concentrations. In this experiment, infant rhesus monkeys were raised under rigorously controlled nursery conditions and fed either standard formula or formula supplemented with physiological concentrations of AA (0.8%) and DHA (0.8%) for the first six months of life. Within 14 days, plasma concentrations of AA and DHA were markedly decreased among the standard formula group. Motor and visual orientation scores were significantly improved among the supplemented infants at days 7 and 14 with a ceiling effect noted by day 21. After 6 months of age, both groups were switched exclusively to diets containing DHA and AA. CSF 5-HIAA concentrations were lower among the DHA/AA supplemented group during formula feeding. At one year of age, heart rate variability (HRV) was markedly greater among the supplemented group. These differences persisted into adolescence, at three years of age. The magnitude of the stable increases in HRV were as great as the transient increases induced with ketamine administration. Low heart rate variability is an indicator of low autonomic arousal and is well established as a longitudinal predictor of disruptive and antisocial behaviors. These data indicate that DHA/AA supplementation results in physiological improvements in heartrate variability that persist into adolescence.

Key Words: Docosahexaenoic, Heart Rate Variability, CSF 5-HIAA

■ Is There a Substrate Preference in Mitochondrial Phosphatidylserine Decarboxylation? J. Hamilton and H.-Y. Kim*, National Institute on Alcohol and Abuse and Alcoholism.

We have previously shown that phosphatidylserine (PS) contents are particularly high in neuronal cells, owing to accumulation of 18:0,22:6-PS, and can be specifically decreased by both n-3 fatty acid deficiency and ethanol. In animal cells PS is synthesized from pre-existing phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by a base-exchange reaction and can then be decarboxylated to PE by phosphatidylserine decarboxylase (PSD). Before PS can be decarboxylated to PE, it must be transported from its site of synthesis in the endoplasmic reticulum to the mitochondria. Specificity in any of these processes will influence the PE and PS status in cell membranes and may attribute to tissue-specific lipid profiles. Although we reported that 22:6n-3 containing phospholipids are preferred in PS biosynthesis, there have been no studies to address the substrate specificity of PSD. In the present study, experiments were designed to determine if PSD has a substrate preference for specific molecular species of PS in liver and brain mitochondria. Unilamellar vesicles containing deuterium-labeled 18:0,18:1-PS, 18:0,22:6-PS and 18:0,20:4-PS were incubated with rat brain cortex or liver mitochondrial fractions, and decarboxylation to the corresponding deuterium-labeled PE was monitored by reversed phase HPLC/electrospray mass spectrometry. Liver mitochondria showed the substrate preference in the order of 18:0,18:1-PS > 18:0,22:6-PS > 18:0,20:4-PS, while in brain cortex mitochondria, the substrate preference was 18:0,22:6-PS > 18:0,18:1-PS > 18:0,20:4-PS. In both tissues PS decarboxylation occurred in a concentration and time-dependent manner. This preference was maintained at all concentrations and time points examined, establishing for the first time that there exists a tissue-specific substrate preference in PSD.

Mitochondrial levels of 18:0,22:6, 18:0,18:1 and 18:0,20:4 PS and PE remained the same after 45 minutes of incubation with the deuterium-labeled PS, indicating that endogenous mitochondrial PS did not significantly interfere with the decarboxylation of exogenously added PS substrates. The levels of 18:0,22:6-PE, 18:0,18:1-PE, and 18:0,20:4-PE in brain cortex or liver mitochondria did not reflect the observed PS molecular species preference, suggesting that the PE status in mitochondrial membranes may be maintained primarily through other pathways such as *de novo* synthesis using a CDP-ethanolamine or ethanolamine base-exchange reaction. Alternatively, it is also possible that not all molecular species of PS are equally transported into the mitochondria, as PS synthesized in microsomes must first be transported to the mitochondria before decarboxylation.

Key Words: Phosphatidylserine Decarboxylase, Docosahexaenoic Acid, Phosphatidylserine

■ Antiapototic Effect of Docosahexaenoic Acid in Neuronal Cells. H.Y. Kim[1], M. Akbar, A. Lau, and L. Edsall, National Institutes of Health, Rockville, MD 20852.

Docosahexaenoic acid (22:6n-3) is the major long chain polyunsaturated fatty acid in neuronal cells. Growing evidence supports the essential role of 22:6n-3 in neuronal function although the underlying biochemical mechanisms are not clearly understood. In this study we investigated the effect of 22:6n-3 on neuronal apoptosis in relation to its capacity to modulate phosphatidylserine (PS) accumulation. When Neuro 2A cells were enriched with 25 μM 22:6n-3, DNA fragmentation induced by serum starvation was significantly inhibited in comparison to nonenriched control, oleic acid (18:1n-9)- or arachidonic acid (20:4n-6)-enriched cells. Consistently, 22:6n-3 enrichment prevented the increase of Caspase-3 activity as well as the cleavage of Pro-Caspase-3 to the active 17-KD fragment upon serum starvation. The protective effect of 22:6n-3 was apparent only after at least 24 h of enrichment, and prolonged treatment for up to 48 h exhibited further protection. During 48 h of incubation, incorporation of 22:6n-3 into PS increased steadily, resulting in a significant increase of the total PS content in Neuro 2A cells. During the same enrichment period, nonenriched control or 18:1n-9-treated cells did not show any significant increases in the PS contents. Membrane translocation of Raf-1 in Neuro 2A cells was significantly enhanced in 22:6n-3-treated cells. *In vitro* biomolecular interaction analysis using PS/PE/PC liposomes and Raf-1 indicated the requirements of PS for their interaction, and this interaction was PS concentration-dependent. When Neuro 2A cells were enriched in a serine-free medium, PS accumulation as well as the protective effect was significantly diminished. Collectively, enrichment of neuronal cells with 22:6n-3 increased the PS accumulation and the membrane localization of Raf-1, down-regulated Caspase-3 activity, and prevented apoptotic cell death under serum-free conditions. The protective potential of 22:6n-3 was sensitive to the extent of PS accumulation, suggesting the observed antiapoptotic effect of 22:6n-3 may be mediated at least in part through the enhanced PS accumulation in neuronal membranes.

Key Words: Docosahexaenoic Acid, Apoptosis, Phosphatidylserine

■ Higher Maternal Docosahexaenoic Acid During Pregnancy Is Associated with More Mature Infant Central Nervous System. C.J. Lammi-Keefe, S.R. Cheruku, H.E. Montgomery-Downs, S.L. Farkas, and E.B. Thoman, University of Connecticut, Storrs, CT, USA.

Functional outcome for the infant with respect to docosahexaenoic acid's (DHA) effect on the developing central nervous system (CNS) during pregnancy remains unexplored. Sleep–wake states of newborns are uniquely appropriate for assessing the functional integrity of the CNS. We hypothesized that the integrity of the infant's CNS measured by sleep recordings during the neonatal period would be associated with maternal long chain polyunsaturated (LC-PUFA), especially DHA, status. Pregnant women ($n$=17) were recruited at parturition; venous blood was collected. Plasma phospholipid (PL) fatty acids were analyzed by gas chromatography. In the hospital, infants' sleep–wake states were measured with the non-intrusive Motility Monitoring System: From a pressure sensitive pad placed under the infants' bedding, infants' body movements and respiratory patterns were logged and scored for sleep–wake states on the first (P1) and second (P2) postnatal days. Maternal plasma PL DHA ranged from 1.91 to 4.5%. Based on previously published data and the median DHA wt% for this population (3.0 wt%), the women were divided into high DHA (Hi) and low DHA (Lo) groups. On P1 infants of Hi had a lower Active/Quiet Sleep Ratio (AS/QS), less Active Sleep (AS), more Quiet Sleep (QS). On P2 they had lower AS/QS, less AS, less Transitional Sleep (T), and more Wakefulness. Maternal n-3/n-6 LC-PUFA (wt%) on P1 was associated negatively with QS and positively with Arousals in Quiet Sleep (Ar/QS). On P2 the n-3/n-6 LC-PUFA was associated positively with AS, T, and AS/QS. Ar/QS is defined as QS fragmentation, and its positive association with higher n-6/n-3 LC-PUFA is significant because Ar/QS is associated with CNS compromise. Thus, the QS fragmentation seen with the higher n-6 to n-3 ratios may be a risk factor for developmental dysfunction. These findings suggest that differences in prenatal supply of LC-PUFA, especially DHA, modify brain PLs and affect neural function. [Supported by grants from NIH (HD 32903), USDA (93-37200-8876), Donaghue Medical Research Foundation, and UCONN Research Foundation.]

Key Words: Docosahexaenoic Acid, Infant Sleep, Central Nervous System

■ Omega-3 Polyunsaturated Fatty Acids (PUFA) Do Not Affect Growth, Energy Expenditure, Body Composition, or Expression of Genes Controlling Fatty Acid Oxidation and Thermogenesis in Piglets. A. Lapillonne[1], C.M. Nelson[2], J.C. DeMar[1], W.C. Heird[1], and S.D. Clarke[2], [1]USDA/ARS Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX, [2]Division of Nutr. Sciences and Institute for Cell and Mol. Biol., University of Texas, Austin, TX.

Background: Studies in human adults and infants as well as in rodents suggest that n-3 PUFA affect the rate and composition of weight gain as well as rates of fatty acid oxidation and energy expenditure. In addition, carnitine palmitoyl-transferase-1 (CPT-1) and acyl-coA oxidase (AOX) activities as well as transcription of genes encoding for CPT-1, AOX, and thermogenic uncoupling proteins (UCP)-2 and -3 are increased by n-3 PUFA. Based on these findings, we hypothesized that the molecular changes induced by n-3 PUFA increase peroxisomal oxidation and uncoupled mitochondrial oxidation and thus reduce the efficiency of fuel utilization and, thereby, increase energy expenditure. This, in turn, results in a lower energy balance and, hence, lower rates of fat deposition and weight gain. To address this issue, 6-day-old pigs were fed diets differing only in quantity of n-3 PUFA for 2 weeks, and the rates of weight gain and energy expenditure as well as body composition and the skeletal muscle and/or liver contents of mRNAs of CPT-1, AOX, and UCP-3 were determined. Methods: Two groups of 6 piglets were fed isocaloric diets differing only in PUFA composition from day 6 to day 20 of life. Group 1 received a corn oil-enriched diet (18.6% of total fatty acid as 18:2n-6 and 1.0% as 18:3n-3), and group 2 received a flaxseed oil-enriched diet (12.7% of total fatty acid as 18:2n-6 and 9.0% as 18:3n-3). Food intake and body weight were monitored daily. Energy expenditure (24-h indirect calorimetry) was assessed on day 5 and day 18 of life. Body composition (dual energy X-ray absorptiometry), plasma FA composition (gas chromatography), and gastrocnemius muscle as well as liver content of the relevant mRNAs (Northern blot or semiquantitative RT-PCR) were determined on day 20 of life. Results: Plasma 18:3n-3, 20:5n-3, 22:5n-3 and 22:5n-3 were higher in pigs fed the diet enriched with flaxseed oil. Neither weight gain (161 g/kg/d vs. 164 g/kg/d in the corn oil and flaxseed oil groups, respectively), energy expenditure (95 kCal/kg/d vs. 93 kCal/kg/d), nor body composition (fat mass = 5.9% vs. 5.7% of body weight) differed between the 2 groups. The mRNAs of CPT-1 and UCP-3 compared to AOX were low in the muscle of both groups. There were no statistically significant differences between groups in either muscle or liver mRNA levels of AOX, CPT-1, or UCP-3. Conclusions: Feeding piglets a diet with 9.0% vs. 1% of total fatty acids as 18:3n-3 from day 6 to day 20 of life did not affect expression of key enzymes of lipid metabolism, weight gain, energy expenditure, or body composition. The greater expression of AOX vs. CPT-1 in skeletal muscle of both groups suggests that peroxisomal oxidation is much greater than mitochondrial fatty acid oxidation in piglets. These data, in contrast to the data in humans and rodents, raise questions concerning the validity of the piglet as a model for fatty acid oxidation.

Key Words: Gene Regulation, n-3 PUFA, Piglets

■ The Effect of Maternal Fish Oil Supplementation During Lactation. L. Lauritzen[1], M.H. Jørgensen[2], S.F. Olsen[3], and K.F. Michaelsen[1], [1]Research Department of Human Nutrition, The Royal Veterinary and Agricultural University, [2]Department of Neonatology, Copenhagen University Hospital, [3]Danish Epidemiology Science Centre, Statens Serum Institut.

Docosahexaenoic acid (DHA) levels in milk vary considerably, but the effect on the infant is unknown. We have previously, in a small cross-sectional study, observed a significant association between milk DHA and visual acuity in breast-fed infants. We have performed a double-blinded intervention study in which we randomized mothers with a habitual low fish intake to receive 1 g DHA from microincapsulated fish oil or olive oil placebo starting the first week after delivery until 4 months. A control group of mothers with a high habitual fish intake was included. Methods: DHA content of milk samples and infant RBC membranes at 4 months, SWEEP-VEP visual acuity at 2 and 4 months, growth, and parental registration of sleep patterns were measured. Ninety-seven infants completed the intervention (44 olive oil, 53 DHA), and 47 completed examinations in the control group. The mean (SD) ha-

bitual DHA intake during pregnancy was 0.7 (0.4) g/d in the high fish group and 0.2 (0.1) g/d in the low fish group. The last 4 month examination was completed early June 2000. Results. Preliminary data analysis on a subsample ($n$=71) showed that the mean (SD) RBC DHA at 4 months was 9.1% (1.8) of fatty acids in the DHA group and 5.9% (1.9) in the placebo group ($P$=0.001), whereas it was 6.5% (1.9) in the "habitual high fish" group. Visual acuity at 4 months was significantly positively associated with infant RBC DHA ($P$=0.026). Awaiting further analysis, these data suggest an effect on the breast-fed infant of maternal DHA intake. The study was supported financially by The Danish Research and Development Programme for Food Technology (FÿTEK) and BASF Health and Nutrition A/S.

■ Analysis of *in situ* Chemical Constituents of the Retina by Fourier Transform Infrared Microspectroscopy. S.M. LeVine[1], J.D. Radel[1], and D.L. Wetzel[2], [1]University of Kansas Medical Center, Kansas City, Kansas, [2]Kansas State University, Manhattan, Kansas.

Fourier transform infrared (FT-IR) microspectroscopy enables the characterization and semiquantitation of chemical functional groups in microscopic regions of tissue sections. Sections of normal or diseased tissues can be analyzed. In diseased tissues, FT-IR microspectroscopy can reveal chemical changes that are associated with discrete regions of lesion sites, which can provide insights into the chemical mechanisms of disease processes. In the first set of studies, FT-IR microspectroscopy was used to analyze sections of retina from normal (pigmented) and albino rats. The outer segments of pigmented animals were found to have unusually strong absorption values for C=C unsaturation and carbonyl functional groups. Docosahexaenoic acid (DHA), a major constituent of lipids in the outer segments, also had particularly high absorption values for these functional groups, which suggests that it is responsible for those enhanced absorption values. Absorbance values for the unsaturation and carbonyl functional groups were substantially reduced in the outer segments of retinas from albino animals. This finding, together with data from other studies on light-induced oxidative events in the retina, indicates a loss of DHA by a light-induced mechanism in albino animals. In a second set of studies, chemical changes were characterized in the photoreceptor outer segments following exposure to iron-catalyzed oxidative stress. A reduction of unsaturation and carbonyl functional groups was observed in the outer segments of iron-injected eyes compared to vehicle-injected eyes at 3 days following injection, which is prior to major histological changes that occur by 7 days. DHA contains a series of six *cis*-conjugated double bonds, which are vulnerable to free radical attack, and the reduction of these unsaturation group absorptions suggests that DHA was degraded and/or removed from the outer segments of iron-injected eyes. In summary, FT-IR microspectroscopy has been used to detect high concentrations of unsaturation and carbonyl chemical groups in the outer segments of the retina and the concentration of these functional groups' decrease in various disease states that involve oxidative stress.

Key Words: Infrared Microspectroscopy, Retina, Docosahexaenoic Acid

■ Pentadecanoic and Heptadecanoic Acid in Red Blood Cell Phospholipids as Biomarkers of Dietary Milk Fat Intake in School Children. F. Lehner[1], H. Demmelmair[1], J. Linseisen[2], S. Verwied-Jorky[1], G. Wolfram[2], and B. Koletzko[1], [1]Dr. von Haunersches Kinderspital, Ludwig-Maximilians University, Germany, [2]Institut für Ernaehrungswissenschaft, Technische University Muenchen, Germany.

Background: The fatty acid pattern of erythrocyte membranes is widely used as a biomarker of dietary fatty acid intake. Since rumen microorganisms synthesize odd-numbered fatty acids, ruminant milk fat is one of the main dietary sources of pentadecanoic acid (C15:0) and heptadecanoic (C17:0) acid. Aim: To evaluate red blood cell (RBC) C15:0 and C17:0 contents as biomarkers for milk fat intake in children. Subjects and methods: Fasting blood samples were obtained from participants of the "Family Intervention Trial (FIT)" (Erlangen, Germany). Eighty-two 6–7-year-old school children (girls $n$=43; boys $n$=39) and their mothers ($n$=68) completed a 3-d weighed-food record. Food and nutrient intake calculations were performed by means of PRODI 4.4 using the German food composition database Bundeslebensmittelschlüssel II.1. The fatty acid pattern of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from erythrocyte membranes were analyzed by gas–liquid chromatography. Pairwise associations between RBC fatty acids and dietary intake of milk fat were examined by Spearman's rank correlation analysis (SPSS for windows, Vers. 9.0.1). Results: In girls the dietary intake of fat delivered from milk and milk products (without cheese) was positively related to the proportions of PC 15:0 ($r$=0.58; $P$<0.001), PC 17:0 ($r$=0.33; $P$=0.030) and PE 15:0 ($r$=0.49; $P$=0.001). In boys, the dietary intake of butter showed strong positive correlations with PC 15:0 ($r$=0.63; $P$<0.001), PC 17:0 ($r$=0.43; $P$=0.007), PE 15:0 ($r$=0.53; $P$<0.001), and PE 17:0 ($r$=0.34; $P$=0.037). Moreover, a positive association between milk fat intake and the proportion of PC 17:0 ($r$=0.32; $P$=0.047) was found. In mothers, the dietary intake of milk/milk products and fat delivered from milk/milk products was positively related to PC 15:0 ($r$=0.25; $P$=0.037 and $r$=0.31; $P$=0.011, respectively); significant associations between the intake of butter and butterfat and PC 17:0 were found ($r$=0.29; $P$=0.016), too. In boys and girls, the proportions of PC 15:0 revealed a significant correlation to the dietary intake of C4:0, C6:0, C10:0, and C14:0. PC 15:0 of maternal erythrocytes was positively related to dietary C4:0 and C6:0. In all children, PC 15:0 correlated with consumption of butter ($r$=0.45; $P$=0.001), fat from milk/milk products ($r$=0.42; $P$<0.001), butter fat ($r$=0.45; $P$<0.001), and fat delivered from all milk products ($r$=0.26; $P$=0.020). Less close but significant correlations were also found for PC C17:0 and PE C15:0, while PE C17:0 was only related to fat delivered from milk/milk products. Conclusion: These data demonstrate that C15:0 and C17:0 in red blood cell phospholipids can be used as biomarkers of milk fat intake with C15:0 revealing the strongest correlations. The relationship was found to be closer in children than in adults, probably due to the higher percentage of beef fat in the mothers' diet.

Key Words: Pentadecanoic Acid, Biomarker, Milk

■ Dose Response Effect of Docosahexaenoate Ethyl Ester and Egg-Phosphatidylcholine on Maze-learning Ability and Fatty Acid Composition of Plasma and Brain Lipids in Mice. S.-Y. Lim[1] and H. Suzuki[2], [1]National Institute on Alcohol Abuse and Alcoholism, NIH, [2]National Food Research Institute, Japan.

Our previous work showed that the intakes of docosahexaenoate (DHA, 22:6n-3), ethyl ester (EE), and egg phosphatidylcholine (PC) improved learning ability in both young and old mice but old mice had a poorer performance than young mice. In the present study, we investigated the dose-response effect of dietary DHA and PC on maze-learning ability and fatty acid composition of plasma and brain lipids in mice. Male Crj:CD-1 mice aged 3 mo were fed (i) a diet containing 5 g palm oil/100 g diet (control group); (ii) a diet containing 0.5 g DHA ethyl ester/100 g diet plus 4.5 g palm oil/100 g diet (DHA-EE 0.5% group); (iii) a diet containing 1 g DHA ethyl ester/100 g diet plus 4 g palm oil/100 g diet (DHA-EE 1% group); (iv) a diet containing 2 g DHA ethyl ester/100 g diet plus 3 g palm oil/100 g diet (DHA-EE 2% group); (v) a diet containing 1 g egg-PC/100 g diet plus 4 g palm oil/100 g diet (egg-PC 1% group); (vi) a diet containing 2.5 g egg-PC/100 g diet plus 2.5 g palm oil/100 g diet (egg-PC 2.5% group); (vii) a diet containing 5 g egg-PC/100g diet (egg-PC 5% group) for 4 mo. Maze-learning ability was assessed 3 mo after the start of the experiment. The time required to reach the maze exit and the number of times that a mouse strayed into blind alleys in the maze were measured in three trials carried out at 4-day intervals. In trial one, the DHA-EE 0.5%, 1%, and 2% groups required less ($P$<0.05) time to reach the maze exit, and the DHA-EE 2% and egg-PC 2.5% groups strayed ($P$<0.05) into blind alleys fewer times than the control group. In trial three, performed 4 days after the second trial, the DHA-EE 2% and egg-PC 5% groups needed less ($P$<0.05) time to find the exit and made fewer ($P$<0.05) entries into blind alleys than did the control group. In the total lipids of plasma and brain of mice fed DHA-EE, increasing dietary DHA resulted in an increase in DHA levels with a corresponding decrease in arachidonic acid (AA, 20:4n-6). Improved maze-learning ability in mice fed 2% DHA-EE was associated with higher DHA levels in brain. However, brain DHA was unrelated in the groups with various doses of egg-PC. Our results suggest that the intakes of 2% DHA-EE and 5% egg-PC diets improved learning ability. However, the mechanisms by which the DHA and PC diets improved learning appear to differ.

Key Words: Docosahexaenoic acid (DHA), Egg-phosphatidylcholine (PC), Maze-learning Ability

■ Spatial Task Performance Depends upon the Level of the Brain Docosahexaenoic Acid. T. Moriguchi, J. Loewke, and N. Salem, Jr., National Institute on Alcohol Abuse and Alcoholism, NIH.

Docosahexaenoic acid (DHA) is rapidly deposited during brain development in the perinatal and early postnatal period and appears to play a critical role in the nervous system. Previously, we found that a dietary n-3 fatty acid deficiency resulted in impaired acquisition of a spatial task in adult rats after two generations of n-3 fatty acid deficiency. The present experiments were designed to determine whether the alterations in brain function were reversible at various stages of development from birth through adulthood. Long-Evans hooded rats were fed an n-3-adequate (n-3 Adq) or n-3-deficient (n-3 Def) diet for three generations. When the n-3 Def male rats of the F3 generation were 0 (birth), 3 (weaning), and 7 (adulthood) wks old, the diet was switched to the n-3 Adq diet, a diet that included both 2.6% alpha-linolenic acid and 1.3% DHA. Behavioral experiments (the Morris Water Maze) were conducted when the animals were either 9 or 13 weeks of age. The n-3 Def group exhibited significantly lower brain DHA, longer escape latency, and a delay in the acquisition of this task as compared with the n-3 Adq group. The diet reversal groups that consumed an n-3 Adq diet for less than 6 weeks prior to testing had only partially recovered their brain DHA and spatial task performance. This indicated a good correlation of learning ability with brain DHA concentration. The groups that had diet repletion for more than 9 weeks contained the same level of brain DHA as in the n-3 Adq group irrespective of the age at which diet reversal had occurred. However, a slight difference in performance on the spatial tasks was evident between the various groups that appeared to be related to the age at the time of diet reversal. These results indicated that the recovery effects of the n-3 Adq diet reversal depended not only on the period over which an n-3 Adq diet was consumed but also, secondarily, on the age at which the diet reversal occurred. This reversibility suggests that the learning deficit in the n-3 Def group is directly related to the neuronal membrane fatty acid compositional change. This study has clear relevance to public health, as it demonstrates the importance of adequate n-3 fatty acids in the infant diet in order to achieve optimal brain development. It also demonstrates that DHA feeding subsequent to a deficiency state can potentially restore nervous system function during early development and potentially up to the time of young adulthood.

Key Words: Docosahexaenoic Acid (DHA), Spatial Task Performance, Recovery

■ Effect of DHA and Cholesterol on Receptor-G Protein Coupling. S. Niu, D.C. Mitchell, and B.J. Litman, NIH, Rockville, MD.

The neuronal and retinal tissues are highly enriched with PUFAs, in particular with 22:6n3 (DHA). DHA deficiency in these tissues is associated with impairment of neuronal function and vision. The goal of this study is to characterize the role of DHA and cholesterol on modulating signal transduction in G protein-coupled receptor systems at molecular levels. The visual transduction system, one of the best-characterized members in the superfamily of G protein-coupled signal transduction systems, which consists of receptor rhodopsin (Rh), G protein, and effector PDE, serves as a model in this study. Rh was reconstituted into lipid bilayers consisting of di-22:6PC (DDPC), 18:0,22:6PC (SDPC), and 18:0,18:1PC (SOPC) to vary the levels of DHA and unsaturation in the acyl chains of the phospholipid. Cholesterol levels varied from 0 to 30% (mol) at each lipid composition. The coupling of Rh and G protein was characterized by measuring the equilibrium binding of metarhodopsin II (MII), the active agonist-bound state that binds and activates G protein, to the G protein. In the absence of G protein, a metastable equilibrium between MI and MII is established within a few milliseconds of photon absorption. The equilibrium is shifted toward MII in the presence of G protein due to MII-G complex formation, and the extent of the shift in equilibrium is dependent on G protein concentration. By varying the G protein concentration, the association constant of MII-G can be determined spectroscopically. The association of MII-G, which in fact is a protein–protein interaction, was affected by the lipid composition in such a way that more efficient coupling was observed in DHA containing phospholipids. Weaker association of MII-G was found in all lipid bilayer compositions in the presence of 30% (mol) cholesterol. The nature of the influence on MII-G coupling by lipid bilayer composition and cholesterol needs further investigation, yet it is plausible that lipid bilayer composition and cholesterol can change the conformation of MII and G, which in turn affects the MII-G coupling. The receptor-G protein coupling is an essential step in the signaling cascade. Our findings demonstrate that lipid and cholesterol composition can mediate the extent of receptor-G protein coupling in the visual transduction system. These findings, if extended to other members of the superfamily of G protein-coupled receptors, suggest that a loss in efficiency of signaling is the mechanism whereby cognitive and visual losses occur in DHA deficiency.

Key Words: PUFA, Receptor, Signal Transduction

■ The Nutritional Impact of Including Egg Yolks in the Weaning Diet of Healthy Infants. M. Makrides[1], J.S. Hawkes[2], M.A. Neumann[2], and R.A. Gibson[2], [1]University Dept. Obstetrics and Gynaecology, Women's and Children's Hospital, [2]Child Nurition Research Centre, Flinders Medical Centre, Flinders University.

The primary objective of our trial was to determine the nutritional value of including egg yolks in the weaning diet of healthy breast- and formula-fed infants and to investigate some of the perceived benefits and risks associated with consuming eggs.

At 6 months, healthy breast-fed and formula-fed infants were randomly allocated to receive either no dietary intervention, four regular egg yolks per week or four omega-3 enriched (high in docosahexaenoic acid, DHA) egg yolks per week. Separate randomization schedules were used to allocated breast- and formula-fed infants. Dietary intake, compliance, and growth were assessed at 6, 9, and 12 months of age. Plasma DHA, iron status, plasma cholesterol, and plasma markers of allergy were assessed when infants were 6 and 12 months of age.

Of 82 breast-fed infants recruited, 24/27 (omega-3 eggs), 23/27 (regular eggs), and 23/28 (no intervention) infants completed the trial. Similarly, of 79 formula-fed infants enrolled, 20/26 (omega-3 eggs), 24/26 (regular eggs), and 23/27 (no intervention) infants completed the trial. The egg intervention was well tolerated. Only one breast-fed infant and one formula-fed infant were withdrawn from the trial because the infant appeared to be unsettled on eggs. Mothers with infants allocated to eggs reported a mean (±SD) egg yolk consumption of 4±1 yolks, while mothers with infants in the no-intervention groups reported using 1±1 yolks. Compliance with the dietary intervention was further highlighted by a 40% increase in plasma DHA levels in both breast- and formula-fed infants allocated to omega-3 egg yolks compared with the respective no-intervention (control) groups. In this well-nourished group of infants, consumption of egg yolks had no effect on the clinical markers of iron status nor any effect on growth.

Within the breast-fed and formula-fed cohorts, there were no differences in plasma cholesterol between the three dietary groups at 6 or 12 months. At 6 months and before dietary intervention, breastfed infants had a higher plasma cholesterol level than the formula-fed infants (4.3±0.8 vs. 3.8±0.7 mmol/L, $P<0.005$). This difference disappeared by 12 months of age. At 12 months, when data from all infants were combined and adjusted for mode of feeding and 6-month values, there was no significant effect of egg yolk intervention on cholesterol levels. We could not detect an effect on indices of allergy as a result of increasing the number of egg yolks included in infant diets. Total plasma IgE levels, the percentage of infants with egg yolk-specific antibodies or egg white-specific antibodies were similar between infants allocated to the egg and control groups.

Egg yolks can safely be included into the weaning diets of healthy breast-fed and formula-fed infants. As egg yolks contain a range of vitamins, minerals, proteins, and fats, they can be included as part of a well-balanced diet for infants and toddlers.

Key Words: Docosahexaenoic Acid (DHA), Infant Weaning Diet, Eggs

■ Dietary Polyunsaturated Fatty Acids (PUFA) and Pregnancy Outcome. M. Makrides[1], L. Duley[2], and S.F. Olsen[3], [1]Child Nutrition Research Centre, Child Health Research Institute, North Adelaide, Australia, [2]Resource Centre for Randomised Trials, Institute of Health Sciences, Oxford, UK, [3]Maternal Nutrition Group, Danish Epidemiology Science Centre, Statens Serum Institut, Denmark.

Pre-eclampsia is a common complication of pregnancy and can result in preterm birth and intrauterine growth restriction (IUGR). Pre-

eclampsia, preterm birth, and IUGR are all associated with increased morbidity for both mother and child.

The use of fish oil supplements, and possibly other polyunsaturated fatty acids (PUFA) such as gamma-linolenic acid (GLA), have been proposed as a possible strategy to prevent pre-eclampsia and preterm birth and to increase birth weight. The hypotheses that fish oil could prevent or ameliorate these conditions were initially developed following population comparisons and more recently have been tested in randomized controlled trials.

Our systematic review aims to estimate the overall benefits and hazards of supplementation with dietary PUFA, including n-3 fatty acids and GLA, during pregnancy.

All randomized trials, with adequate concealment of allocation, of dietary PUFA supplementation during pregnancy, were selected for the review. Relevant trials were identified from the Medline database, the Pregnancy and Childbirth Group's Specialized Register and the Cochrane Controlled Trials Register.

We have identified five trials involving over 2,500 women. The intervention in most trials was fish oil with 2.7 g of n-3 fatty acids per day, while one trial had a combination of evening primrose and fish oils (300 mg GLA and 220 mg n-3 fatty acids per day). The individual results of trials suggest that intervention with dietary PUFA had little or no effect on pre-eclampsia and pregnancy-induced hypertension. However, some trials suggest a modest increase in birth weight and length of gestation. The meta-analysis of these trials will be presented and discussed.

Key Words: PUFA, Pregnancy Outcome, Fish Oil

■ Dietary Long Chain Polyunsaturated Fatty Acids Alter Intestinal Fatty Acid Profiles, but Do Not Impact Digestive Function in Neonatal Piglets. M.C. McCarthy[1], A.D. Beaulieu[2], J.K. Drackley[2], H. Mangian[1], K.A. Tappenden[1], and S.M. Donovan[1], [1]Dept. of Food Science Human Nutrition, [2]Dept. of Animal Sciences, University of Illinois, Urbana, IL 61801.

Recent data suggest that dietary arachidonic acid (AA) and docosahexaenoic acid (DHA) benefit brain and retinal development of infants; however, their impact on intestinal function in neonates is less well described. Herein, the effects of dietary AA and DHA on intestinal fatty acid composition and digestive function were assessed. Piglets (2 day old) were randomized to receive formula containing a standard PUFA blend (n-6; $n=6$) or a blend with added 1.2% AA and 0.9% DHA (LC-PUFA; $n=8$) for 12 d. Weight gain, intestinal weight, and intestinal histomorphology were similar between groups. Plasma and red blood cell membrane DHA, but not AA, were increased ($P<0.05$) in piglets fed the LC-PUFA diet. Brush border phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were separated by thin-layer chromatography and fatty acid profiles determined by gas chromatography. The content of DHA in PE but not PC was increased ($P<0.05$) by dietary LC-PUFA, whereas AA content of intestinal brush border PE and PC were unchanged. Transmural resistance and sodium-coupled glutamine and glucose transport were determined in Ussing chambers. Barrier function, nutrient transport, and sodium/glucose co-transporter (SGLT-1) mRNA abundance were not affected by LC-PUFA. Jejunal lactase mRNA abundance and activity were also unaffected. These data suggest that dietary DHA is incorporated into intestinal membrane PE of neonatal piglets, but does not alter digestive function. Therefore, it appears that AA and DHA can be added to infant formulas without detrimentally impacting intestinal development.

Key Words: Intestine, Pig, Neonate

■ Arachidonic and Eicosapentaenoic Acid-Derived Prostaglandins Regulate the TH1 Versus TH2 Cytokine Balance: Implications for Atopic Disease. E. A. Miles, L. M. Aston, and P.C. Calder, University of Southampton, Southampton, UK.

T-helper (TH) lymphocytes have been divided into two groups according to the cytokines they produce. Typically, TH1 cells produce interleukin (IL-2) and interferon (IFN)-γ, while TH2 cells produce IL-4, IL-5 and IL-10. TH1 cytokines bias toward a cell-mediated immune response to eliminate intracellular pathogens, while TH2 cytokines promote B cell antibody responses. An imbalance in these cytokines underlies certain diseases such that a bias toward TH1 cytokines is present in patients with chronic inflammatory diseases (e.g., rheumatoid arthritis), while a bias toward TH2 cytokines is present in patients with atopic diseases. Eicosanoids are thought to have a role in regulation of the TH1/TH2 cytokine balance, but this has only been studied in detail for $PGE_2$ and the 4-series leukotrienes. The influence of eicosanoids synthesised from eicosapentaenoic acid on the TH1/TH2 profile has not been well studied. Therefore, in this study we investigate the effects of $PGE_2$ and $PGE_3$ on TH1 and TH2 cytokine production in human whole blood cultures. Whole human blood was diluted 1:5 in RPMI medium containing 0.75 mM glutamine and antibiotics and cultured for 48 h in the presence of concanavalin A (50 μg/mL) with or without the eicosanoids. $PGE_2$ and $PGE_3$ were introduced in ethanol (final concentration 0.1%) at $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$, and $10^{-10}$ M. Both prostaglandins suppressed IL-2 production (by between 30–40%) and IFN-γ production (by 70–75%) in a concentration-dependent manner. Conversely, both prostaglandins promoted IL-4 production at concentrations of $10^{-7}$ M and greater with a maximum of 1.5–1.6 times the control level. The addition of prostaglandins to the cultures did not affect IL-10 production when compared with the control. Thus, the addition of $PGE_2$ or $PGE_3$ to concanavalin A-stimulated human whole blood cultures results in reduced production of the TH1 cytokines IL-2 and IFN-γ and increased production of the TH2 cytokine IL-4, so changing the TH1/TH2 balance. This alteration in cytokine profile toward a TH2 bias may have important implications for increasing the development of allergic disorders. Dietary intervention with fish oil containing eicosapentaenoic acid is well known to markedly decrease production of $PGE_2$. There are reports that such an intervention results in only a small increase in production of $PGE_3$. Thus, overall, provision of eicosapentaenoic acid might result in a cytokine environment, which is less favorable toward the development of atopic disease.

Key Words: Prostaglandin, Cytokine, Atopic Disease

■ Effects of Acyl Chain Unsaturation and Cholesterol on the Rate of Receptor-G Protein Coupling. D.C. Mitchell[1], S. Niu[1], and B.J. Litman[1], [1]LMBB/NIAAA/NIH, Bethesda, MD, USA.

Visual signal transduction is a prominent member of the ubiquitous family of G protein-coupled signal transduction systems. In all of these systems an activated receptor passes an external signal to an internal effector enzyme *via* a guanine nucleotide binding protein, or G protein. The G protein is bound to the membrane *via* an isoprenoid tail that is believed to penetrate into the bilayer. Thus, the rate of signal transfer from the activated receptor to the effector depends on the two-dimensional diffusion rates of G protein and receptor. We have utilized rhodopsin and the visual G protein reconstituted in large, unilamellar vesicles to examine the effect of membrane composition on the rate of diffusional coupling of G protein and receptor. The form of photolyzed rhodopsin that binds G protein, meta II, is stabilized by bound G protein in the absence of GTP. Kinetics of both meta II formation and meta II-G protein complex formation at 20°C were measured for rhodopsin in native rod disk membranes and in bilayers consisting of 18:0,18:1 PC, 18:0,22:6n-3 PC, or di-22:6n-3 PC with and without 30 mol% cholesterol. The time constant for meta II in the reconstituted systems was 1 to 3 ms less than that measured in the native bilayer. At a mole ratio of G protein to photolyzed rhodopsin of approximately 1:3, the shortest time constant for complex formation was 7.4 ms in 18:0, 22:6 PC, and the longest time constant was 41 ms in 18:0,18:1 PC/30% cholesterol. Cholesterol increased the time constant for meta II-G protein complex formation in 18:0,18:1 PC by a factor of 4, but increased it by only 15% in 18:0,22:6 PC and di-22:6 PC. In order to isolate the effects of the two-dimensional diffusion process on the overall kinetics of G protein binding, we determined the ratio of the time constants for G protein-receptor complex formation and meta II formation. This ratio, $R_\tau$, indicates the number of periods, equal to its time of formation, that an activated receptor must wait before the diffusion of G protein and receptor results in a G protein-receptor complex. In native disk membranes and di-22:6 PC $R_\tau$ is 2.0, in 18:0,22:6 PC it is 1.5 and in 18:0,18:1 PC it is 2.6. Cholesterol more than doubles $R_\tau$ in 18:0,18:1 PC, but increases it only slightly in 18:0,22:6 PC or di-22:6 PC. These results demonstrate that 22:6 acyl chains in the bilayer increase the rate of meta II-G complex formation, indicating an increase in the rates of lateral diffusion for G protein and/or receptor. In bilayers lacking 22:6 acyl chains, addition of cholesterol severely decreased

the rates of lateral diffusion, however, in bilayers where 50% or more of the acyl chains were 22:6 the effect of cholesterol was minimal. These results demonstrate that acyl chain composition is a critical factor in determining the response time to the appearance of an agonist in a G protein-coupled signaling system.

Key Words: Docosahexaenoic Acid, G Protein, Receptor

■ Fatty Acid Composition of Human Milk in Poland. H. Mojska[1], J. Socha[2], and L. Szponar[1], [1]National Food and Nutrition Institute, [2]The Children's Memorial Health Institute.

There are few data on fatty acid composition of human milk in Poland. Subject and methods: The fatty acid composition of human milk was determined by capillary gas chromatography at different stages of lactation. Samples of milk were taken between 3 to 4 days post partum ($n$=50), the 5th to 6th week of lactation ($n$=38) and the 9th to 10th week of lactation ($n$=34). The fatty acid composition was expressed as weight percentage. Results and conclusion: The major fatty acid fraction in milk lipids consisted of saturated fatty acids. The percentage of total saturated fatty acids decreased with duration of lactation from 45.65% to 42.31% to 40.93%. In the same time period, polyunsaturated fatty acids increased from 9.35% to 11.11% to 11.29%. No statistically significant difference in milk monounsaturated fatty acids was seen in colostrum and mature milk. The median value of *trans* fatty acids in mature milk (2.6%) was higher than in colostrum (1.4%). With regard to the essential fatty acids, the linoleic acid (18:2n-6) content of mature milk was 9.61% and alpha-linolenic acid (18:3n-3) was 1.03%. The percentage of linoleic and alpha-linolenic acid increased whereas long-chain polyunsaturated fatty acids decreased in mature milk in comparison to colostrum. The median values of arachidonic acid (0.28%) and docosahexaenoic acid (0.11%)in mature milk were lower than those reported in other studies of lactating women in Europe.

Key Words: Fatty Acids, Human Milk, Stage of Lactation

■ Use of an Artificial Rat Breast and Artificial Rat Milk for the Complete Control of the Rodent Diet; Induction of n-3 Fatty Acid Deficiency in the First Generation. T. Moriguchi[1], R. Greiner[1], B. Lefkowitz[1], S.-Y. Lim[1], J. Loewke[1], J. Hoshiba[2], and N. Salem, Jr.[1], [1]National Institute on Alcohol Abuse and Alcoholism, NIH, [2]Okayama University Medical School, Japan.

Several studies have reported that the level of dietary n-3 fatty acids influence the brain docosahexaenoic acid (DHA) level during postnatal development period when rats are fed using the intragastric method for artificial rearing. However, this method had some disadvantages due to the absence of social interaction and suckling or swallowing behavior. Furthermore, it can begin usually only at day 4–5 of life. Thus, diet cannot be controlled during an important period of neural development. We report here the brain DHA level in newborn rats after exposure to an n-3–deficient diet using a new artificial rearing method that can be employed the first day of life. The newborn rats were separated within 12 hours of birth from their mothers. The dams were maintained on an n-3–adequate diet containing 3% alpha-linolenic acid (LNA) and no DHA. The pups were moved to two rearing chambers and were given an artificial rat milk that was n-3 fatty acid–deficient (n-3 fatty acids were less than 0.002% of total fatty acids) or –adequate (addition of 3% LNA) using an artificial nipple and a nursing bottle after the method of Hoshiba. The n-3 fatty acid content of deficient milk was much lower than that of F-2 n-3 fatty acid–deficient mothers' milk (0.05% total n-3 fatty acid). During the first three days, pups were trained by feeding artificial milk from a nursing bottle with a silicon nipple every 2–3 hours by hand. After three days, the pups were able to find the nipples and suckle by themselves. At various times from day 0 to weaning, animals were killed and the fatty acid composition of various tissues were determined. After about 3 days, rats in the n-3 deficient milk group exhibited a gradually decreasing brain DHA level yielding about a 60% decline by day 17 of life relative to the level of the n-3 adequate milk group. The dam-reared pups were slightly higher in brain DHA than the artificial reared group receiving n-3 adequate milk due to the dams' high DHA intake and the high DHA level in their milk. This method offers a method for obtaining rats with a marked loss of central nervous system DHA in a short period of time and will be useful for the evaluation of alterations in brain function.

Key Words: n-3 Fatty Acid Deficiency, Artificial Rearing, Brain Docosahexaenoic Acid (DHA)

■ Differential Effects of Fatty Acid Binding Proteins on Cellular Fatty Acid Uptake and Lipid Metabolism. E. Murphy, University of North Dakota.

Although fatty acid binding proteins have similar binding affinities for fatty acids *in vitro,* these proteins have differential effects on fatty acid uptake and trafficking. Two proteins in particular, I-FABP and L-FABP, demonstrate these differential effects on cellular fatty acid metabolism when stably transfected in L-cell fibroblasts. In these cells, L-FABP stimulates a 1.7-fold increase in fatty acid uptake, whereas I-FABP has no effect on fatty acid uptake. Both proteins target fatty acids to different lipid pools. L-FABP targets fatty acids for esterification into phospholipids and triglycerides, whereas I-FABP only targets fatty acids for esterification into neutral lipids. Both proteins increase the apparent cytoplasmic diffusion coefficient, demonstrating that these proteins are involved in intracellular fatty acid trafficking, supporting the targeting results. In these cells, I-FABP and L-FABP stimulate the apparent cytoplasmic diffusion coefficient 2.6- and 1.9-fold compared to control. Although both proteins stimulate an increase in total phospholipid content, the magnitude of this increase in L-FABP expressing cells is significantly greater than that in I-FABP expressing cells. Similarly, both proteins alter phospholipid acyl chain composition; however, the effect of L-FABP is more robust than that of I-FABP. Hence, expression of I- and L-FABP differentially affect fatty acid uptake, targeting, phospholipid mass, and phospholipid acyl chain composition when expressed in L-cell fibroblasts.

Key Words: Fatty Acid Binding Proteins, Fatty Acid Uptake, Lipid Trafficking

■ Plasma Essential Fatty Acids (EFA), Vitamin E, and Vitamin A in Relation to Alcohol Intake in African American Women at Twenty-Four Weeks Gestation. M. Murthy[1], S. Martier[2], J. Whitty[2], R. Sokol[2], J. Hannigan[2], and N. Salem, Jr.[1], [1]LMBB/NIAAA, National Institute of Health, Rockville, MD, [2]Wayne State University School of Medicine, Detroit, MI.

High levels of alcohol intake affect the metabolism of long-chain polyunsaturated fatty acids (LC-PUFA). More specifically, pregnant women being exposed to alcohol may risk having lower levels of n-3 LC-PUFA in the maternal and fetal circulation. Further evidence from several urban sites suggests that African American women may be at increased risk for fetal alcohol-related birth effects (ARBE) relative to other populations. We hypothesize that alcohol and/or diet low in n-3 LC-PUFA in this population may contribute to lower maternal levels of n-3 fatty acids, suboptimal brain docosahexaenoic acid (DHA) levels in the fetus, and adverse pregnancy outcomes. To address this hypothesis, an inner city African American sample was chosen based on the high incidence of ARBE and a socio-economic profile consistent with potentially suboptimal intake of n-3 fatty acids. Pregnant women presenting at an antenatal clinic were screened and recruited into one of the following three groups based on their self-reported levels of absolute alcohol intake per day (AAD): Light ($0.00<AAD<0.05$ ounces), Moderate ($0.05\leq AAD<1.00$ ounces) and Heavy ($AAD\geq 1.00$ ounces). In this first wave of data at twenty-four weeks gestation, we have attempted to establish statistical correlations between alcohol intake and three nutritional variables, including plasma EFA, vitamin E and vitamin A. In addition, we are collecting data on other dependent variables that reflect both nutritional and perinatal outcomes [e.g., birth weight, head circumference, dysmorphologic features, and fetal alcohol syndrome (FAS) diagnosis], which will be reported in later papers. We expect that results from this study may for the first time lead to a proposal for testing a simple dietary approach to help normalize n-3 LC-PUFA levels in drinking African American mothers and potentially reduce alcohol-related birth effects in the newborn (supported by the Office of Minority Health and NIAAA).

Key Words: Alcohol, Essential Fatty Acids, Pregnancy

■ Polyunsaturated Fatty Acids and the Ketogenic Diet: Seizure Protection and Tissue Lipids in an Animal Model. K. Musa, C. Dell, S.S. Likhodii, M. Burnham, and S.C. Cunnane, University of Toronto, Toronto, Ontario, Canada.

The ketogenic diet (KD) is a very high fat, very low carbohydrate (CHO) diet that is used to treat children with intractable epilepsy. The anticonvulsant mechanism of the KD is currently unknown. An animal study assessing the effects of an n-3 polyunsaturated fatty acid (PUFA)-enriched KD on tissue lipids and seizure protection was undertaken. Thirty-six male albino Wistar rats were weaned at 20 d of age and randomly assigned to a control or ketogenic diet group. Control rats were fed the AIN-93G diet and ketotic rats were fed a 3.5:1 (ratio of fat:protein + CHO, by weight) KD. After 48 d of dietary treatment, six rats from each group were sacrificed for tissue lipid analysis and the remaining twelve rats in each group underwent a threshold pentylenetetrazol (PTZ) seizure test (50 mg/kg, s.c.). Ketotic rats weighed 6% less than control rats (318 ± 6 g vs. 340 ± 26 g, $P<0.05$). Compared to control rats, ketotic rats had significantly lower blood glucose (6.3 ± 0.3 mM vs. 6.9 ± 0.7 mM, $P<0.01$), and significantly higher plasma β-hydroxybutyrate (0.8 ± 0.3 mM vs. 0.1 ± 0.03 mM, $P<0.001$). The two dietary interventions resulted in significantly different responses to the PTZ threshold testing, where 100% seizure incidence was observed in controls and only 50% seizure incidence was observed in ketotic rats ($P<0.05$). In comparison to controls, the brains of ketotic rats had 6% less stearic acid (18:0), 14% less arachidonic acid (AA, 20:4n-6), and a twofold increase in linoleic acid (18:2n-6) ($P<0.05$). The brains of ketotic rats also contained trace amounts of α-linolenic acid (ALA, 18:3n-6) and eicosapentanoic acid (EPA, 20:5n-3), which were not detected in the brains of control rats. Plasma levels of palmitic acid (16:0) and docosahexaenoic acid (DHA, 22:6n-3) in ketotic rats were twofold lower than in control rats ($P<0.05$). The plasma of ketotic rats contained 30% more stearic acid, seven times more ALA, and six times more EPA ($P<0.05$). The total plasma fatty acid concentration was approximately 1.5 times lower in ketotic rats compared to controls rats (15.9 ± 5.5 mg/dL vs. 24.1 ± 3.9 mg/dL, $P<0.05$). Control and ketotic rats did not differ in plasma concentrations of cholesterol. The greater amounts of brain and plasma n-3 and n-6 PUFA in ketotic rats may be important in conferring seizure protection by acting as more readily oxidized substrates for ketone body formation and by increasing membrane stability and repair. The lower total plasma fatty acids and similar cholesterol levels observed in ketotic rats may implicate a PUFA-enriched KD as being less atherogenic, while eliciting beneficial effects on seizure control. Acknowledgements: Bloorview MacMillan Centre, Dairy Farmers of Canada, National Sciences and Engineering Research Council of Canada, and Stanley Thomas Johnson Foundation are thanked for supporting this study. Mary Ann Ryan is thanked for technical support.

Key Words: Ketogenic Diet, Polyunsaturated Fatty Acids, Epilepsy

■ Modulation of the Signaling in the Visual Transduction Pathway by DHA Containing Phospholipids. B. Litman, D. Mitchell, S.-L. Niu, and A. Polozova, National Institute on Alcohol Abuse and Alcoholism, NIH, Rockville, MD, USA.

The retinal rod outer segment disks and the membranes of neuronal tissue contain as much as 50% of the phospholipid acyl chains as DHA, 22:6n-3. We have undertaken a study of the visual transduction system, employing the protein components of this pathway reconstituted in defined lipid systems so as to understand the functional and structural role of the DHA containing phospholipids. The formation of metarhodopsin II (MII), the form of rhodopsin that binds and activates the visual G protein, was greatest in DHA containing phosphatidylcholines (PC), relative to more saturated PC. The kinetic and equilibrium properties for the formation of the MII-G protein complex, were also studied. An important parameter of signaling is the rate of appearance of MII relative to the rate of formation of the MII-G complex, since these relative rates determine the kinetics of initiation of the signaling cascade. These rates were very similar in disk membrane and 18:0,22:6PC bilayers. However, the rate of MII formation exceeded that of MII-G complex formation by about a factor of five in 18:0,18:1PC bilayers at 37°C, indicating a lag time in the initial MII-G protein coupling. Measurements of the cGMP phosphodiesterase (PDE) activity reflect the activity of the integrated pathway. Here again, the PDE activity in a 22:6-containing PC was greater than that measured in a more saturated lipid. In mixed lipid bilayers, rhodopsin was found to separate into microdomains enriched in di22:6PC. The function of the visual pathway, a G protein-coupled system, appears to be optimized in DHA containing bilayers. If the function of other members of the superfamily of G protein-coupled receptors, such as several neurotransmitter receptors, is also optimized in DHA-containing bilayers, then these results provide a basis for explaining the loss of visual and cognitive skills associated with DHA deficiency.

Key Words: DHA, Visual Signaling, G Protein-Coupled Receptors

■ Biophysical Mechanisms of Docosahexaenoic Acid Influence on Membrane Receptor Function—Insights from Deuterium NMR Studies. I.V. Polozov, A.M. Safley, and K. Gawrisch, LMBB NIAAA National Inst. of Health.

Phospholipids of neural and retinal membranes are rich in polyunsaturated fatty acyl chains, particularly in docosahexaenoic acid (DHA, 22:6n3). It is likely that the high content of this fatty acid modulates physical properties of membranes, creating an environment that is optimal for function of neuronal receptors. We investigated this hypothesis by solid-state NMR methods. A unique membrane probe—perdeuterated DHA—was synthesized and incorporated into the lipid matrix. Six distinct order parameters and their corresponding signal intensities were measured. Partial order parameter assignments were made using magic angle spinning NMR techniques. In comparison to saturated chains, DHA order parameters were low, reflecting both a change in bond geometry and an increase in angular fluctuation amplitudes. This is contrary to the current belief that high concentrations of double bonds result in less flexible hydrocarbon chains. Using $^{2}$H-labeled lipids we compared lipid order parameters in the absence, and in the presence of rhodopsin, the primary visual receptor, and a member of G-protein coupled receptor family. Rhodopsin was reconstituted into fully hydrated, solid-supported oriented multi-bilayer samples. With this novel approach we obtained, for the first time, highly resolved spectra from deuterated acyl chains in membranes containing reconstituted integral membrane protein at physiologically sound conditions. Oriented samples also improve NMR sensitivity enabling work with milligram-size samples. The presence of rhodopsin induced differential changes in DHA order parameters along the acyl chain, which suggest a change in the average conformation. We compared rhodopsin effects on the deuterium order parameter profile of saturated chain in mono- and polyunsaturated lipid systems. Protein incorporation decreased the order parameters of polyunsaturated PC, while not affecting that of the monounsaturated PC. The data suggest that rhodopsin preferentially interacts with polyunsaturated lipids resulting in lateral phase separation within the lipid matrix.

Key Words: DHA, Rhodopsin, NMR

■ Role of DHA in Formation of Membrane Domains: Specific Association of Rhodopsin with DI22:6PC. A. Polozova and B.J. Litman, National Institute on Alcohol Abuse and Alcoholism.

It is widely acknowledged that cellular membranes have highly specialized microdomains enriched with specific lipids and proteins. One intriguing hypothesis is that microdomain formation, facilitated by a delicate balance in lipid composition, is critical for proper cell functioning. The goal of this study was to determine the role of polyunsaturated lipids in formation of microdomains. It was shown previously that activation of G-protein coupled photoreceptor rhodopsin is extremely sensitive to lipid polyunsaturation. The lateral segregation of rhodopsin with polyunsaturated lipids was proposed as a possible native mechanism regulating rhodopsin functional activity in retina. To test this hypothesis, we designed a method based on fluorescent resonance energy transfer (FRET). Purified rhodopsin was reconstituted into membranes consisting of phosphatidylcholines (PC) with polyunsaturated (di22:6PC) and saturated (di16:0PC) acyl chains and cholesterol. Trace levels of lipid with similar acyl chains, labeled with fluorophore pyrene (Py), either di22:6-PE-Py or di16:0-PE-Py, were introduced into the membranes. Rhodopsin is a very efficient acceptor of pyrene fluorescence. In reconstituted membranes composed of di22:6PC/di16:0PC/cholesterol at 3:7:3 proportion, the FRET efficiency was significantly higher for di22:6PE-Py, compared to di16:0PE-Py. This result is consistent with presence of microdomains enriched with di22:6PC centered on rhodopsin molecules, as higher FRET efficiency is a direct indication of co-localization of donors and acceptors. Theoretical evaluation of data showed that such microdomains contain at least three lipid layers around each rhodopsin and are enriched

threefold in di22:6PC. Microdomain formation was sensitive to lipid composition. No microdomain formation was found in bilayers containing different proportions of di22:6PC or upon removal of cholesterol. Acute ethanol exposure severely disrupted microdomain structures, as indicated by altered FRET efficiencies for both lipid probes. These results suggest, that DHA lipids, and their proportion are critical for formation of microdomains around receptors. Disruption of such receptor microenviromnents in DHA deficiency may contribute to associated functional losses.

Key Words: Membrane Domains, Rhodopsin, Lipid–Protein Interactions

■ Increasing Dietary Intake of Docosahexaenoic Acid Affects Long-Chain Polyunsaturated Fatty Acid Composition Differently According to Brain Areas in 2-Mo-Old Rat. C. Poumés-Ballihaut[1], S. Vancassel[1], S. Chalon[2], G. Durand[1], Ch. Latgé[3], and G. Guesnet[1], [1]Institut National de la Recherche Agronomique (INRA), [2]Institut National de la Santé et de la Recherche Médicale (INSERM), [3]Blédina S.A., Groupe Danone.

To ensure optimal brain development of the newborn infant, infant formulas in some countries have been supplemented with docosahexaenoic acid (DHA,22:6n-3), the long-chain polyunsaturated fatty acid (LC-PUFA) derived from alpha-linolenic acid (18:3n-3). However, the link between the DHA content in the diet and the fatty acid content of specific regions of the brain has received little study. For this reason, we studied the effects of increasing amounts of dietary DHA on several lipid membrane components in frontal cortex, striatum, hippocampus, and cerebellum in the 2-mo old rat. One week after mating, Wistar female rats deficient in n-3 fatty acids (2nd generation) were randomly divided into eleven groups. Ten groups received DHA from 0 to 1600 mg/100 g diet and one group received 300 mg of 18:3n-3/100 g of diet (control). All diets contained 7% by weight fat and 1.2 g of 18:2n-6/100 g, the precursor of the n-6 family. At weaning, male rat pups continued to receive the same diet as their mother until 2-mo of age. Cholesterol and total phospholipid content of brain regions was not affected by DHA intake, but they differed in concentration among the brain areas studied. Compared to other brain, regions, cholesterol was lowest in frontal cortex and hippocampus (1.0 vs. 1.3 mg/100 mg tissue) and total phospholipids were lowest in hippocampus (3.2 vs. 4.4 mg/100 mg tissue). Neither the cholesterol to phospholipid ratio nor the proportions of phospholipid classes changed or differed among brain areas (weight %): phosphatidylcholine (PC), 47%; phosphatidylethanolamine (PE), 36%; phosphatidylserine (PS), 11%; phosphatidylinositol (PI), 4%; and sphingomyelin (SM), 2%. Whatever the DHA intake, DHA and arachidonic acid (AA, 20:4n-6) levels were specific to the class of phospholipid and the brain area. In PE and PS, the DHA content was similar, higher in frontal cortex and the lower in striatum (31 and 22% of total fatty acids, respectively, in rats fed 1600 mg DHA). In PC, the highest DHA content was in cerebellum (10%). For AA, hippocampus had the highest concentration in PC, PE, and PS. The DHA supply affected proportions of 22:4n-6, 22:5n-6 but mainly DHA and AA. In PC, PE, and PS of each brain area studied, the DHA incorporation followed a logarithmic curve ($P<0.001$) with a rapid increase up to 100 mg DHA/100 g diet, from 7 to 23% in PE of frontal cortex. This increase was followed by a slower one, from 23 to 30% in PE of frontal cortex. During this same interval, AA fell dramatically up to 200 mg DHA/100 g diet, from 14.5% to 11.5% AA in PE of frontal cortex. This decline was followed by a slower decrease, from 11.5% to 7%($P<0.001$). The different DHA and AA contents in brain regions might suggest different needs for those LC-PUFA. All brain areas studied showed an inverse relationship between DHA and AA in PC, PE, and PS as dietary 22:6n-3 increased. This must be carefully considered given the importance of those two LC-PUFA in cerebral functions.

Key Words: Docosahexanoic Acid, Brain Areas, Rat

■ Maternal Diet and Human Brain Evolution. S. Robson, University of Utah.

Animal products make up a larger portion of human than non-human diets. Humans also have larger brains, both absolutely and relative to body size. It has often been proposed that increased carnivory during human evolution provided the nutritional support needed to underwrite greater encephalization. Comparative analyses show that at birth, human brains are the same fraction of neonatal weight as the average primate but are a much smaller portion than expected of mature brain size. This means that postnatal, not fetal, growth accounts for our departure from the general primate pattern. During the period of most rapid postnatal brain growth, infants are provisioned primarily (if not exclusively) by lactation. If increased carnivory is responsible for our larger brains, human breast milk should differ from that of other primates by displaying a higher proportion of specific lipids which are critical components of brain matter. Breast milk in humans whose diets vary in meat content should also differ in predictable ways. Multiple lines of evidence suggest that meat in the maternal diet has little impact on breast milk quantity or quality. In addition, human breast milk does not appear to differ in composition from that of other anthropoid primates. If the proportion of meat in maternal diets does not affect breast milk composition (specifically LC-PUFA) among humans, then increased carnivory is unlikely to account for the human pattern of encephalization.

Key Words: Encephalization, Breast Milk, Long-Chain Polyunsaturated Fatty Acids

■ Medium-Chain (MC), Intermediary-Chain (IC) and Polyunsaturated (PU) Fatty Acid (FA) Supplies with Mature Human Milk of Three Different African Populations. G. Rocquelin[1], S. Tapsoba[2], J. Kiffer[1], N. Thiombiano[3], C. Bouda[4], O.N. Zougmoré[5], and A. Traoré[3], [1]IRD, Montpellier, France, [2]ANSA, Ouagadougou, Burkina Faso, [3]FAST, Université de Ouagadougou, Burkina Faso, [4]Faculté de Médecine, Université de Ouagadougou, Burkina Faso, [5]FLASHS, Université de Ouagadougou, Burkina Faso.

Objective: To measure the FA supply from breast milk as part of nutritional surveys of the essential fatty acid (EFA) status of 5-month-old African infants.

Settings: Urban Congo (UC), urban Burkina Faso (UBF), and rural Burkina Faso (RBF).

Subjects: Random samples of healthy mothers nursing 5-month-old infants ($n=100$) living in suburban districts of Brazzaville and Ouagadougou, or in a rural area of Burkina Faso.

Data collection procedures: Mothers were questioned about their socio-econonomic status and dietary habits, and their body mass index (BMI) was measured. Samples of milk (5 to 10 mL) were collected at 2 specific times in the day by manual expression from both breasts. Milk lipid contents were measured and FA analyzed by high-resolution gas chromatography.

Results: Compared with breast milk from various countries, Congolese and Burkinabe women's mature milk was low in lipid. Milk fat content was higher in UBF (33.42 g/L) and RBF (31.42 g/L) than in UC (28.70 g/L). MC and ICFA contents (C8:0–C14:0) were several-fold greater than in most countries, particularly in RBF where they accounted for one third of total FA (33.52%). UC milk was noticeably rich in n-3 PUFA (2.39% of total FA), particularly 18:3 (1.19%) and 22:6 (0.55%) whereas UBF milk was high in n-6 PUFA (21.74%) particularly 18:2 n-6 (19.80%). RBF milk had levels intermediate between UC and UBF milk. In the 3 groups, MC and ICFA were strongly negatively related with oleic acid levels but not related to n-6 or n-3 PUFA. Also a highly significant positive correlation was found between 18:2n-6 and 18:3n-3. Ratios of 18:2n-6/18:3n-3 and n-6/n-3 long-chain PUFA varied respectively from 12:1 in UC to 52:1 in UBF, and from 1:1 in UC to almost 5:1 in UBF. In general, the dietary habits of mothers explained these differences. Data from food frequency questionnaires showed that Congolese and Burkinabe mothers' diets were very high in carbohydrates and low in fat therefore enhancing de novo synthesis of MC and ICFA in the mammary gland. Also in the Congo high consumption of foods providing n-3 PUFA such as green leafy vegetables and fish led to enrichment of milk in these FA. On the other hand, high levels of 18:2n-6 in UBF milk were due to frequent consumption of cottonseed oil (high in linoleic acid) and cereals (millet, sorghum, corn, wheat) higher in 18:2n-6 than tubers (casava) that were preferentially consumed in the Congo.

Conclusions: FA supplies from breast milk to 3 different populations of 5-month-old African infants were much different and their impact on infant EFA status may vary. Studies are needed, for instance, to explore the influence in breast milk of 25% to 33% of total FA as MC and ICFA

on energy balance or else the effect of unbalanced n-6/n-3 PUFA ratios on utilization and metabolism of these PUFA by infants.

Key Words: Breast Milk, Essential Fatty Acid, Infant Nutrition

■ Synthesis of n-6 Long-Chain Polyunsaturated Fatty Acids in Preterm Infants Fed Medium-Chain Triacylglycerides. M. Rodriguez[1], H. Demmelmair[1], M. Fink[1], S. Kiss[2], M. Turini[3], G. Crozier[3], and B. Koletzko[1], [1]Ludwig-Maximilians University of Munich, Germany, [2]Children Hospital Pecs, Hungary, [3]Nestec, Vevey, Switzerland.

The effects of medium-chain triglycerides (MCT) in preterm infant formulas on essential and long-chain polyunsaturated fatty acid (LC-PUFA) status and metabolism were investigated using stable uniformly $^{13}C$-labeled isotopes. Nineteen premature infants (gestational age≤37 wk) who were exclusively formula fed were enrolled in the study. Infants were randomly assigned to receive for 7 days an infant formula with 40% of fat as MCT ($n$=10) or without MCT ($n$=9), with otherwise similar composition and linoleic and α-linolenic acid contents. At study day 5, infants received 2 mg/kg body weight of uniformly $^{13}C$-labeled linoleic acid. Tracer oxidation was assessed by analyzing $^{13}C$ content in breath $CO_2$. Fatty acid composition of plasma lipids and $^{13}C$ content in plasma phospholipids was determined 48 h after tracer application. The results showed that in the MCT group oxidation of linoleic acid was lower (tracer recovery in breath was 3.0% vs. 5.6% of the applied dose, $P$≤0.01) and plasma linoleic acid concentrations were higher (not significant) than in the control group. LCP concentrations were also higher in infants fed MCT, particularly in plasma triglycerides (57.07±4.36 mol/L vs. 37.93±4.75 mol/L, $P$≤ 0.05). As indicated by the tracer distribution, no significant differences in the relative conversion of linoleic acid to arachidonic acid were observed ($^{13}C$20:4n-6/$^{13}C$18:2n-6 was 0.107 and 0.085 in the control and the MCT group, respectively). Thus, we conclude that MCT have a positive effect on the n-6 LCP status, possibly through a decreased oxidation of linoleic acid and its conversion products.

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Key Words: Long-Chain Polyunsaturated Fatty Acids, Medium-Chain Triglycerides, Premature Infant

■ Effect of DHA on Gene Expression in Human Fetal Retinal Explants. C. Rojas[1], D. Hoffman[2], J. Martinez[1], and R. Uauy[1,2], [1]INTA University of Chile, Santiago-Chile, [2]Retina Foundation of the Southwest, Dallas, TX.

Docosahexaenoic acid (DHA) has a profound effect on functional maturation of the retina. To explore the possible effect of DHA on gene expression during human fetal retinal maturation, we compared mRNA expression from retinal explants with and without added DHA using gene microarray methodology. Total RNA was obtained on day 14 from fetal retina (14–18 wk gestation) cultured in serum-free Waymouth's media with 20 μM DHA in BSA. Control explants were cultured in lipid-free BSA. cDNA was synthesized with labeled nucleotides and hybridized to the microarray. Relative expression analysis of 2400 genes was based on signal amplification using fluorescent reporters. The +DHA/Control ratio of expression was unchanged in 80% of genes (i.e., several ribosomal proteins and GADPH). Decreased expression (ratio < 0.33) was observed in 4% of genes (i.e., human brain fatty acid binding proteins, cytoskeletal proteins, PI kinase, leukotriene hydrolase). 15% of the genes showed increased expression (ratio > 3.0). These included genes for fatty acid oxidation and desaturation, PAF acetylhydrolase, LDL receptor, lipoprotein lipase mitochondrial oxidation processes, differentiation and apoptosis-related protein kinases, transcription factors, neurotransmitter receptors, and IGF-binding proteins. We conclude that DHA action during retinal development is mediated not only by its known effects on membrane biophysical properties but also by significant modulation of gene expression. Funded by Catedra Presidencial RU-96.

Key Words: DHA, Gene Expression, Retina

■ Docosahexaenoic Acid Protects Retinal Photoreceptors from Apoptosis Induced by Oxidative Stress. N. Rotstein, Instituto de Investigaciones Bioquimicas, UNS-CONICET.

Oxidative stress induces apoptosis both during the normal development of the nervous system and in neurodegenerative diseases. The retina, especially in newborns, is particularly prone to oxidative damage. One of the reasons is its high content of polyunsaturated fatty acids (PUFA). However, it has recently been demonstrated that docosahexaenoic acid (DHA), the most abundant PUFA in photoreceptor cells, can postpone the onset of the apoptosis of these neurons, which otherwise inexorably starts after 4 days in culture (Rotstein *et al.,* 1997, *J. Neurochem. 69*, 504; 1998, *Invest. Ophthalmol. Vis. Sci. 39,* 2750). This protective effect correlates with an active esterification and accumulation of DHA in neuronal lipids. To investigate the effect of oxidative stress on retinal neurons and whether addition of DHA enhanced oxidative damage, 3-day rat retinal neurons were incubated with Paraquat (24 μM), a generator of reactive oxygen species. After 24 hours, this oxidant induced apoptosis in both amacrine and photoreceptor neurons, the two major neuronal types in the culture: the percentages of apoptotic cells raised from 3.2% to 35.0% of the total amacrine neurons and from 23.6% to 67.4% of the total photoreceptors, in control and Paraquat-treated cultures, respectively. Apoptotic death was only slightly affected by a Paraquat concentration ranging from 2.4 to 240 μM. Addition of Paraquat led to the increased expression of the proapototic protein p53 and to the fragmentation of the nuclear lamins, characteristic of apoptotic processes. DHA supplementation at day 1 in culture slightly diminished the percentage of apoptotic photoreceptor at day 4, compared to control conditions. This addition reduced almost by half photoreceptor apoptosis induced by Paraquat: only about 35% of photoreceptors were apoptotic in this condition. This effect was specific for photoreceptors, since induction of apoptosis in amacrine neurons was unaffected by DHA addition. These results suggest that oxidative damage is highly lethal to retinal neurons in the early stages of their development in culture. Surprisingly, a higher content of DHA in their cell membranes does not increase neuronal sensitivity to this damage. On the contrary, it has a protective effect on photoreceptor neurons, enhancing their survival and preventing apoptosis. This suggests that, apart from its well-known structural role, DHA may also act as a signal molecule, triggering intracellular pathways that protect photoreceptors from apoptotic degeneration.

Key Words: Docosahexaenoic Acid, Retinal Photoreceptors, Oxidative Damage

■ Study of Essential Fatty Acid Metabolism Using Multiple Stable-Isotope Labeled Precursors. Y. Lin and N. Salem, Jr., LMBB, NIAAA, NIH, Rockville, MD.

An important issue in nutrition is whether it is adequate to supply only dietary fats like linoleate (LA, 18:2n-6) and alpha-linolenate (LNA, 18:3n-3) or whether it is important to also supply their preformed metabolites. Therefore, comparison was made between 18- and 20-carbon essential fatty acids (EFA) with respect to their metabolism to longer chain, more unsaturated fatty acids (long-chain polyunsaturated fatty acids) in adult rats. A newly developed, multiple stable isotope technique was employed together with isotope dilution gas chromatography/negative chemical ionization mass spectrometry. Deuterium-labeled-LNA (D5-18:3n-3), carbon-13-U-labeled eicosapentaenoate (20:5n-3), carbon-13-U-labeled-LA and deuterium-labeled-dihomo-gamma linolenate (D5-20:3n-6) were used as tracers. These isotope-labeled fatty acids in vehicle oil were simultaneously given as a single oral dose with an 18C/20C ratio of 5:1 (w/w). Rat blood and tissues were collected at various time points after dosing. In rat plasma and liver, the isotopomers of the precursors and their main metabolites, including the 13C-labeled and D-labeled, can be detected simultaneously in the same sample with good resolution due to their differences in mass and retention time. Both 18C and 20C precursors reached their maximal concentration after 4 hr in plasma, but after 8 hr in liver and brain. They were largely eliminated by about 96 hr except in brain, where the precursors were still detectable after 10 d. In plasma, maximal concentrations of D-22:5n-3 and $^{13}C$-22:5n-3 were reached after 8–12 hr, while D5-22:6n-3 and $^{13}C$-22:6n-3 were maximal after 24 hr. However, in the brain, the peak concentrations of isotope-labeled 20:4n-6 and 22:6n-3 occurred at 10 d and were still detected after 30 d. Retroconversion of D-18:2n-6 and D-18:3n-6 from D-20:3n-6 was observed in rat plasma

and liver. Even though the $^{13}C$ enrichment of 20:5n-3 was much greater than that of the deuterium in plasma and liver, the 22:5n-3 metabolite contained a similar enrichment of the two isotopes while the 22:6n-3 had a greater enrichment of deuterium. Similarly, there was evidence that 20:3n-6 molecules that originated from $^{13}C$-18:2n-6 were selectively metabolized to 20:4n-6 in comparison to preformed D5-20:3n-6. This unexpected behavior may be the result of an isotope effect, a different subcellular localization or a different lipid class form of the substrates when derived from either the 18- or 20-carbon fatty acids. It may also indicate coupling between the various desaturase/elongase steps in EFA metabolism.

Key Words: Essential Fatty Acid Metabolism, Stable Isotope Tracers, GC–MS Analysis, Isotope Enrichment

■ Effect of Increasing Dietary Long-Chain Polyunsaturates (LCP) with High Vitamin E on Lipid Peroxidation and Susceptibility to Oxidation of Piglet Erythrocytes, Plasma and Liver. E. Sarkadi Nagy, M.C. Huang, R. Kirwan, A. Chao, C. Tschanz, and J.T. Brenna, Cornell University, Ithaca, NY.

The purpose of this study was to determine the effects of increasing doses of dietary docosahexaenoic acid (DHA) and arachidonic acid (AA) supplementation on lipid peroxidation and *in vitro* susceptibility to oxidation in piglet tissues. Twenty-four one-day old piglets ($n$=6 per group) were bottle-fed a sow milk replacement formula containing one of four experimental treatments for four weeks: no long-chain fatty acid ("Diet 0") and three different levels of DHA/AA at 1-fold (0.3%/0.6% FA;"Diet 1") 2-fold (0.6%/1.2% FA;"Diet 2") and 5-fold (1.5%/3% FA; "Diet 5") concentration of some human infant formulas. All sow milk formulas had vitamin E at 260 mg/kg diet. Red blood cell (RBC) membrane total lipid DHA and AA increased in a dose-dependent manner while 18:2n-6 (linoleic acid) and 18:3n-3 (linolenic acid) concentrations decreased. These modifications resulted in a concomitant dose-dependent increase in the membrane unsaturation index. Lipid peroxidation was assessed by measuring conjugated diene and glutathione (GSH) levels in the liver, and thiobarbituric acid-reactive substances (TBARS) in plasma. There were no significant differences between the groups in any of these parameters. Oxidative stability was assessed by determination of TBARS concentration after exposure of RBC to 10 mM $H_2O_2$, and liver homogenates to various concentrations of $H_2O_2$ (0, 1, 5, 10 mM) for 30 minutes. TBARS levels of the erythrocyte membranes increased in a dose-dependent manner when *in vitro* oxidation was induced. The TBARS levels of the liver homogenates of the Diet 5 group were significantly different from those of the membranes of the Diet 0 group when the *in vitro* oxidation was induced with only the highest concentration of $H_2O_2$ (10 mM). We conclude that there was no increase in oxidation due to high dietary and tissue LCP with high vitamin E. The susceptibility of RBC and liver of piglets in the Diet 5 group to *in vitro* oxidation was increased compared to the Diet 0 group only under extreme oxidative conditions. Overall, the results suggest that the dietary vitamin E effectively prevented oxidation at the LCP concentrations investigated.

Key Words: Lipid Peroxidation, Long-Chain Polyunsaturates, Vitamin E

■ The Effect of Essential Fatty Acid Metabolite Supplementation During Pregnancy and Lactation on Growth and Development of Vervet Monkeys *(Cercoopithecus aethiops)*. H.Y. Tichelaar, M.C. Mdhluli, C.M. Smuts*, and A.J.S. Benade, Medical Research Council, Tygerberg, South Africa.

Nonhuman primates are suitable models of human physiology to study the effect of multiple pregnancies on growth and development. We examined the changes in anthropometry and fatty acids of vervet infants from females ($n$=12), aged 6–17 years who had 2–9 previous live births. Vervets were fed a high-carbohydrate diet (low in n-3 fatty acids) that was either supplemented (experimental group) with a DHA-rich oil (Scotia Pharmaceuticals; 320 mg n-3 fatty acids/day, which included 280 mg DHA) for at least one year before and during pregnancy and lactation (based on the Canadian Recommendations for pregnant women), or not supplemented (control group). Following an overnight fast (18 hours), the female vervets were sedated with ketamine HCl (10 mg/kg intramuscular). Blood (2 mL) and anthropometric measurements were taken at birth, after 1, 3 and 6 months when infants were weaned. Plasma and red blood cell (RBC) total phospholipid (TPL) fatty acids were extracted with chloroform/methanol (2:1; vol/vol) and analyzed by GLC for fatty acid composition. Plasma (11.19% vs. 4.92%), RBC (11.14% vs. 4.53%) TPL DHA and milk DHA (3.51% vs. 1.65%) was higher ($P$<0.001) after 6 months of lactation in infants from n-3 fatty acid-supplemented females, compared to the control infants. Experimental infants had higher weights after 3 and 6 months (698 g vs. 573 g and 947 g vs. 722 g, respectively; $P$<0.05); were longer after 1 month (327 mm vs. 312 mm; $P$<0.05); had longer lower leg length after 6 months of lactation (108 mm vs. 101 mm; $P$<0.01) and had larger head circumferences after 3 (202 mm vs. 194 mm; $P$<0.05) and 6 months (208 mm vs. 195 mm; $P$<0.01) than the control infants. The results suggest that n-3 fatty acid supplementation of pregnant and lactating female vervets had profound effects on their offspring. The findings of this study are encouraging and should be used to design a clinical trial of n-3 fatty acid supplementation with pregnant women to measure growth and development of their infants.

Key Words: DHA, Growth, Vervet Monkeys

■ Relative Effects of Women's DHA Status Entering the Third Trimester of Pregnancy and Third Trimester DHA Intake on Infant DHA Status at Birth. C.M. Smuts[1] and S.E. Carlson[2], [1]Medical Research Council, Tygerberg, South Africa, [2]University of Kansas Medical Center, Kansas City, KS.

Background: The last intrauterine trimester is important for fetal brain docosahexaenoic acid (DHA, 22:6n-3) accumulation, but little is known about the role that maternal DHA status, including the portion related to maternal DHA intake, plays in infant DHA status. Objective: To determine the relative effects of maternal DHA status at the beginning of the 3rd trimester and varied DHA intake during the 3rd trimester on maternal and infant DHA status at birth. Methods: As part of a pilot study to determine if eggs could be used to increase DHA status of women and their infants, we investigated these relationships. Pregnant women were randomly assigned to eat either regular (~27 mg/egg, $n$=19) or high-DHA (~135 mg/egg, $n$=18) eggs (up to 1 dozen per week) during the last trimester of pregnancy. A third group of pregnant women, who indicated in the initial interview that they routinely ate few if any eggs, was included as a non-randomized group ($n$=16). Women in all groups consumed a range of eggs, and, consequently, there was a range of DHA intake from 50 mg/day (obtained from regular diet without eggs) to ~1700 mg/day. Blood was drawn from mothers at enrollment and delivery, and blood was drawn from the cord to determine newborn DHA status at birth. Lipids from plasma and RBC were extracted with chloroform/methanol (2:1; vol/vol) and analyzed by GLC for fatty acid composition. We have already reported that at birth the maternal plasma triglyceride (TG) and total phospholipid (PL), and the RBC PL DHA levels (% wt/wt) did not differ significantly between the treatment groups despite large differences in DHA intake (245.6 and 1445.6 mg/study week). However, the total DHA intake/day (from diet and eggs) was significantly correlated with maternal plasma TG, PL and RBC PL DHA at birth when controlled for weeks in the study ($r$ = 0.475; $r$ = 0.350 and $r$ = 0.294, respectively). Total DHA intake also correlated with the baby plasma PL DHA levels ($r$ = 0.247; $P$ = 0.049). In turn, maternal DHA status at birth (positively) and gravidity (negatively) accounted for 10–39% of the variability in infants' DHA status at birth. Maternal DHA at birth was explained mainly by maternal DHA status at enrollment and dietary DHA intake, which together accounted for 20% (TG), 47.3% (Plasma PL) or 34% (RBC PL) of the variability in maternal DHA at birth. However, maternal DHA at enrollment was the stronger predictor of DHA levels in plasma and RBC PL at delivery. Conclusion: It is important to know and control for prior maternal DHA status in studies of maternal DHA supplementation that test the influence of DHA on outcomes of pregnancy and infant development. Supported by OmegaTech, Inc., Boulder, CO.

Key Words: DHA, Eggs, Pregnancy

■ Essential Fatty Acids in Maternal and Newborn Phospholipids from a Primarily African American Population in the United States. C.M. Smuts[1], M.I. Huang[2], D. Mundy[3], and S.E. Carlson[2], [1]Medical Research

Council, Tygerberg, South Africa, [2]University of Kansas Medical Center, Kansas City, KS, [3]University of Missouri–Kansas City, Kansas City, MO.

Reports from The Netherlands have established characteristics of maternal and cord blood as well as umbilical arterial and venous vessel wall phospholipid (PL) fatty acids. The purpose of this study was to compare those data to results from a group of predominantly African American, lower SES women from Missouri (USA) who ate a range of DHA (50 to 1620 mg DHA/wk, Block Food Frequency Questionnaire, 24–28 wks gestation) and delivered at term ($n$=39). The women were the first to deliver from a larger group enrolled to study relationships of dietary DHA intake to later DHA status and pregnancy outcome. At delivery, cord and maternal venous blood were obtained and the cord arterial and venous vessel walls were isolated. Lipids from maternal and cord plasma, as well as from umbilical artery and vein vessel walls, were extracted with chloroform/methanol (2:1; vol/vol) and PL isolated and transmethylated ($BF_3$). FAME were analyzed by GLC with an SP-2560 100 meter capillary column. PL fatty acids expressed as g/100 g are shown below. The U.S. women and infants were compared to the range of means from two analogous studies in The Netherlands (*Early Hum. Dev. 24*, 239–248, 1990; *Eur. J. Clin. Nutr. 51,* 232–242, 1997). The mean (SD) maternal age, gestational age at delivery and weights of infants at birth were 19.8 (3.3) years, 39.3 (1.2) weeks, and 3149 (442) g, respectively, in the U.S. group compared with 27–29 years, 40.0–40.2 weeks and 3333–3451 g in The Netherlands groups. For PL fatty acid composition of mother and cord plasma, and the walls of umbilical vessels see table.

| | Mother at birth | | Cord venous blood | |
|---|---|---|---|---|
| | USA | Netherlands | USA | Netherlands |
| 18:2n-6 | 17.3(3.2) | 20.7–23.1 | 7.2(2.1) | 7.3–7.8 |
| 20:4n-6 | 12.8(1.9) | 8.7–9.4 | 21.0(2.0) | 17.1–19.8 |
| 22:6n-3 | 4.4(1.1) | 3.8–3.9 | 6.9(1.5) | 6.5–6.7 |
| ∑n-6 | 40.1(.18) | 34.5–36.8 | 37.6(2.1) | 32.2–35.6 |
| ∑n-3 | 5.3(1.2) | 5.1–5.7 | 7.5(1.7) | 7.3–8.6 |
| ∑n-7≠n-9 | 10.1(1.2) | 13.0–15.7 | 8.6(1.5 | 12.0–15.2 |

| | Vessel arterial wall | | Vessel venous wall | |
|---|---|---|---|---|
| | USA | Netherlands | USA | Netherlands |
| 18:2n-6 | 1.5(0.4) | 1.1–1.9 | 2.5(0.6) | 1.8–2.8 |
| 20:4n-6 | 15.6(2.9) | 11.4–13.6 | 19.8(2.9) | 15.9–18.0 |
| 22:6n-3 | 6.0(1.3) | 4.7–5.3 | 4.7(1.1) | 4.5–5.2 |
| ∑n-6 | 28.3(3.9) | 21.5–22.4 | 34.7(3.8) | 29.2–30.6 |
| ∑n-3 | 6.6(1.2) | 5.2–5.6 | 5.1(1.2) | 5.3–5.5 |
| ∑n-7+n-9 | 19.1(4.0) | 23.5–28.1 | 12.4(2.7) | 16.1–19.4 |

Compared to The Netherlands, USA mothers were much younger, delivered ~1 wk earlier and had infants who were appropriately lighter given an anticipated fetal gain of 30 g/day toward the end of gestation. Despite these differences and the differences in fat intake inferred from lower 18:2n-6 and higher 20:4n-6 in maternal plasma PL, cord plasma and vessel PL fatty acids from the USA and The Netherlands were most remarkable for their similarity. Only Σn-7 and n-9 and 20:4n-6 appeared to differ. The former were much lower and the latter somewhat higher in pregnant women from the USA compared to The Netherlands. Supported by OmegaTech, Inc., Boulder, CO.

Key Words: EFA Status, Newborn, Umbilical Vessels

■ Low Docosahexaenoic Acid in Brain but Not in Liver, Plasma and Red Blood Cells in Newborn Zellweger Syndrome Mouse. H.-M. Su[1], A.B. Moser[1], H.W. Moser[1], and P.L. Faust[2], [1]Kennedy Krieger Institute, Johns Hopkins Medical Institutions, [2]Columbia University.

Zellweger syndrome is a genetic peroxisomal biogenesis disorder. Peroxisomal β-oxidation plays an important role in the catabolism of very long chain saturated fatty acids, and synthesis of docosahexaenoic acid (DHA, 22:6n-3). We report the fatty acid profiles in newborn, 5, 11, and 13 days old PEX2 peroxisome assembly gene knockout mice, compared with their control littermates. The PEX2 -/- is a model of human Zellweger syndrome. Whole brain, liver, plasma, and red blood cell (RBC) were collected. Lipids were extracted by the Folch method, and fatty acid composition was determined by gas chromatography. At birth, DHA was reduced in the PEX2 -/- brain (10%" vs. 14%"), but normal in RBC (9%"), and, surprisingly, higher than control in plasma (11%" vs. 6%") and liver (14%" vs 12%"). In 5, 11, and 13 days old mice, the DHA was low in all tissues. Ratio of C26:0/C22:0 was higher in PEX2 -/- brain, liver, plasma, and RBC, compared to control littermates at all ages. Plasmalogens were less than 1%" of control in PEX2 -/- brain and RBC at all ages. The brain nervonic acid (24:1n-9) was normal in the newborn and 5-day-old PEX2 -/- mice, and below normal thereafter. Findings in the brain of the PEX2 -/- mouse model in general parallel those in human Zellweger syndrome.

Key Words: DHA, Zellweger Syndrome

■ Dietary n-3 Fatty Acid Alters Rhodopsin Function of Developing Retina. M. Suh and M.T. Clandinin, University of Alberta, Edmonton, Alberta, Canada.

The effect of diets differing in n-3 fatty acid composition on fatty acid content, rhodopsin content, rhodopsin phosphorylation, and rhodopsin regeneration in retina was investigated. Weanling rats were fed diets containing 20% (w/w) fat with either high (5% w/w) DHA or low (1% w/w) n-3 fatty acid without DHA. After 6 wks of feeding, half of the animals in each group were exposed to 48 hrs of continuous light at 850 lux or were kept in complete darkness. After light exposure, animals fed a high n-3 fatty acid diet showed reduction in 22:6n-3 as well as in n-6 and n-3 fatty acid from 24 to 34 carbons (VLCFA) in PC and PS. Diet low in n-3 fatty acid increased rhodopsin content and rhodopsin phosphorylation compared to diet in high n-3 diet. However, low n-3 fatty acid diet caused greater rhodopsin loss after light exposure, resulting in less phosphorylation. Rhodopsin in animals fed a high n-3 fatty acid diet disappeared in a relatively short time with bleaching. Rhodopsin regeneration *in vitro* was increased by feeding a high n-3 fatty acid. It is concluded that the fatty acid composition, rhodopsin content and function in retina is influenced by dietary n-3 fatty acid and light exposure. Feeding a high n-3 fatty diet with DHA may play a role in conserving rhodopsin during or after light exposure.

Key Words: Dietary n-3 Fatty Acid, Rhodopsin, Retina

■ Do Neuronal Receptors Sense Lipid Matrix Curvature Elastic Stress from Polyunsaturated Fatty Acids in Surrounding Lipid Bilayer? W. Teague and K. Gawrisch, National Institute on Alcohol Abuse and Alcoholism. Rockville, MD.

Phosphoethanolamine (PE) comprises more than 40% of retinal and synaptisomal membranes and, within these membranes, more than 50% of all fatty acids in PE are the sixfold unsaturated docosahexaenoic acid (DHA). Increased levels of polyunsaturated PE are known to significantly boost the activation of membrane-bound rhodopsin. PE has the tendency to form phases with small area per molecule near the lipid water interface and larger area per molecule near the terminal methyl groups of the hydrocarbon chains. When forced into a lamellar arrangement, such membranes are under considerable curvature elastic stress. We are investigating quantitatively the influence of polyunsaturation on the coefficient of membrane monolayer curvature elasticity for PE. We alter the membrane curvature of purified polyunsaturated phospholipids by the application of osmotic stress on the inverse hexagonal phase state. The parameters of lipid chain packing and membrane free energy are then determined through solid state NMR and diffraction methods. Preliminary results indicate that PE containing polyunsaturated fatty acids has a tendency to form monolayers with increased levels of curvature elastic stress. This suggests a link between the activity of transmembrane proteins and the degree of polyunsaturation of lipid hydrocarbon chains in the retinal and synaptisomal membranes. We propose that a high concentration of DHA in the PE phospholipids of the retinal and synaptisomal membranes facilitates protein structural transitions during receptor activation. Polyunsaturated PE may serve to balance the curvature stress placed on the membrane due to the presence of tilted transmembrane protein helices.

Key Words: Biophysics, Membrane, Phospholipid

■ PUFA in Pregnancy and Lactation: New Directions. A.C.v. Houwelingen, S.J. Otto, and G. Hornstra, NUTRIM, Dept. Human Biology, Maastricht University.

Pregnancy is accompanied by maternal hyperlipidemia, which might be a physiological adaptation to meet the increased energy demands due to pregnancy and to improve the fetal access to essential fatty acids (EFA). The fetus needs EFA for incorporation into the proliferating membranes, and especially docosahexaenoic acid (DHA, 22:6n-3) for the CNS and the retina. The fetus must obtain its EFA through placental transfer and is, consequently, dependent on the maternal EFA status. Any limitation in the EFA transfer to the fetus may have functional consequences. Therefore, the maternal EFA status in general, and the n-3 EFA status in particular, has to be adequate to ensure optimum conditions for structural and functional tissue maturation.

The course of the maternal EFA status in the very beginning of pregnancy, until the 10th week of gestation, shows an increase in maternal plasma DHA, probably representing an enhanced incorporation of DHA in phospholipids.

In a prospective longitudinal study performed in pregnant Dutch women it was shown that the overall maternal EFA status steadily declines during normal, uncomplicated pregnancy. This holds for the DHA status also. These data were obtained by measurements of the DHA levels from the 10th week of gestation until delivery.

For most EFA levels, normalization after delivery is complete within 32 weeks. The percentages of plasma 20:4n-6 increased, whereas the percentages of DHA decline significantly after delivery. In contrast to the n-6 EFA status, the normalization of the postpartum course of maternal n-3 EFA status differs between lactating and nonlactating women.

To investigate whether the steady decline in the maternal EFA status during pregnancy observed in pregnant Dutch women is a local or general phenomenon, the EFA status of healthy women from The Netherlands, Hungary, Finland, England, and Ecuador was measured during uncomplicated, singleton pregnancies. Although considerable differences between these groups were observed in the maternal EFA status, the change in the absolute as well as relative amounts of the EFA followed a similar course in the five populations during pregnancy. It seems that the reduction in maternal EFA status during pregnancy is a general phenomenon, and is largely independent of differences in dietary habits and ethnic origin. The neonatal EFA profiles reflected the differences found in maternal plasma during pregnancy and at delivery. Correlations were found, particularly between the neonatal and the maternal n-3 EFA status. The functional implications of the pregnancy-associated reduction in the maternal EFA status for the fetal and neonatal development are not obvious and need to be further elucidated. Since strong correlations were found between the maternal and neonatal n-6 and n-3 EFA at birth, maternal supplementation can be expected to influence the EFA status of the infant.

Key Words: Essential Fatty Acids, Lactation, Pregnancy

■ The Effects of Dietary *Trans* Fatty Acids Combined with a Marginal Essential Fatty Acid Status During the Pre- And Postnatal Period on Growth, Brain Fatty Acid Composition, and Behavioral Development in B6D2F2 Mice. I. Wauben, H.-C. Xing, D. McCutcheon, and P. Wainwright, University of Waterloo.

The objective of this study was to investigate whether dietary *trans* fatty acids (TFA) during the pre- and postnatal period would exacerbate the effects of marginal essential fatty acid (EFA) status on growth, brain long-chain polyunsaturated fatty acids (LC-PUFA), and behavioral development in B6D2F2 mice. Pregnant B6D2F1 females were randomly assigned to one of three diets: Marginal EFA (mEFA) plus 22% *trans* 18:1 (mEFA+TFA); mEFA diet; and control diet (CON). The total 18:1 contents in all diets were similar. The offspring were weaned and maintained on the same diets. Sensory and motor development were assessed at d12, and one male from each litter was tested on acquisition and reversal learning in a T-water maze at 7 weeks. Brains were excised and the phosphatidyl-ethanolamine (PE) and phosphatidylcholine (PC) fractions were analyzed for LC-PUFA. mEFA and mEFA+TFA reduced growth and brain weight compared to CON, but did not differ from each other. As expected, mEFA and mEFA+TFA reduced docosahexaenoic acid [DHA (22:6n-3)] and increased 22:5n-6 concentrations in brain PC and PE compared to CON, but again did not differ from each other. Motor and sensory development were not significantly affected by either mEFA or mEFA+TFA compared to CON. Reversal learning in the T-water maze, however, was significantly slower in mEFA+TFA compared to mEFA, and both were slower compared to CON. These findings suggest that TFA do not exacerbate the effects of mEFA on growth or brain LC-PUFA. However, possible long-term effects of dietary TFA on behavioral development and neural function should be investigated in future studies.

Key Words: Isomeric Fatty Acids, Brain Fatty Acid Composition, Behavioral Development

■ Perinatal Omega-3 Status Affects Adult Blood Pressure, Body Fluid Homeostasis, and Retinal Function. H.S. Weisinger[1], R.S. Weisinger[2], A.J. Vingrys[3], J.A. Armitage[2,3], P. Burns[2], and A.J. Sinclair[1], [1]RMIT University, [2]Howard Florey Institute of Experimental Physiology and Medicine, [3]University of Melbourne.

The omega-3 fatty acid, docosahexaenoic acid (DHA) is the most prevalent lipid in the nervous system, comprising up to 7% of the dry weight of the brain grey matter and retina. This study investigated the importance of perinatal omega-3 supply in the control of blood pressure, osmotic homeostasis and retinal function in adult Sprague-Dawley rats. Animals raised on an omega-3 deficient diet had higher blood pressure, decreased retinal function and affected osmotic homeostasis, despite subsequent restoration of tissue DHA levels. Our findings indicate that inadequate levels of DHA in the peri-natal period may result in hypertension later in life, possibly as a result of altered osmotic control.

Key Words: Blood Pressure, Hypertension, n-3 Fatty Acids

■ Efficacy of Dietary Triglyceride and Phospholipid Arachidonic Acid for Tissue Accretion in Primate Neonates. V. Wijendran[1], G.Y. Diau[1], M.C. Huang[1], G. Boehm[2], G. Sawatzki[2], G. Kohn[2], P.W Nathanielsz[1], and J.T. Brenna[1], [1]Cornell University, Ithaca, NY, [2]Numico Research Group, Germany.

We measured the relative and absolute accretion of dietary arachidonic acid (AA) as a component of phosphatidylcholine (PC) or as triglyceride (TG) in a long-chain polyunsaturate (LCP)-containing formula using $^{13}C$ tracers in baboon neonates. Term neonates were delivered by Caesarean section and immediately placed on formula containing the LCP AA and docosahexaenoic acid, with an n-6/n-3 PUFA ratio of about 10:1. In formula, the fraction of AA in TG was 92% with the remaining 8% in phospholipid (PL). [U-$^{13}C$]-AA (AA*) was chemically synthesized at the *sn*-2 position in either TG or PC, with unlabeled palmitic acid in the remaining positions. TG-AA* ($n$=3) or PC-AA* ($n$=4) was given once orally at 18–19 days of age. Tissues were collected 10 days post-dose, and isotopic enrichment was measured using high-precision isotope ratio mass spectrometry. Results are shown in the Table. As a percentage of dose, AA incorporation from PL-AA was significantly greater than from TG-AA for liver and brain. However, because of the predominance of TG in formula, the total tissue AA accretion was greater from TG-AA expressed by weight. Overall, we estimate that 3.2, 2.3, and 0.002% of dietary AA was incorporated in the liver, brain, and retina, respectively. We conclude that (i) dietary AA as a component of PC is more efficiently incorporated into tissues than AA contained in TG, and (ii) in this fat blend, with a similar TG-AA/PL-AA ratio as human milk, about 5.6-fold more TG-AA appears in tissues compared to PL-AA.

| | Liver | Brain | Retina |
|---|---|---|---|
| TG-AA(%)[†] | $3.1^{a} \pm 0.7$ | $2.1^{a} \pm 0.4$ | $0.0018 \pm 0.0006$ |
| PL-AA(%)[†] | $4.8^{b} \pm 0.7$ | $4.0^{b} \pm 1.3$ | $0.0031 \pm 0.0015$ |
| TG-AA(μg AA/day)[‡] | $765^{a} \pm 181$ | $521^{a} \pm 98$ | $0.47^{a} \pm 0.07$ |
| PL-AA(μg AA/day)[‡] | $124^{b} \pm 17$ | $103^{b} \pm 36$ | $0.08^{b} \pm 0.03$ |

Mean ± SD; [†]% tracee AA in tissue; [‡]tracee AA (wt.) in tissue; [a,b]All means within cells are significantly different ($P \leq 0.05$) except for retina (%).

Key Words: Arachidonic Acid, Neonate, Triglyceride/Phospholipid

■ The Effect of DHA on Growth and Development of Newborn Rat Cerebral Neurons. J. Zhao[1] and F. Wang[2], [1]Institute of Health and Environment Medicine, [2]Institute of Basic Medical Science.

Docosahexaenoic acid (DHA) normally accounts for greater than one-third of the total fatty acids of the brain gray matter and the retina of the eye. Animal and clinical experiments study have shown that DHA is essential for functional integrity of the visual process, and there is an

impairment of learning ability in rats reared on DHA-deficient diets. We investigated the results of DHA on cellular level. Objective: To study the effects of DHA on the growth and development of cerebral neurons. Methods: Primary cultures of cerebral neurons from newborn rats make out *in vitro* by using serum-free medium (Neurobasal Medium, 1%N2, 2% B27, all were purchased from Gibco). Cells were divided into two groups, experimental and control. DHA (99% cell culture tested, Sigma) was added to the experimental group. After the neurons were cultured, the morphological states of the neurons were investigated. The positive rate of 3-(4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium-bromide (MTT), Neurone specific enolase (NSE) and protein value were measured using immunohistochemical method. Results: Both the survival of cells and the outgrowth of neurites were increased by DHA. In the presence of DHA, the diameter and volume of neurons (see Table 1) as well as the content of protein in the cells and the activity of NSE and MTT were markedly increased in image analysis. Conclusion: It is suggested that DHA might play a promotive role in the growth and development of cerebral neurons.

**TABLE 1**
**Effects of DHA on Soma Area, Body Maximum and Minimum Diameter (X ± S, *n* = 30)**

| | Control | | |
|---|---|---|---|
| Culture (d) | Soma area $(\mu m)^2$ | Maximum diameter (μm) | Minimum diameter (μm) |
| 3 | 65.4 ± 18.2 | 12.3 ± 1.4 | 6.4 ± 0.8 |
| 7 | 81.4 ± 25.4 | 13.2 ± 1.5 | 7.5 ± 1.2 |
| 14 | 111.2 ± 30.4 | 16.2 ± 1.7 | 8.1 ± 2.1 |
| 21 | 143.8 ± 29.9 | 18.8 ± 3.4 | 9.4 ± 1.8 |
| 28 | 146.3 ± 39.8 | 19.3 ± 2.9 | 10.6 ± 2.8 |
| | DHA | | |
| Culture (d) | Soma area $(\mu m)^2$ | Maximum diameter (μm) | Minimum diameter (μm) |
| 3 | 65.9 ± 16.8 | 13.1 ± 1.6 | 6.9 ± 1.9 |
| 7 | 99.2 ± 28.1b | 14.9 ± 2.4a | 8.9 ± 2.1a |
| 14 | 136.0 ± 34.1b | 19.2 ± 2.8a | 9.8 ± 1.6a |
| 21 | 196.0 ± 15.5b | 21.4 ± 3.1a | 10.9 ± 2.4a |
| 28 | 202.0 ± 29.4b | 23.2 ± 2.7b | 14.2 ± 3.3b |

$^aP<0.05$, $^bP<0.01$ compared with control

**TABLE 2**
**Effects of DHA on the Protein Synthesis, NSE, and MTT Activity**

| Group | *n* | Protein (mg/$10^6$ cell) | NSE (U/$10^6$ cell) | MTT (U/$10^6$ cell) |
|---|---|---|---|---|
| Control | 30 | 7.2 ± 2.6 | 8.4 ± 2.2 | 0.10 ± 0.03 |
| DHA | 30 | 13.8 ± 1.6b | 16.4 ± 3.4b | 0.40 ± 0.09b |

Note: same as Table 1.
NSE, neurone-specific enolase; MTT, 3-[4,5-dimethythiazol-2yl(-2,5-diphenyl tetrazolium-bromide)].

Key Words: Cerebral Neurons, DHA, Serum-Free Medium

# Mixed-Chain Phospholipids: Structures and Chain-Melting Behavior

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**ABSTRACT:** It has long been established that diacyl phospholipids isolated from animal cell membranes are predominantly of a mixed-chain variety, meaning that the *sn*-1 and *sn*-2 acyl chains are saturated and unsaturated acyl chains, respectively. In general, monoenoic and dienoic acids are found in the *sn*-2 acyl chain of phosphatidylcholine (PtdCho), whereas polyenoic acids are in phosphatidylethanolamine (PtdEth). These unsaturated chains contain only *cis*-double bonds, which are always methylene-interrupted. In recent years, the structures and the chain-melting behavior of mixed-chain PtdCho and PtdEth have been systematically studied in this laboratory. Specifically, we have examined the effects of chain unsaturation of the *sn*-2 acyl chain on the phase transition temperature ($T_m$) of many PtdCho and PtdEth by high-resolution differential scanning calorimetry (DSC). The $T_m$ values, for instance, obtained from all-unsaturated mixed-chain PtdEth derived from a common precursor can be grouped together according to their chemical formula to form a $T_m$-diagram. Hence, all the $T_m$ values can be compared simply, systematically, and simultaneously using the $T_m$-diagram. In addition, the energy-minimized structures of mixed-chain phospholipids containing different numbers/positions of methylene-interrupted *cis*-double bonds have been simulated by molecular mechanics calculations (MM). In this review, the results of our MM and DSC studies carried out with various mixed-chain phospholipids are summarized. In addition, we emphasize that the combined approach of MM and DSC yields unique information that can correlate the various $T_m$-profiles seen in the $T_m$-diagram with the structural variation of mixed-chain lipids as caused by the introduction of different numbers/positions of methylene-interrupted *cis*-double bonds.

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Diacyl phospholipids are amphipathic lipid molecules that can be found in all cell and organelle membranes. These lipid molecules serve not only as a basic structural component of cell and organelle membranes but also as the precursors in lipid-mediated signal transduction. In animal cells, membrane phospholipids are structurally an extremely diverse group of lipid molecules (Fig. 1) which includes principally phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEth), phosphatidylinositol (PtdIns), phosphatidylglycerol (PtdGro), cardiolipin ($Ptd_2Gro$), and phosphatidylserine (PtdSer). Of these various classes, PtdCho and PtdEth are quantitatively the most

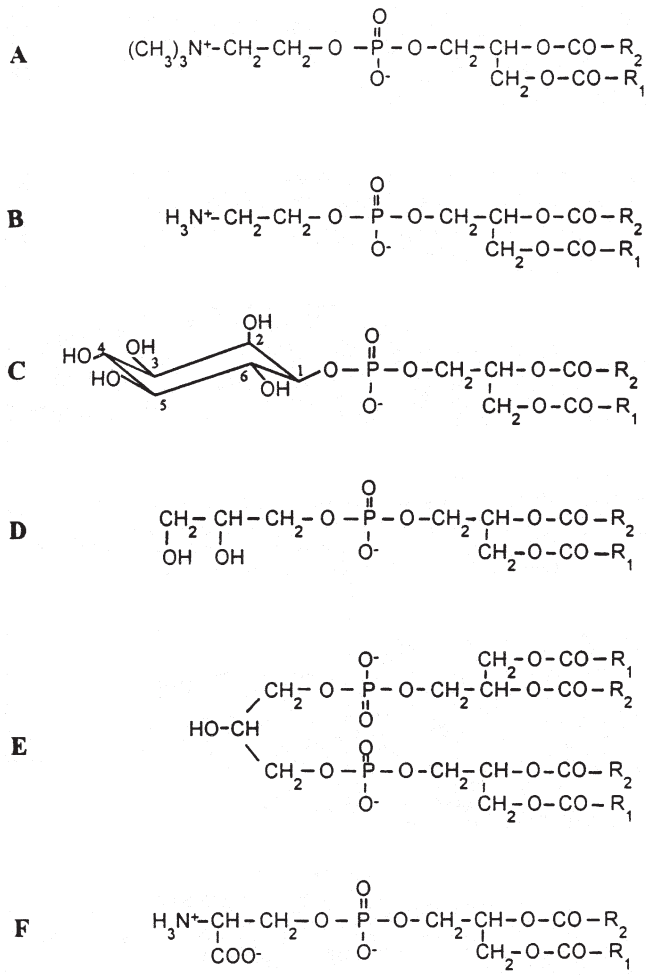


**FIG. 1.** The chemical formulas of diacyl phospholipids that are commonly found in membranes of animal cells. (A) Phosphatidylcholine (PtdCho), (B) phosphatidylethanolamine (PtdEth), (C) phosphatidylinositol (PtdIns), (D) phosphatidylglycerol (PtdGro), (E) diphosphatidylglycerol ($Ptd_2Gro$), and (F) phosphatidylserine (PtdSer). $R_1$ and $R_2$ refer to hydrocarbon chains of fatty acids esterified at carbons 1 and 2 of the glycerol backbone in diacyl phospholipids.

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Abbreviations: ATS, all-*trans* segment; ΔC, effective chain length difference, as measured in C–C bonds, between the *sn*-1 and the *sn*-2 acyl chains, an indication of chain asymmetry; $\Delta C_{ref}$, effective chain length difference of the reference state [or, effective chain length difference between the *sn*-1 and *sn*-2 acyl chains]; CL, effective chain length of the longer of the two acyl chains; DSC, differential scanning calorimetry; MM, molecular mechanics; N, distance, in C–C bond lengths, between the two carbonyl oxygens of the *sn*-1 acyl chains in a trans-bilayer dimer of C(X):C(Y)PtdCho; PtdCho, phosphatidylcholine; PtdEth, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; $Ptd_2Gro$, cardiolipin (= diphosphatidylglycerol); PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; $T_m$, main phase transition temperature.

important species. The basic structure of a given class of diacyl phospholipids has customarily been considered to consist of three regions: the polar headgroup, the interfacial region, and the hydrocarbon tail. As shown in Figure 1, the structural features that are common to all classes of phospholipids are the tetrahedrally arranged phosphorus atom in the polar headgroup, the glycerol backbone moiety in the interfacial region, and the long fatty acyl chains in the hydrocarbon tail (1).

In most animal cells, diacyl phospholipids are predominantly of a mixed-chain variety, meaning that the two acyl chains are structurally different. Specifically, the two fatty acids esterified at the *sn*-1 and *sn*-2 positions of the glycerol backbone are originated *in vivo* from saturated and unsaturated fatty acyl-CoA, respectively (2). In particular, the saturated *sn*-1 acyl chain contains an even number of carbon atoms ranging from 14 to 22, predominantly 16 and 18 carbons. The unsaturated *sn*-2 acyl chain may have 16–22 carbons; in addition, it contains 1–6 *cis*-double bonds (Δ-bonds). Interestingly, two or more Δ-bonds in the *sn*-2 acyl chains are always methylene-interrupted, indicating that two neighboring *cis*-double bonds are invariably separated from each other in the *sn*-2 acyl chain by a methylene unit (3). It should be pointed out that (i) methylene-interrupted *cis*-double bonds are the hallmark of membrane lipids originated from animal cells, and (ii) these structural elements are absent in other basic biological molecules such as protein, nucleic acid, and carbohydrate.

Over the last several decades, a wide variety of biochemical and biophysical studies have offered productive approaches for investigating the structure, dynamics, and properties of membrane phospholipids self-assembled, in excess $H_2O$, in the lipid bilayer (4,5). Most of these studies have been concerned with synthetic phospholipids that contain two identical saturated fatty acids. Occasionally, mixed-chain phospholipids containing various polyenoic fatty acids at the *sn*-2 position have been studied (6–12). These studies have been concerned exclusively with mixed-chain PtdCho. In biological membranes, however, mixed-chain PtdCho usually contain one or two Δ-bonds in the *sn*-2 acyl chains. In contrast, polyenoic fatty acids are present most abundantly in mixed-chain PtdEth. Consequently, our current knowledge about the structural and physicochemical properties of naturally occurring phospholipids, particularly mixed-chain PtdEth with saturated *sn*-1 and polyunsaturated *sn*-2 acyl chains, is limited. To approach a broader understanding of the structures/properties of naturally occurring phospholipids, we have in recent years synthesized a large number of mixed-chain phospholipids, including PtdCho, PtdEth, PtdGro, and phosphatidylethanol, that have the same structures as those of naturally occurring phospholipids (13,14). Specifically, these synthesized mixed-chain phospholipids contain the same number and position of methylene-interrupted *cis*-double bonds in the *sn*-2 acyl chain as those that are commonly found in membrane phospholipids of animal cells. Subsequently, we have carried out a systematic and comprehensive study of the structure/chain-melting behavior of the lipid bilayer comprised of the synthesized mixed-chain phospholipids. In these studies, computational molecular mechanics (MM) simulations and differential scanning calorimetry (DSC) are employed. I shall summarize some of our recent studies obtained with mixed-chain PtdCho and PtdEth in this review. These studies give unique information relating the energy-minimized structures of mixed-chain PtdCho and PtdEth to the chain-melting behavior of lipid bilayers composed of the corresponding PtdCho and PtdEth.

## THE STRUCTURE AND PHASE TRANSITION BEHAVIOR OF SATURATED C(X):C(Y)PtdCho

To examine the structure and the chain-melting behavior of the lipid bilayer composed of mixed-chain phospholipids with saturated *sn*-1 and unsaturated *sn*-2 acyl chains, let us first examine the structure and the chain-melting behavior of saturated mixed-chain phospholipids such as C(X):C(Y)PtdCho packed in the lipid bilayer. Here, the abbreviation C(X) designates the total number of carbons in the *sn*-1 acyl chain and C(Y) designates the total number of carbons in the *sn*-2 acyl chain. Both the *sn*-1 and *sn*-2 acyl chains contain only saturated hydrocarbon chains.

Figure 2 shows the energy-minimized structure of C(16):C(16)-PtdCho obtained with MM calculations using Allinger's MM3(92) program (15). The atomic coordinates used as the initial input for MM calculations were derived from experimental data obtained by x-ray diffraction (16). Two conformational features of the acyl chains are revealed by the energy-minimized structure. First, the fully extended *sn*-1 and *sn*-2 acyl chains are aligned in the same direction; however, the zigzag planes of the *sn*-1 and *sn*-2 acyl chains are oriented perpendicularly to each other. Second, although the *sn*-1 and *sn*-2 acyl chains have the same total number of methylene units, there is an effective chain length difference between them. This is due largely to the fact that the *sn*-2 acyl chain is bent 90° at C(2). It should be pointed out that the energy-minimized structure corresponds to the structure of lipid packed in the crystalline state. In the gel-state bilayer, the *sn*-2 acyl chain of C(16):C(16)PtdCho is still bent; however, the effective chain length difference between the *sn*-1 and the *sn*-2 acyl chains is smaller, and, is about 1.5 C–C bond lengths according to neutron diffraction measurements (17). This value can be applied to all saturated identical-chain PtdCho packed in the gel-state bilayer, and we designate it as $\Delta C_{ref}$, the effective chain length difference of the reference state, as shown in Figure 2. The effective chain length of the longer of the two acyl chains (CL) is also introduced in Figure 2. In the case of C(16):C(16)PtdCho packed in the gel-state bilayer, the value of CL is 15 C–C bond lengths, which corresponds to the effective chain length of the *sn*-1 acyl chain.

For saturated mixed-chain C(X):C(Y)PtdCho packed in the gel-state bilayer, three structural parameters are graphically illustrated in Figures 3A and 3B (13). ΔC is the effective chain length difference, in C–C bond lengths, between the *sn*-1 and the *sn*-2 acyl chains. It represents the chain asymmetry. The larger the ΔC value, the greater the asymmetry of the lipid's

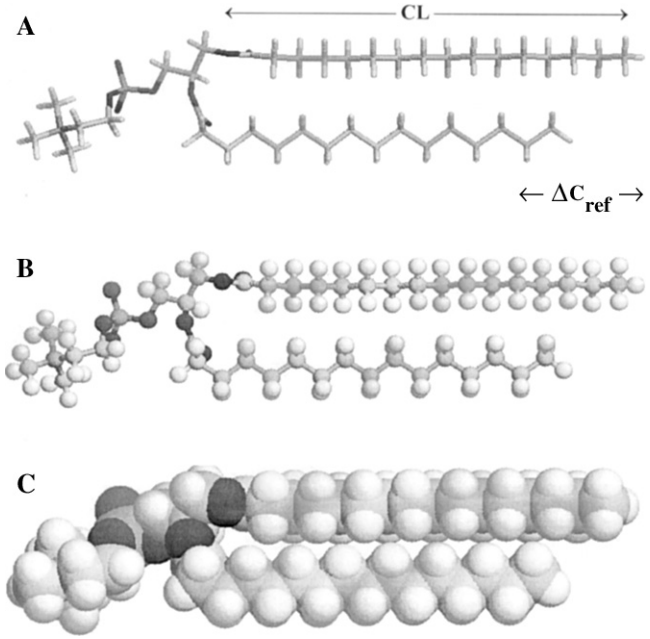


**FIG. 2.** The energy-minimized structure of C(16):C(16)PtdCho obtained by molecular mechanics simulations. This structure corresponds to C(16):C(16)PtdCho packed in the crystalline bilayer. The structural parameter $\Delta C_{ref}$ refers to the effective chain length difference between the *sn*-1 and the *sn*-2 acyl chains, which is about 1.5 C–C bond length for C(16):C(16)PtdCho packed in the gel-state bilayer. This value can, in fact, be applied to all saturated identical-chain PtdCho packed in the gel-state bilayer; hence, $\Delta C_{ref}$ is the effective chain length difference of the reference state. A second structural parameter, CL, refers to the effective chain length of the longer of the two acyl chains. In the case of C(16):C(16)PtdCho packed in the gel-state bilayer, the chain length difference between the two acyl chains, $\Delta C_{ref}$, is 1.5 C–C bond lengths, and the length of the longer chain, CL = X – 1, is 15 C–C bond lengths, or 19 Å, which corresponds to the effective chain length of the *sn*-1 acyl chain. The normalized chain length difference, $\Delta C_{ref}$/CL, is 0.10.

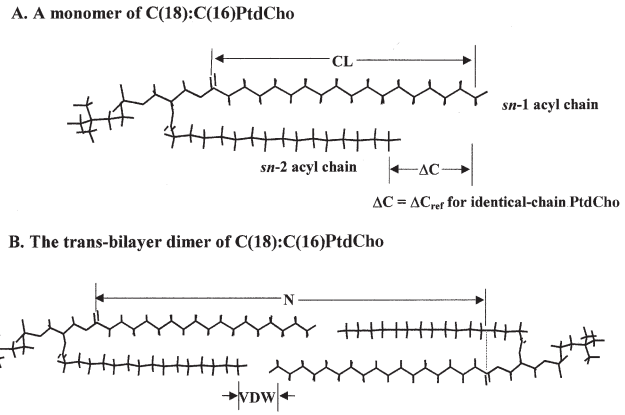


**FIG. 3.** A molecular-graphically depicted diagram illustrating the various structural parameters (ΔC, CL, and N) of C(18):C(16)PtdCho. (A) A monomer of C(18):C(16)PtdCho. ΔC is the effective chain length difference between the two acyl chains along the long molecular axis. The units for ΔC are carbon-carbon bond lengths. $\Delta C_{ref}$ is the ΔC value for identical-chain PtdCho packed in the gel-state bilayer, and $\Delta C_{ref}$ is taken to be 1.5 C–C bond lengths. For C(X):C(Y)PtdCho packed in the gel-state bilayer, $\Delta C = |X - Y + \Delta C_{ref}| = |X - Y + 1.5|$. In the case of C(18):C(16)PtdCho, the value of ΔC is 3.5 C–C bond lengths. CL is the effective length of the longer chain of the two acyl chains, also in units of C–C bond lengths. In the case of C(18):C(16)PtdCho, the value of CL is 17. (B) The trans-bilayer dimer of C(18):C(16)PtdCho with a partially interdigitated packing motif at $T < T_m$, where T is the experimental temperature and $T_m$ is the main phase transition temperature. N is the effective hydrophobic thickness of the dimer, corresponding to the separation distance between the two carbonyl oxygens of the *sn*-1 acyl chains in the two opposing leaflets of the bilayer. VDW is the van der Waals contact distance between the two opposing methyl termini in the bilayer interior, and is assumed to be 3 C–C bond lengths in the gel-state bilayer. The structural parameter N is related to X and Y in C(X):C(Y)PtdCho as follows: N = (X – 1) + VDW + (Y – 2.5) = X + Y – 0.5. For the gel-state bilayer of C(18):C(16)PtdCho, the value of N is 33.5 C–C bond lengths.

acyl chains. The second structural parameter, CL, has the same meaning as that illustrated for C(16):C(16)PtdCho shown in Figure 2. The distance N, in C–C bond lengths, between the two carbonyl oxygens of the *sn*-1 acyl chains in a trans-bilayer dimer of C(X):C(Y)PtdCho is introduced in Figure 3B. N represents the effective hydrocarbon-core thickness of the lipid bilayer composed of C(X):C(Y)PtdCho. These three structural parameters, in C–C bond lengths, are related to X and Y in C(X):C(Y)PtdCho as follows (13):

$$\Delta C = |X - Y + \Delta C_{ref}| \quad [1]$$

$$CL = (X - 1) \quad [2]$$

$$N = X + Y - 0.5 \quad [3]$$

The crystalline structure of C(X):C(Y)PtdCho, as represented by C(18):C(16)PtdCho, is drawn in Figure 3 for the purpose of simplicity. All three structural parameters are actually defined for C(X):C(Y)PtdCho packed in the gel-state bilayer with a partially interdigitated packing motif. In this packing motif, the methyl terminus of the *sn*-1 acyl chain of one lipid molecule in the bilayer is juxtaposed with the methyl end of the *sn*-2 acyl chain of another lipid molecule from the opposing bilayer leaflet as shown diagrammatically in Figure 3B. It is well established that C(X):C(Y)PtdCho with ΔC/CL < 0.42 can, in excess water, self-assemble into the partially interdigitated bilayer at $T < T_m$ where T = experimental temperature and $T_m$ = main phase transition temperature (18).

Figure 4 shows some representative DSC curves obtained with aqueous lipid dispersions prepared from a homologous series of mixed-chain C(X):C(Y)PtdCho. These mixed-chain lipids share a common value of (X + Y), which corresponds to 32 carbons (19). Hence, the N value for the gel-state bilayer prepared from each lipid species of this lipid series is identical. However, the ΔC value obtained with each lipid in this series increases stepwise by 1.0 C–C bond length as the lipid species changes progressively from C(15):C(17)PtdCho to C(10):C(22)PtdCho. Figure 4 clearly shows that the $T_m$ exhibited by C(X):C(Y)PtdCho with ΔC/CL < 0.42 decreases steadily as the value of ΔC increases. This figure demonstrates that, for a series of C(X):C(Y)PtdCho packed in the partially interdigitated bilayer, $T_m$ decreases with increasing ΔC value when N is held constant. In addition, for a homologous series of saturated identical-chain PtdCho (ΔC/CL < 0.42), ranging from C(13):C(13)PtdCho to C(21):C(21)PtdCho, with a common ΔC

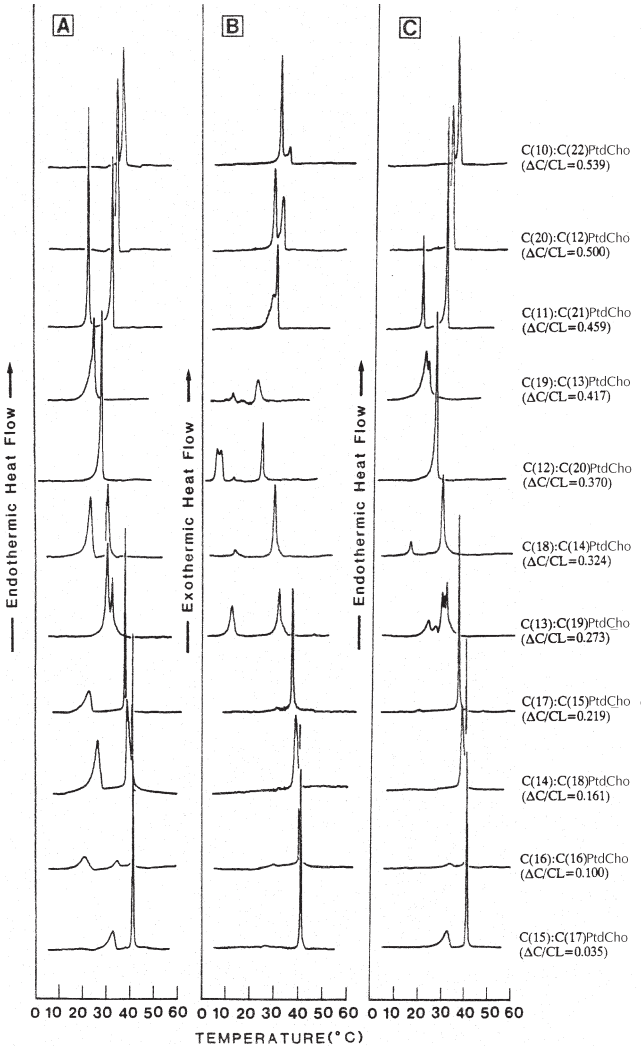


**FIG. 4.** The differential scanning calorimetry (DSC) curves obtained with aqueous lipid dispersions prepared from a homologous series of mixed-chain C(X):C(Y)PtdCho. These mixed-chain lipids share a common value of (X + Y), which corresponds to 32 carbons. The ΔC/CL represents the normalized chain length difference. (A) The initial heating scans, (B) the first cooling scans, and (C) the second heating scans. The DSC scans were obtained from a Microcal MC-2 microcalorimeter (Microcal, Inc., Northampton, MA). Scan rate: 15°C/h. Prior to DSC experiments, each lipid sample was incubated at 0°C for a minimum of 24 h. For abbreviations see Figures 1 and 3.

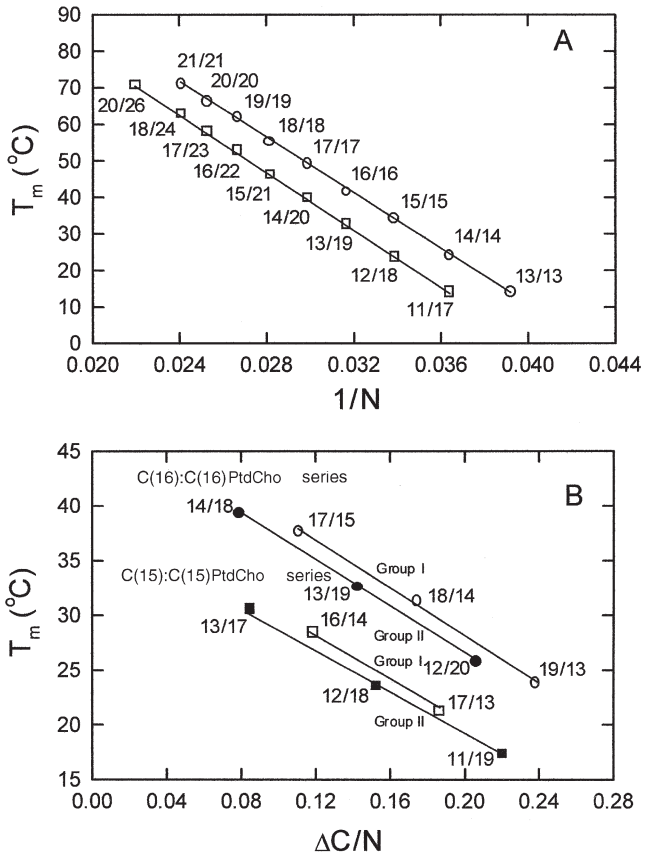


**FIG. 5.** Plots of the phase transition temperature, $T_m$, vs. the structural parameter(s). (A) $T_m$ values from two series of saturated PtdCho with ΔC = 1.5 and 4.5, respectively, are plotted against 1/N. The numerical numbers of X/X or X/Y next to the data points denote the numbers of carbons in the *sn*-1/*sn*-2 acyl chains of the corresponding phosphatidylcholine species. (B) $T_m$ values of lipids with identical N values vs. ΔC/N. The data (○,●) connected by the top two lines were obtained with saturated PtdCho having a constant N value of 31.5 C–C bond lengths. These lipids thus belong to the C(16):C(16)PtdCho series. The data (□,■) in the bottom two lines were obtained with saturated PtdCho having a constant N value of 29.5 C–C bond lengths; hence, these lipids belong to the C(15):C(15)PtdCho series. Within each series, lipids with an effective longer *sn*-1 acyl chain length (X ≥ Y – 1.5) are grouped into Group I lipids, and those with longer effective *sn*-2 acyl chain (Y – 1.5 > X) are classified as Group II lipids.

value of 1.5 C–C bond lengths, the $T_m$ value exhibited by the lipid dispersion prepared individually from them decreases as the value of 1/N increases (Fig. 5). Moreover, the same trend is observed in Figure 5 for another homologous series of mixed-chain C(X):C(X + 6)PtdCho (ΔC/CL < 0.42) with a common ΔC value of 4.5 C–C bond lengths (20). Based on DSC data presented in Figure 5 and elsewhere, the $T_m$ value of C(X):C(Y)-PtdCho (ΔC/CL < 0.42) with X ≥ Y can be related to the structural parameters ΔC and N in a simple first-order manner (13):

$$T_m = 161.75 - 3706.06\,(1/N) - 278.75\,(\Delta C/N) + 239.94\,[\Delta C/(N + \Delta C)] \quad [4]$$

A similar equation (Eq. 5) is also derived for C(X):C(Y)-PtdCho with X < Y (13):

$$T_m = 155.11 - 3534.31(1/N) - 245.78(\Delta C/N) + 199.04\,[\Delta C/(N + \Delta C)] \quad [5]$$

These two equations not only show the fundamental antagonistic effect between N and ΔC in determining the $T_m$ value but also can be employed to predict the $T_m$ value for C(X):C(Y)PtdCho with ΔC/CL < 0.42. The prediction of $T_m$ value based on the chemical formula of C(X):C(Y)PtdCho is possible because the N and ΔC values can be readily calculated from X and Y (Eqs. 1–3). Predicted $T_m$ values ($n$ = 207) for various C(X):C(Y)PtdCho, obtained on the basis of Equations 4 and 5, are presented in Table 1, along with 54 $T_m$ values observed calorimetrically. Clearly, the predicted and the observed $T_m$ values agree well. An important consequence of this excellent agreement is that the number of carbon atoms in the *sn*-1 (or *sn*-2) acyl chain, X (or Y), can be determined

**TABLE 1**
**Predicted $T_m$ Values for Fully Hydrated Samples of Saturated Diacyl C(X):C(Y)PtdCho with Varying Degrees of Acyl Chain Length Asymmetry[a]**

| Total carbons in the *sn*-1 acyl chain | Total carbons in the *sn*-2 acyl chain | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
| 26 | | | | | | | | | | | | 68.3 | 72.5 | 76.4 | 79.8 | 83.0 | 85.9 | 88.5 |
| 25 | | | | | | | | | | | 63.8 | 68.3 | 72.5 | 76.2 | 79.7 | 82.7 | 85.5 | 88.0 |
| 24 | | | | | | | | | | 58.8 | 63.7 | 68.2 | 72.3 | 76.0 | 79.3 | 82.3 | 84.9 | 83.2 |
| 23 | | | | | | | | | 53.3 | 58.7 | 63.6 | 68.0 | 72.0 | 75.6 | 78.8 | 81.6 | 80.2 | 80.6 |
| 22 | | | | | | | | 47.2 | 53.1 | 58.4 | 63.3 | 67.6 | 71.5 | 74.9 | 78.0 | 76.9 | 77.4 | 77.7 |
| | | | | | | | | | | | | | | ***74.8*** | | | | ***77.8*** |
| 21 | | | | | | | 40.3 | 46.8 | 52.7 | 58.0 | 62.8 | 67.0 | 70.7 | 74.1 | 73.3 | 73.9 | 74.3 | 74.5 |
| | | | | | | | | | | | | | ***71.1*** | | | | | |
| 20 | | | | | | 32.6 | 39.9 | 46.4 | 52.2 | 57.4 | 62.0 | 66.1 | 69.7 | 69.3 | 70.1 | 70.5 | 70.9 | 71.0 |
| | | | | | | ***33.2*** | | | | 56.8 | | ***66.4*** | | | | | | ***70.7*** |
| 19 | | | | | | 32.0 | 39.2 | 45.7 | 51.4 | 56.5 | 61.0 | 64.9 | 65.0 | 65.8 | 66.4 | 66.8 | 67.1 | 67.2 |
| | | | | | | ***31.8*** | ***39.0*** | | | | ***61.8*** | | | | | | | |
| 18 | | | | | 23.0 | 31.1 | 38.3 | 44.7 | 50.3 | 55.3 | 59.6 | 60.2 | 61.2 | 61.9 | 62.4 | 62.8 | 62.9 | 63.0 |
| | | | | | | ***31.2*** | ***38.1*** | ***44.4*** | ***50.4*** | ***55.3*** | | ***59.7*** | | ***62.2*** | | ***62.7*** | | ***63.9*** |
| 17 | | | | | 21.9 | 30.0 | 37.1 | 43.4 | 48.9 | 53.7 | 54.8 | 56.0 | 56.9 | 57/5 | 58.0 | 58.3 | 58.4 | 58.4 |
| | | | | | ***21.2*** | | ***37.7*** | ***43.2*** | ***49.0*** | | | | | | ***57.9*** | | | |
| 16 | | | | 11.2 | 20.4 | 28.5 | 35.6 | 41.7 | 47.1 | 48.9 | 50.3 | 51.3 | 52.1 | 52.7 | 53.1 | 53.3 | 53.4 | 53.4 |
| | | | | ***11.3*** | | ***28.4*** | | ***41.4*** | ***46.2*** | ***48.8*** | | | | ***52.8*** | | ***53.2*** | | ***53.3*** |
| 15 | | | –1.1 | 9.4 | 18.6 | 26.6 | 33.6 | 39.5 | 42.1 | 43.8 | 45.1 | 46.1 | 46.8 | 47.3 | 47.7 | 47.9 | 48.0 | |
| | | | | | ***18.8*** | | ***34.0*** | | ***41.7*** | | ***44.8*** | | ***46.1*** | | | | | |
| 14 | | –15.7 | –3.5 | 7.1 | 16.3 | 24.2 | 31.0 | 34.5 | 36.5 | 38.0 | 39.2 | 40.2 | 40.8 | 41.3 | 41.7 | 41.9 | | |
| | | | | | | ***24.1*** | ***30.7*** | ***34.9*** | | ***39.2*** | | ***39.8*** | | | | ***43.3*** | | |
| 13 | | –18.7 | –6.4 | 4.3 | 13.3 | 21.1 | 25.7 | 28.1 | 30.0 | 31.5 | 32.7 | 33.6 | 34.3 | 34.8 | | | | |
| | | | | | ***13.9*** | | ***25.8*** | | ***30.5*** | | ***32.6*** | ***33.1*** | ***34.1*** | | | | | |
| 12 | | –22.4 | –9.9 | 0.7 | 9.6 | 15.5 | 18.5 | 20.8 | 22.7 | 24.2 | 25.3 | 26.2 | | | | | | |
| | | | | | | | | ***21.7*** | | ***23.5*** | | ***25.6*** | | | | | | |
| 11 | –41.8 | –26.9 | –14.4 | –3.9 | 3.6 | 7.3 | 10.2 | 12.5 | 14.4 | 15.9 | 17.1 | | | | | | | |
| | | | | | | | | | ***13.9*** | | ***17.3*** | | | | | | | |
| 10 | –47.7 | –32.6 | –20.1 | –10.5 | –5.9 | –2.3 | 0.6 | 3.0 | 4.9 | | | | | | | | | |
| 9 | –55.0 | –39.8 | –27.5 | –21.7 | –17.1 | –13.4 | –10.4 | | | | | | | | | | | |

[a]Experimental $T_m$ values are also indicated in boldface italic. Predicted $T_m$ values are calculated in part from Equation 4 (see text) and the relationships between ΔC, N, X, and Y as indicated in Figure 3. Abbreviations: PtdCho, phosphatidylcholine; $T_m$, main phase transition temperature; ΔC, effective chain length difference as measured in C–C bonds; N, distance, in C–C bond lengths, between the two carbonyl oxygens of the *sn*-1 acyl chains in a trans-bilayer dimer of C(X):C(Y)phosphatidylcholine; X, total carbons in the *sn*-1 acyl chain; Y, total carbons in the *sn*-2 acyl chain.

accurately provided that the $T_m$ value and the number of carbon atoms in the *sn*-2 (or *sn*-1) acyl chain, Y (or X), of C(X):C(Y)PtdCho are known. This important relationship will be reiterated later when I discuss the structure/chain-melting behavior of mixed-chain lipids with saturated *sn*-1 and unsaturated *sn*-2 acyl chains.

## THE STRUCTURES AND THE PHASE TRANSITION BEHAVIOR OF PtdCho AND PtdEth WITH SATURATED *sn*-1 AND MONOUNSATURATED *sn*-2 ACYL CHAINS [C(X):C(Y:1$\Delta^n$)PtdCho AND C(X):C(Y:1$\Delta^n$)PtdEth]

The six atoms in the immediate vicinity of a *cis*-double bond, C–CH=CH–C, in a long hydrocarbon chain are coplanar (Fig. 6). Although the rotational flexibility of the *cis*-double bond is highly restricted at physiological temperatures, paradoxically the carbon-carbon single bond preceding or succeeding the *cis*-double bond is rotationally highly flexible, even at –10°C (18). Results of our MM calculations indicate that the carbon-carbon single bond next to the *cis*-double bond prefers to adopt the *skew* ($s^{\pm}$) conformation with torsion angle of about ±110° (Fig. 6). As a result, the most stable structure of a hydrocarbon chain containing a single *cis*-double bond has a twisted boomerang-like conformation with a $s^{\pm}\Delta s^{\pm}$ sequence around the Δ-bond (Fig. 7). Such a twisted boomerang-like conformation, however, is unlikely to be adopted by the *sn*-2 acyl chain in the gel-state bilayer composed of monounsaturated mixed-chain lipids because the all-*trans sn*-1 acyl chain would impose steric constraints on the boomerang-like conformation of the neighboring *sn*-2 acyl chain. Results from our detailed MM calculations indicate that in the presence of an all-*trans sn*-1 acyl chain, the *sn*-2 acyl chain of the monoenoic PtdCho is able to pack favorably in the gel-state bilayer with a kinked crankshaft-like conformation (18,21). Specifically, two parallel chain segments separated by a small kink sequence are observed in this kinked *sn*-2 acyl chain; moreover, these two parallel segments are in close van der Waals contact with the all-*trans sn*-1 acyl chain.

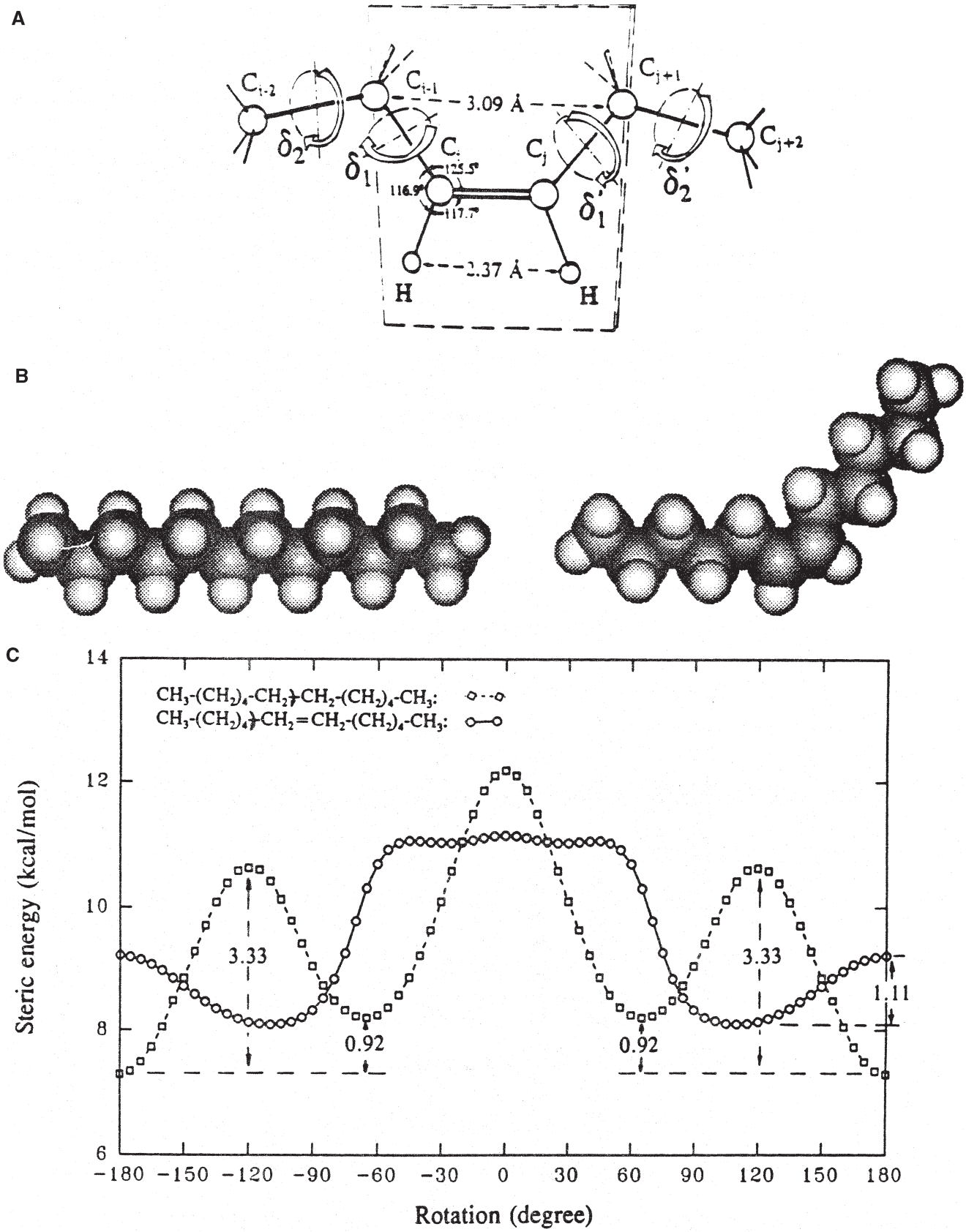


**FIG. 6.** Structure of a *cis*-double bond in a segment of hydrocarbon chain is shown in the top (A). The introduction of a *cis*-double bond into an all-*trans* hydrocarbon chain transforms the linear conformation into a boomerang-like conformation (B). In (C) the energy profiles arising from rotations of a single C(6)-C(7) bond in dodecane (□) and of a single C(4)-C(5) bond in dodecene-6 (○) are shown. Molecular mechanics (MM) calculations were performed with the MM2(85) program (15).

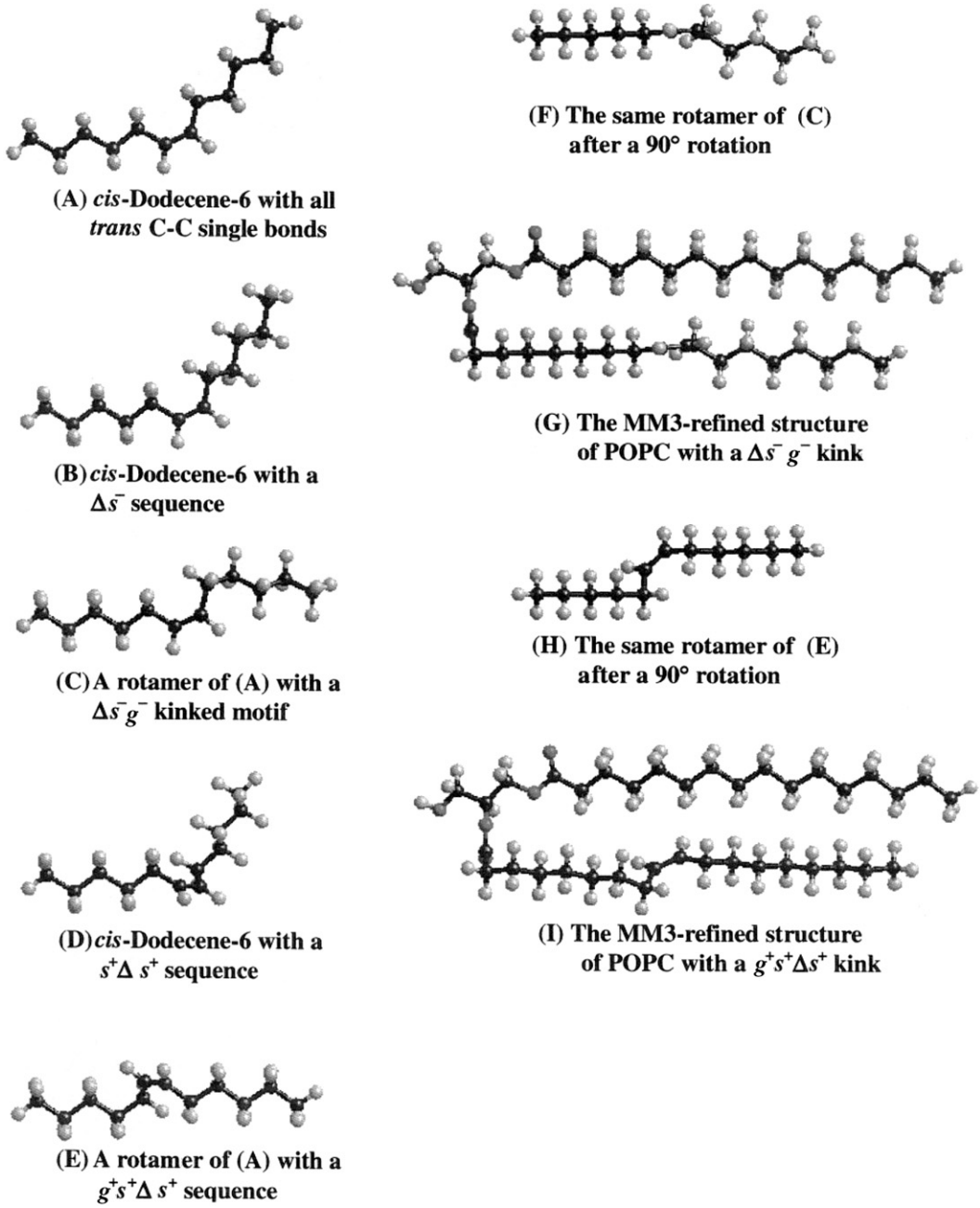


**FIG. 7.** Computer-graphic representations of the hydrocarbon chain with boomerang-like, twisted boomerang-like, and kinked crankshaft-like motifs. Two kinked conformations with identical low-energy for C(16):C(18:1$\Delta^9$)PtdCho as obtained by MM calculations are also shown. POPC, 1-palmitoyl-2-oleoyl-PtdCho; for other abbreviations see Figures 1 and 6.

As a result, such a kinked motif has a low steric energy. As illustrated in Figure 7, a monoenoic hydrocarbon chain with kinked crankshaft-like conformation can be readily obtained by the rotational *trans* → *gauche* isomerizations of the C–C single bond next to the twisted boomerang sequence of $s^{\pm}\Delta s^{\pm}$. However, the overall length of the kinked chain is shortened by 0.8 C–C bond length relative to its saturated all-*trans* counterpart. Two of the kinked crankshaft-like lipid structures obtained with C(16):C(18:1$\Delta^9$)PtdCho, 1-palmitoyl-2-oleoyl-phosphatidylcholine, are also illustrated in Figure 7. Here, the abbreviation C(18:1$\Delta^9$) denotes the *sn*-2 acyl chain with a single *cis*-double bond at the ninth carbon ($\Delta^9$) counting from the carbonyl end. In fact, we have shown by MM calculations that 16 kinked crankshaft-like conformations with a similar low steric-energy may exist normally for C(16):C(18:1$\Delta^9$)-PtdCho at $T < T_m$ (18). Our MM calculations thus indicate that many possible low-energy rotational isomers (rotamers) may be adopted by monounsaturated PtdCho, and these rotamers are likely to interconvert rapidly in the bilayer at $T < T_m$. In addition, these low-energy rotamers share a common structural feature characterized by a kinked crankshaft-like motif.

Examining some representative DSC curves exhibited by lipid bilayers composed of monounsaturated phospholipids demonstrates an interesting point (21,22). In Figure 8, the second DSC heating curves obtained with aqueous dispersions of C(20):C(18)PtdCho, C(20):C(18)PtdEth, C(20):C(18:1$\Delta^9$)-PtdCho, and C(20):C(18:1$\Delta^9$)PtdEth in the presence of 50 mM NaCl, 5 mM phosphate buffer (pH 7.4), and 1 mM EDTA are illustrated (13). A sharp, single endothermic transition with $T_m$ = 75.8°C is observed for C(20):C(18)PtdEth. The introduction of a single $\Delta^9$-bond into the *sn*-2 acyl chain reduces the $T_m$ value of C(20):C(18:1$\Delta^9$)PtdEth to 33.9°C. As shown in Figure 8, this $T_m$-lowering effect of acyl chain monounsaturation is also observed for mixed-chain PtdCho. In particular, the $T_m$ values exhibited by C(20):C(18)PtdCho and C(20):C(18:1$\Delta^9$)PtdCho bilayers are 56.8 and 11.0°C, respectively, showing a marked reduction of 45.8°C in $T_m$ as a single $\Delta^9$-bond is introduced into the *sn*-2 acyl chain of C(20):C(18)PtdCho. Such a $T_m$-lowering effect of acyl chain monounsaturation has been previously observed for PtdCho by other investigators (8,12) but not for PtdEth.

Another distinct characteristic associated with the acyl chain monounsaturation is the $T_m$-lowering effect of acyl chain unsaturation, which is critically dependent on the position of the single *cis*-double bond along the *sn*-2 acyl chain (21). The second DSC heating curves for a series of C(18):C(18:1$\Delta^n$)PtdCho with $n$ = 6, 7, 9, 11, 12, and 13 demonstrate this point (Fig. 9). The minimal $T_m$ is observed when $n$ = 11 or when the *cis*-double bond is positioned near the center of the linear segment of the *sn*-2 acyl chain. Further, the $T_m$ value increases progressively as the *cis*-double bond moves away from $\Delta^{11}$ toward either end of the acyl chain. Specifically, the $T_m$ values of C(18):C(18:1$\Delta^n$)PtdCho with $n$ = 6, 7, 9, 11, 12, and 13 are 24.8, 16.7, 5.6, 3.8, 9.1, and 15.9°C, respectively. As a result, when $T_m$ is plotted against the position of the single *cis*-double bond, a V-shaped $T_m$-profile is seen for the various positional isomers of C(18):C(18:1$\Delta^n$)PtdCho. The second DSC heating curves for C(20):C(20:1$\Delta^n$)PtdCho with $n$ = 5, 8, 11, and 13 are also presented in Figure 9 (21). These data have been extended recently (23). The $T_m$ values of lipid bilayers prepared from C(20):C(20:1$\Delta^n$)PtdCho with $n$ = 5, 8, 11, 13, and 17 are 44.9, 30.6, 19.4, 22.1, and 49.7°C, respectively. Again, a roughly V-shaped $T_m$-profile is observed in the plot of $T_m$ vs. the position of the *cis*-double bond in the *sn*-2 acyl chain for this series of C(20):C(20:1$\Delta^n$)PtdCho (23). Similarly, positional isomers of C(18:1$\Delta^n$):C(18:1$\Delta^n$)PtdCho also display a V-shaped $T_m$-profile in which a *cis*-double bond is present in each of the two acyl chains at the same position (6).

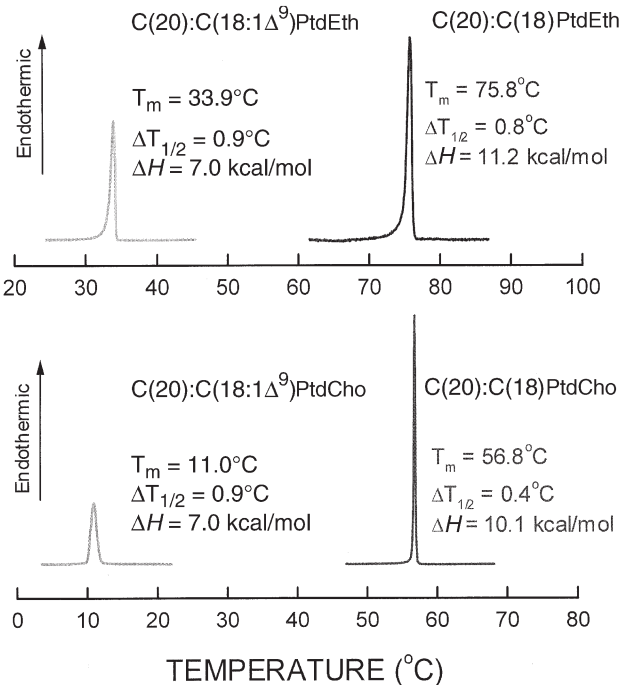


**FIG. 8.** The second DSC heating curves obtained with aqueous dispersions of C(20):C(18)PtdCho, C(20):C(18)PtdEth, C(20):C(18:1$\Delta^9$)PtdCho, and C(20):C(18:1$\Delta^9$)PtdEth. $\Delta T_{1/2}$ represents width of phase transition curve at half-peak height. Scan rate: 15°C/h. Lipid concentration: 3–5 mM. For abbreviations see Figure 1.

Before a molecular model that explains the effects of chain monounsaturation on phospholipids phase transition behavior is presented, let us first examine the energy-minimized structures of some saturated and monounsaturated phospholipids obtained with MM calculations (Figs. 10 and 11). First, compare the monomeric structures of C(18):C(18)PtdCho, C(18):C(17)PtdCho, and C(18):C(18:1$\Delta^{11}$)PtdCho. Clearly, the two acyl chains are more symmetrical, in terms of chain lengths, for C(18):C(18)PtdCho. Quantitatively, the chain-asymmetry parameter, $\Delta C$, is nearly the same for C(18):C(17)PtdCho and C(18):C(18:1$\Delta^{11}$)PtdCho. Based on the relationship between $\Delta C$ and $T_m$ that is known for saturated mixed-chain PtdCho, one would expect the values of $T_m$ exhibited by C(18):C(17)PtdCho and C(18):C(18:1$\Delta^{11}$)-PtdCho to be nearly the same. Yet, the $T_m$ value observed calorimetrically for C(18):C(18:1$\Delta^{11}$)PtdCho is 46.6°C smaller than for C(18):C(17)PtdCho. In comparing the *trans*-dimeric structures of C(18):C(17)PtdCho and C(18):C(18:1$\Delta^{11}$)-PtdCho, the overall *trans*-bilayer thickness is almost indistinguishable, with the structural parameter N being 34.5 and 34.7 C–C bond lengths, respectively. Again, based on the relationship between N and $T_m$ that is known for saturated mixed-chain PtdCho, we would expect a nearly identical value of $T_m$ for these two lipid species. This expectation, however, is not borne out by experimental data as shown in Figure 11. The phase transition behavior observed for unsaturated lipids is thus distinctively different from that of saturated lipids. Therefore, prediction of the chain-melting behavior of unsaturated lipids based solely on the chain-melting behavior of the corresponding saturated lipids cannot be made reliably.

Earlier, it was shown that the $T_m$ value of saturated mixed-chain C(X):C(Y)PtdCho can be quantitatively related to X and Y values (Table 1). Similarly, the value of Y can also be calculated provided that $T_m$ and X are known. The values of $T_m$ and X for C(18):C(18:1$\Delta^{11}$)PtdCho are 3.8°C and 18, respectively. A corresponding saturated mixed-chain PtdCho with a partially interdigitated packing motif can thus be calculated; this fictive

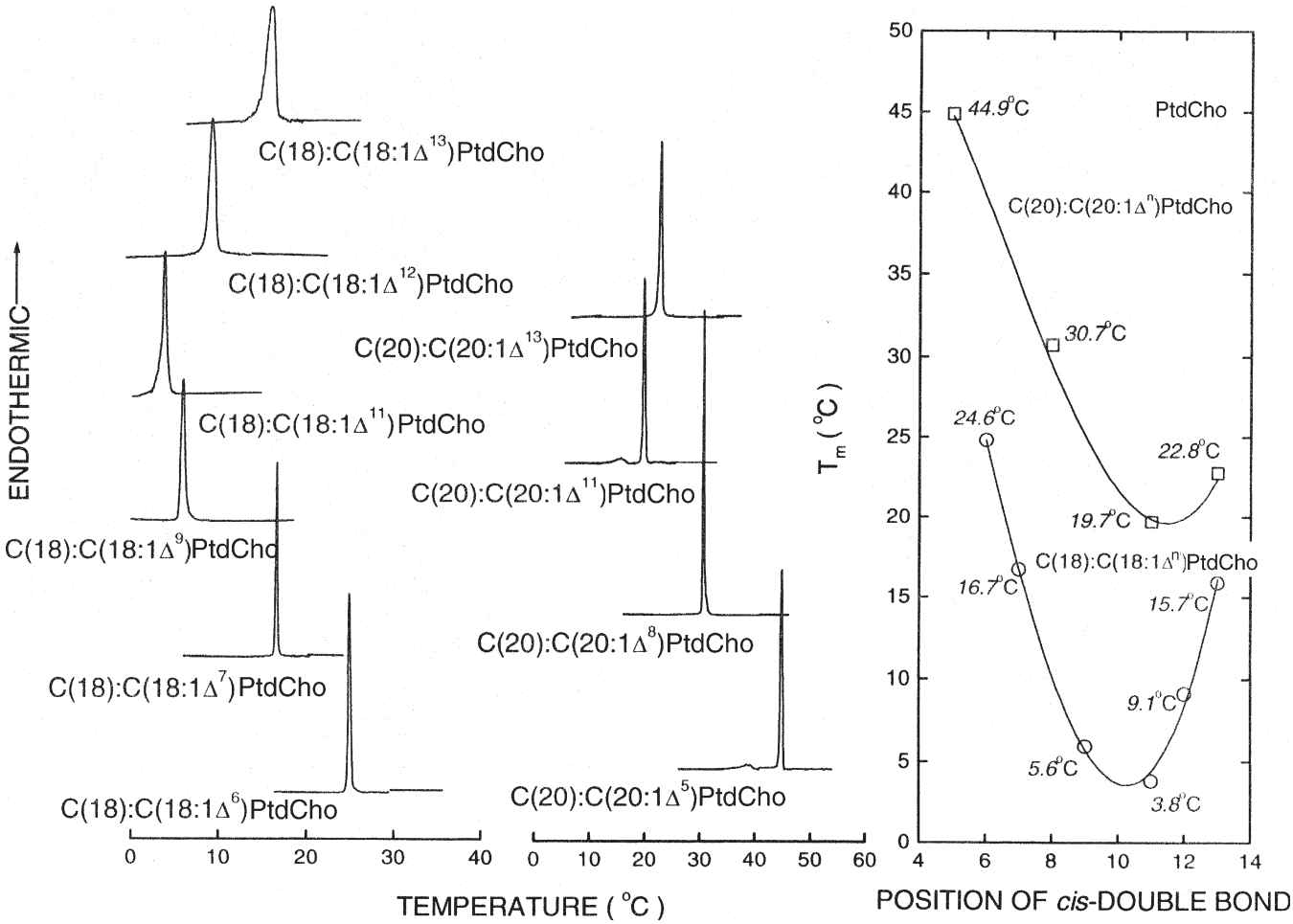


**FIG. 9.** The $T_m$-lowering effect of acyl chain monounsaturation as a function of the position of the single *cis*-double bonds. For abbreviation see Figure 3.

lipid molecule with $T_m$ = 3.8°C is calculated to be C(18):C(11)-PtdCho as illustrated in Figure 12. By comparing the energy-minimized structure of the fictive C(18):C(11)PtdCho with that of C(18):C(18:1$\Delta^{11}$)PtdCho, one can immediately see that the entire length of the *sn*-2 acyl chain of the fictive molecule is virtually identical to the length of the longer upper chain-segment of the kinked *sn*-2 acyl chain in C(18):C(18:1$\Delta^{11}$)PtdCho. At this point, it should be mentioned that the gel-to-liquid crystalline phase transition exhibited by the lipid bilayer at $T_m$ involves fundamentally the rotational *trans* → *gauche* isomerizations of the C–C single bonds in the lipid's acyl chains (24). Based on the energy-minimized structures of the fictive C(18):C(11)PtdCho and C(18):C(18:1$\Delta^{11}$)PtdCho shown in Figure 12, we have proposed a molecular model for monounsaturated phospholipids with the following assumptions (13,21): (i) Monounsaturated lipids are assumed to adopt the kinked crankshaft-like motif at $T < T_m$; hence, the kinked *sn*-2 acyl chain consists of a longer chain-segment and a shorter chain-segment at $T < T_m$. (ii) The short chain-segment is assumed to be largely disordered in the gel-state bilayer due to the presence of appreciable *gauche* bonds at $T \leq T_m$. As a result, this short chain-segment does not contribute significantly to the rotationally disordering process of the *trans* → *gauche* isomerizations of the C–C bonds at $T_m$ (3). (iii) The longer chain-segment in the *sn*-2 acyl chain, at $T < T_m$, is assumed to be composed of *trans* rotamers only and to undergo favorable van der Waals contact interactions with its neighboring all-*trans sn*-1 acyl chains in the gel-state bilayer. Hence, the longer chain-segment in the kinked *sn*-2 acyl chain contributes significantly to the thermally induced chain-melting process at $T_m$.

With the molecular model described above, the drastic $T_m$-lowering effect of acyl chain monounsaturation can be interpreted. Because the short chain-segment is already disordered at $T < T_m$, the total number of *trans* → *gauche* isomerizations of the C–C bonds at $T_m$ is reduced appreciably for a monounsaturated lipid molecule relative to that of the saturated counterpart, leading to a significantly lower $T_m$ value. We can apply the molecular model to predict the V-shaped $T_m$ profile in the plot of $T_m$ vs. the position of the single *cis*-double bond in the *sn*-2 acyl chain. In this molecular model, the longer chain-segment of the kinked *sn*-2 acyl chain is assumed to undergo favorable van der Waals interactions with its neighboring all-*trans sn*-1 acyl chains. The magnitude of this contact interaction depends on the length of the longer chain-segment. When the Δ-bond is positioned at the center of the *sn*-2 acyl chain, the longer chain-segment has a minimal length,

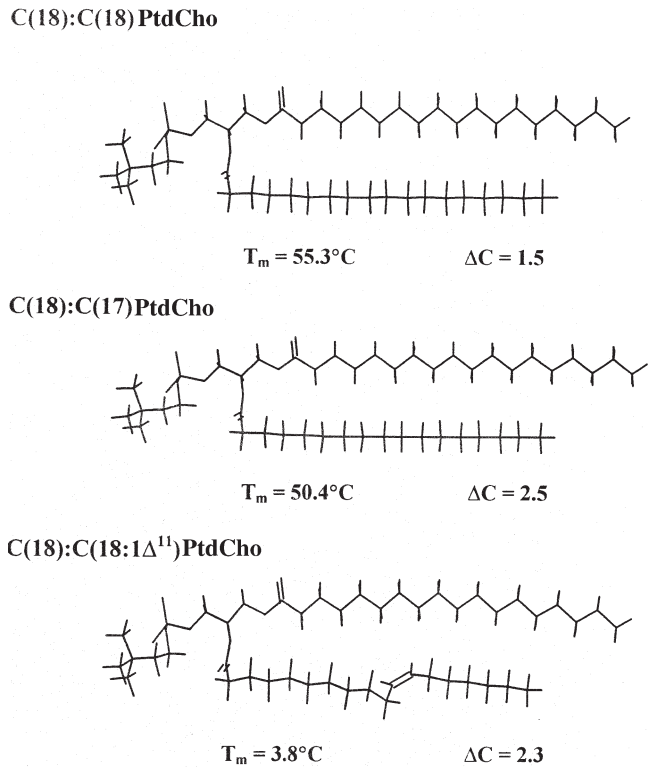


**FIG. 10.** Computer-graphic representations of the energy-minimized structures obtained with the monomers of C(18):C(18)PtdCho, C(18):C(17)PtdCho, and C(18):C(18:1$\Delta^{11}$)PtdCho. For abbreviations see Figure 1.

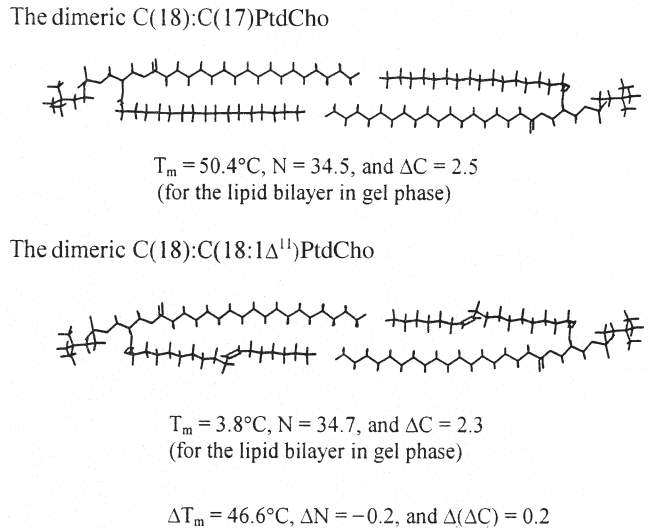


**FIG. 11.** Computer-graphic representations of the energy-minimized structures obtained with the *trans*-dimers of C(18):C(17)PtdCho and C(18):C(18:1$\Delta^{11}$)PtdCho. For abbreviation see Figure 1.

which is almost equal to that of the shorter chain-segment. Consequently, the contact interaction with the all-*trans sn*-1 acyl chain is also minimal. As the Δ-bond moves away stepwise from the chain center toward either end, the length of the longer chain-segment is progressively increased, leading to a proportionally increased van der Waals contact interaction with the all-*trans sn*-1 acyl chain. Because the origin of $T_m$ for the gel-to-liquid crystalline phase transition is largely enthalpic, the $T_m$ can be approximately related to the lateral

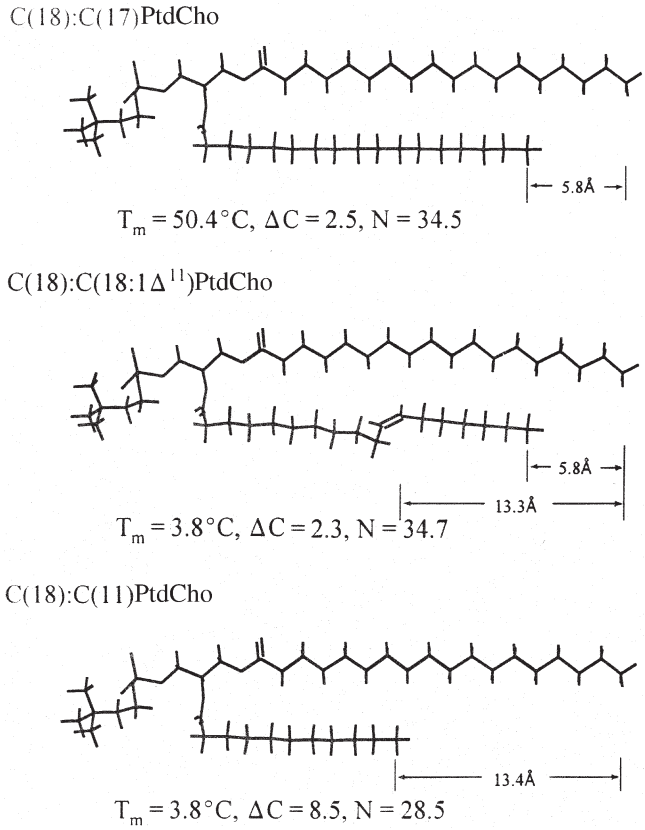


**FIG. 12.** The structure of the saturated fictive PtdCho molecule calculated on the basis of the $T_m$ and X values of C(18):C(18:1$\Delta^{11}$)PtdCho. For abbreviation see Figure 1.

chain-chain contact interaction (13). As a result, the $T_m$ increases as the magnitude of the contact interaction between the *sn*-1 acyl chain and the *sn*-2 acyl chain is increased. Our molecular model thus predicts a V-shaped $T_m$-profile in the plot of $T_m$ vs. the position of the single *cis*-double bond in the *sn*-2 acyl chain, $\Delta^n$, of mixed-chain C(X):C(Y:1$\Delta^n$)PtdCho. Furthermore, the minimal $T_m$ in the V-shaped $T_m$-profile must correspond to the positional isomer of C(X):C(Y:1$\Delta^n$)PtdCho with the Δ-bond positioned at the chain center, because the lateral chain-chain interaction is minimal in this lipid species. Indeed, the $T_m$-profiles observed for the two series of monounsaturated C(18:1$\Delta^n$):C(18:1$\Delta^n$)PtdCho and C(20):C(20:1$\Delta^n$)PtdCho are V-shaped (Fig. 9); in addition, each V-shaped profile is characterized by a minimal $T_m$ corresponding to the anticipated positional isomer.

## THE STRUCTURE AND THE CHAIN-MELTING BEHAVIOR OF PHOSPHOLIPIDS WITH *sn*-1 SATURATED AND *sn*-2 POLYUNSATURATED ACYL CHAINS

As discussed above, the presence of just a single *cis*-double bond in the *sn*-2 acyl chain of a phospholipid molecule is sufficient to exert profound influence on the chain-melting behavior of the lipid bilayer. Now, we shall see how the successive addition of a second, a third, . . . methylene-interrupted *cis*-double bond changes the gel-to-liquid crystalline phase

transition behavior of the lipid bilayer. Specifically, I shall present calorimetric data obtained with mixed-chain PtdEth with saturated *sn*-1 and polyunsaturated *sn*-2 acyl chains, since these lipid molecules are present abundantly in biological membranes of animal cells. Moreover, based on the simple molecular model described earlier for monounsaturated lipids, a more general MM-based molecular model will be described, from which the chain-melting behavior of lipid bilayers composed of polyunsaturated mixed-chain lipids can be rationalized in terms of structural changes of the polyunsaturated acyl chain.

The first and the immediate second DSC heating curves obtained with C(20):C(20)PtdEth and its eight unsaturated derivatives are described (Fig. 13). These unsaturated derivatives with two or more *cis*-double bonds in the $C_{20}$-*sn*-2-acyl chain are seen to contain only methylene-interrupted *cis*-double bonds. The second DSC curves are reproducible upon further repeated reheatings, and the transitions observed in the second heating scans can thus be assigned as the gel-to-liquid crystalline phase transitions. Only the phase transition behavior exhibited by various PtdEth in the second DSC heating scans is discussed in this presentation. For instance, the thermodynamic parameters associated with the gel-to-liquid crystalline phase transition of lipid bilayers prepared from three series of PtdEth are shown in Table 2 (29). More recently, the total numbers of different species of *sn*-1 saturated/*sn*-2 unsaturated PtdEth originated from the common precursors of C(20):C(18)PtdEth and C(20):C(20)PtdEth have been extended in this laboratory to 10 and 15 molecular species, respectively (24,25). In this presentation, I shall limit my discussion of the phase transition behavior of mixed-chain PtdEth to the $T_m$ values of lipid bilayers prepared individually from the 15 lipid species derived commonly from C(20):C(20)PtdEth.

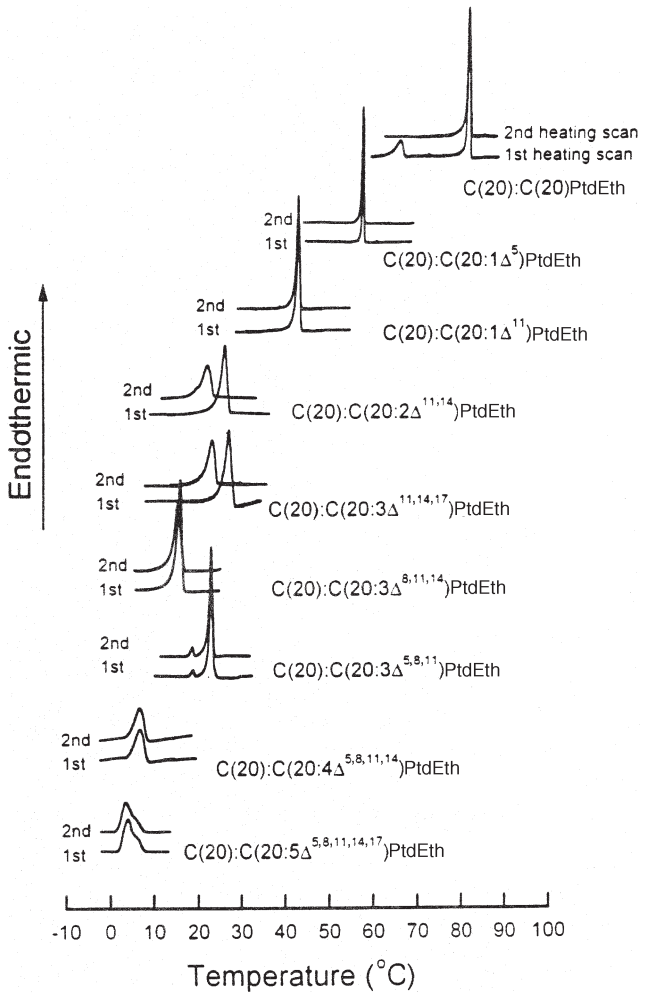


**FIG. 13.** The first and the immediate second DSC heating curves obtained with C(20):C(20)PtdEth and its eight unsaturated derivatives. Scan rate: 15°C/h. Lipid concentration: ~3–5 mM.

The second DSC heating curves for c(20):C(20)PtdEth and its five unsaturated derivatives of C(20):C(20:1$\Delta^{17}$)PtdEth, C(20):C(20:2$\Delta^{14,17}$)PtdEth, C(20):C(20:3$\Delta^{11,14,17}$)PtdEth, C(20):C(20:4$\Delta^{8,11,14,17}$)PtdEth and C(20):C(20:5($^{5,8,11,14,17}$)-PtdEth are illustrated in Figure 14. Here, the first Δ-bond is incorporated at the n-3 (or $\Delta^{17}$)-position in the *sn*-2 acyl chain. The second and all subsequent methylene-interrupted Δ-bonds are introduced into the chain segment located between the n-3-Δ-bond and the carbonyl end. Hence, the *sn*-2 acyl chains of this series of unsaturated PtdEth belong to the n-3-family. In the upper left inset in Figure 14, the $T_m$ value exhibited by each species in the n-3PtdEth series is plotted against the number of Δ-bonds. Clearly, the $T_m$ value decreases continually, but nonlinearly, with increasing number of Δ-bonds. Similarly, like the calorimetric data shown for n-3PtdEth, the $T_m$ value exhibited by each lipid in the unsaturated n-6PtdEth series also decreases

**TABLE 2**
**Thermodynamic Parameters Associated with the Gel-to-Liquid Crystalline Phase Transition of Lipid Bilayers Prepared from Various Phosphatidylethanolamines (PtdEth)**

| Lipid | $T_m$ (°C) | Δ*H* (kcal/mol) | Δ*S* (cal/mol/K) |
|---|---|---|---|
| C(20):C(20)PtdEth | 82.5 | 12.5 | 35.2 |
| C(20):C(20:1$\Delta^{5}$)PtdEth | 58.2 | 8.3 | 25.1 |
| C(20):C(20:1$\Delta^{11}$)PtdEth | 43.3 | 8.1 | 25.6 |
| C(20):C(20:2$\Delta^{11,14}$)PtdEth | 22.4 | 4.5 | 15.2 |
| C(20):C(20:3$\Delta^{11,14,17}$)PtdEth | 23.3 | 6.0 | 20.2 |
| C(20):C(20:3$\Delta^{8,11,14}$)PtdEth | 15.6 | 5.5 | 19.1 |
| C(20):C(20:3$\Delta^{5,8,11}$)PtdEth | 23.0 | 5.8 | 19.6 |
| C(20):C(20:4$\Delta^{5,8,11,14}$)PtdEth | 6.6 | ~5.2 | ~18.6 |
| C(20):C(20:5$\Delta^{5,8,11,14,17}$)PtdEth | 3.5 | 5.7 | 20.6 |
| C(20):C(18)PtdEth | 75.8 | 11.2 | 32.1 |
| C(20):C(18:1$\Delta^{9}$)PtdEth | 33.9 | 7.0 | 22.8 |
| C(20):C(18:2$\Delta^{9,12}$)PtdEth | 7.2 | 3.1 | 11.1 |
| C(20):C(18:3$\Delta^{9,12,15}$)PtdEth | 10.4 | 4.7 | 16.6 |
| C(20):C(18:4$\Delta^{6,9,12,15}$)PtdEth | 3.9 | 5.5 | 19.5 |
| C(18):C(20)PtdEth | 79.1 | 11.5 | 32.7 |
| C(18):C(20:1$\Delta^{11}$)PtdEth | 39.5 | 7.4 | 23.7 |
| C(18):C(20:2$\Delta^{11,14}$)PtdEth | 18.5 | 3.9 | 13.4 |
| C(18):C(20:3$\Delta^{11,14,17}$)PtdEth | 21.0 | 5.4 | 18.4 |
| C(18):C(20:3$\Delta^{8,11,14}$)PtdEth | 11.7 | 5.1 | 17.9 |
| C(18):C(20:3$\Delta^{5,8,11}$)PtdEth | 19.8 | 5.2 | 17.8 |
| C(18):C(20:4$\Delta^{5,8,11,14}$)PtdEth | 1.3 | ~4.5 | ~16.4 |

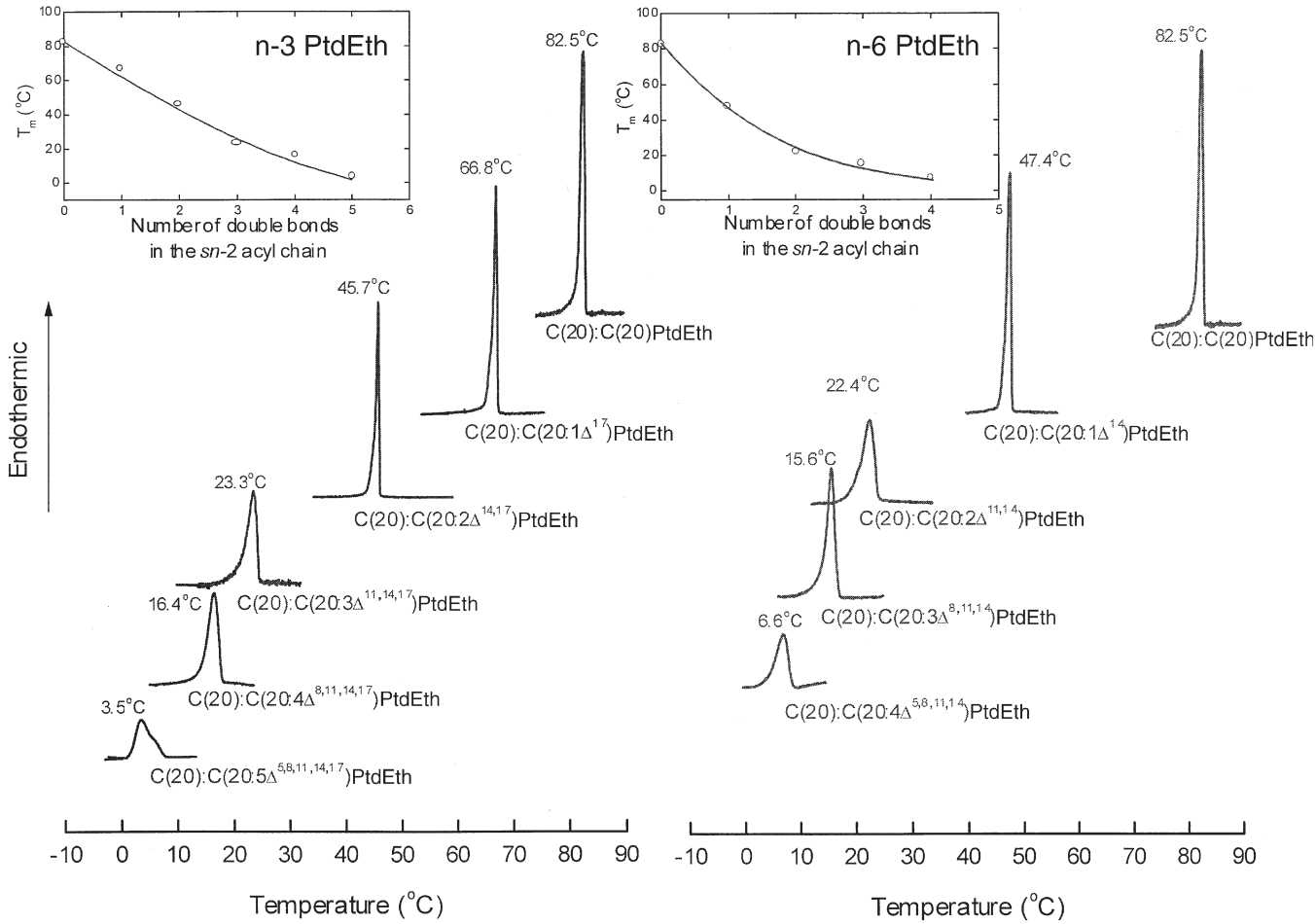


**FIG. 14.** Representative second DSC heating curves for aqueous dispersions prepared individually from C(20):C(20)PtdEth, five unsaturated n-3 derivatives of C(20):C(20)PtdEth, and four unsaturated n-6 derivatives of C(20):C(20)PtdEth. These unsaturated n-3PtdEth containing n-3 fatty acids and n-6PtdEth containing n-6 fatty acids contain 1–5 and 1–4 *cis*-Δ-bonds, respectively, in the lipid's *sn*-2 acyl chain, with the position of the commonly shared double bond being 3 and 6 carbons from the methyl end, respectively. The abbreviated name for each unsaturated lipid species is indicated under each transition curve, and, above the transition curve, the value of $T_m$ obtained with each lipid dispersion is also indicated. Furthermore, the $T_m$ values obtained with unsaturated lipids in each series are plotted against the number of *cis*-Δ-bonds as shown in the two insets.

continually but nonlinearly with increasing number of Δ-bonds, as shown in the upper right inset in Figure 14. It should be noted that the newly added Δ-bond is also placed on the carbonyl side of the existing Δ-bonds in the n-6PtdEth series.

In Figure 15, the DSC curves exhibited by lipids in two additional series of unsaturated PtdEth are depicted. In these two series of unsaturated PtdEth, the first *cis*-double bonds are introduced into the *sn*-2 acyl chain at the $\Delta^8$- and $\Delta^{11}$-positions, respectively. In contrast to the unsaturated lipids shown in Figure 14, the second and subsequent *cis*-double bonds are always inserted into the *sn*-2 acyl chain at positions located between the existing double bond and the methyl end. Hence, the *sn*-2 acyl chains of these two series of unsaturated PtdEth shown in Figure 15 belong to the $\Delta^8$- and the $\Delta^{11}$-series. For the $\Delta^{11}$PtdEth series, the $T_m$ value of C(20):C(20)PtdEth is seen in Figure 15 to decrease from 82.5 to 43.3°C upon the introduction of the first Δ-bond. A further decrease of 20.9°C in $T_m$ is observed in going from C(20):C(20:1$\Delta^{11}$)PtdEth → C(20):C(20:2$\Delta^{11,14}$)PtdEth. In contrast, a slight increase in $T_m$ (0.9°C) is detected as the third methylene-interrupted *cis*-double bond is subsequently added to the dienoic *sn*-2 acyl chain on the methyl end of the $\Delta^{14}$-double bond. Structurally, the C(20):C(20:2$\Delta^{11,14}$)PtdEth → C(20):C(20:3$\Delta^{11,14,17}$)PtdEth conversion, characterized by a small increase in $T_m$, corresponds to an n-6PtdEth → n-3PtdEth conversion. When all $T_m$ values obtained with lipids in this $\Delta^{11}$-PtdEth series are plotted against the number of *cis*-double bonds, a down-and-up $T_m$-profile is observed (upper right inset, Fig. 15). Similarly, for lipids in the $\Delta^8$-PtdEth series, the variation of $T_m$ as a function of the number of Δ-bonds is also characterized by a down-and-up trend (upper left inset, Fig. 15). In this case, the lipid with four Δ-bonds, C(20):C(20:4$\Delta^{8,11,14,17}$)PtdEth, is an n-3PtdEth which displays a higher $T_m$ value than its precursor, C(20):C(20:3$\Delta^{8,11,14,}$)-PtdEth, an n-6PtdEth with three Δ-bonds.

Based on the $T_m$ values shown in Figures 14 and 15 and some other DSC data published previously, a $T_m$ diagram is constructed as shown in Figure 16 (25). Here, nine series of mixed-chain PtdEth with saturated $C_{20}$-*sn*-1 and unsaturated $C_{20}$-*sn*-2 acyl chains derived from the common precursor of

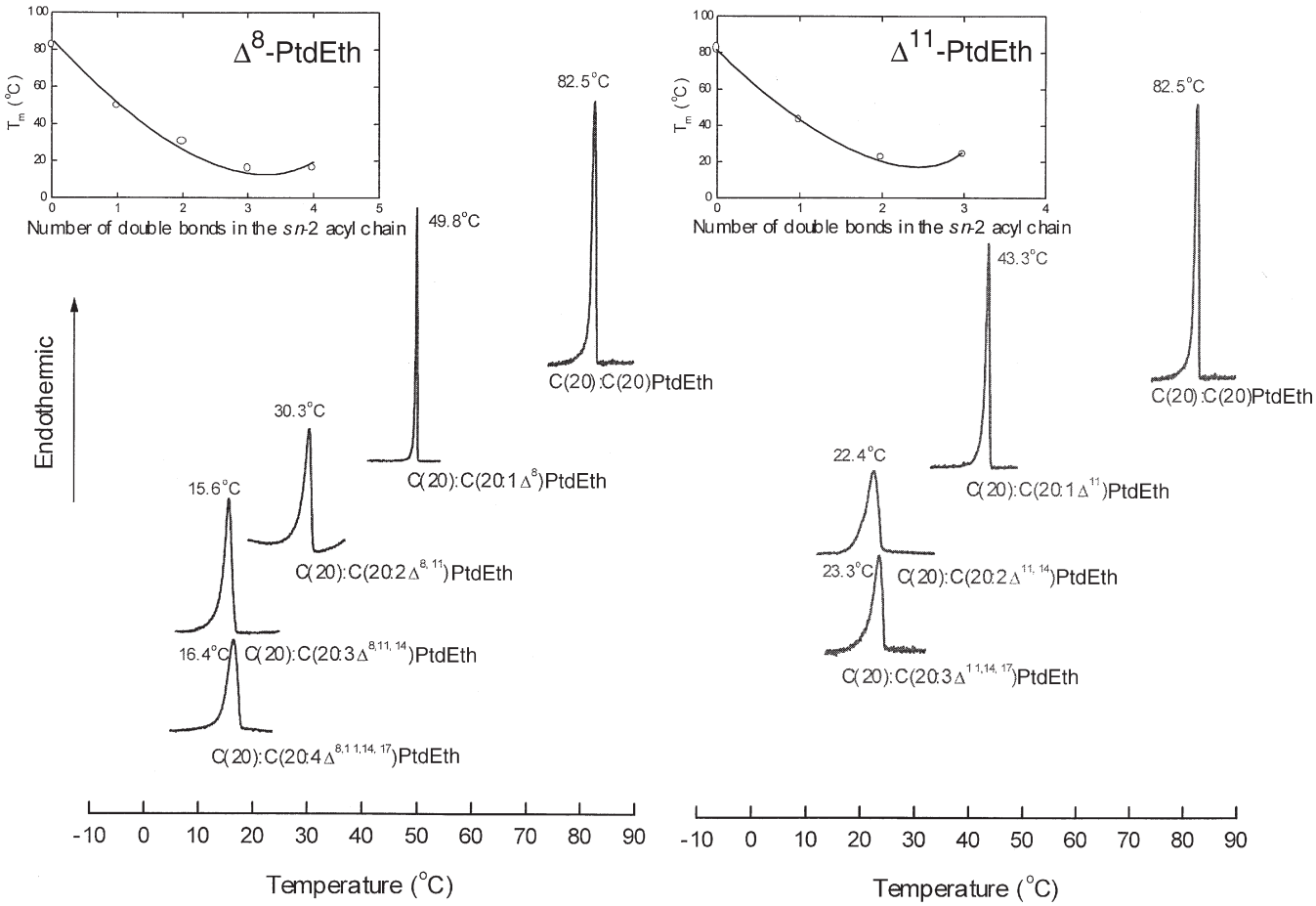


**FIG. 15.** The second DSC heating curves exhibited by lipids in the $\Delta^8$PtdEth- and $\Delta^{11}$PtdEth-series. Here, the superscript number denotes the position of the common *cis*-double bond in the *sn*-2 acyl chain when counting from the carbonyl end.

saturated C(20):C(20)PtdEth are presented. In each of these nine series of PtdEth, there are three or more lipids that share either a common Δ-bond at a fixed position along the *sn*-2 acyl chain or a fixed number of Δ-bonds. In this $T_m$ diagram, all $T_m$ values are placed underneath the appropriate structural formulas. These formulas are arranged systematically in a unifying manner according to their order of complexity. From Figure 16, the effects of the number and position of Δ-bonds on the $T_m$ obtained with lipids in the nine series of mixed-chain unsaturated $C_{20}$-PtdEth can thus be examined simply and simultaneously. Let us first look at the 3-series of PtdEth aligned horizontally in Figure 16; mixed-chain lipids in each of the three n-3 to n-9 PtdEth-series are characterized by a continuously decreasing $T_m$ profile as the number of Δ-bonds increases progressively. Similarly, mixed-chain lipids in the $\Delta^5$-PtdEth series aligned vertically along the rightmost line in the $T_m$-diagram also display a decreasing $T_m$ profile. However, as discussed earlier, the C(20):C(20:2$\Delta^{11,14}$)PtdEth → C(20):C(20:3$\Delta^{11,14,17}$)PtdEth and the C(20):C(20:3$\Delta^{8,11,14}$)-PtdEth → C(20):C(20:4$\Delta^{8,11,14,17}$)PtdEth conversions are coupled with increased values of $T_m$. For the $\Delta^8$- and $\Delta^{11}$-PtdEth series that are also aligned vertically in Figure 16, the $T_m$-profiles are characterized by a down-and-up trend. Finally, three series of mixed-chain PtdEth each with a fixed number of Δ-bonds in the *sn*-2 acyl chain can be seen along the diagonal lines in the $T_m$-diagram (Fig. 16). The monoenoic, dienoic, and trienoic series of mixed-chain PtdEth have five, four, and three positional isomers, respectively. In response to changes in the position of the double bond, the $T_m$ values of mixed-chain lipids with a fixed number of Δ-bonds give rise to a roughly V-shaped profile, a scenario that has been discussed earlier in details for monounsaturated lipids. Interestingly, the two series of positional isomers of PtdEth containing two and three *cis*-double bonds also exhibit V-shaped $T_m$-profiles in the plot of $T_m$ vs. the position of *cis*-double bonds. The most novel feature of the $T_m$-diagram shown in Figure 16 is the following: all the different $T_m$ profiles observed for mixed-chain lipids derived from a common precursor of a saturated lipid species can be seen simultaneously in a simple and unifying manner.

The energy-minimized structures of monounsaturated mixed-chain PtdCho such as C(16):C(18:1$\Delta^9$)PtdCho obtained with MM calculations have been presented. Similar structures can also be simulated for monounsaturated mixed-chain PtdEth based on the single-crystal data of PtdEth (26) and the atomic coordinates of the energy-minimized structures of monoenoic hydrocarbons (22). For instance, a kinked crankshaft-like structure with low energy is simulated for

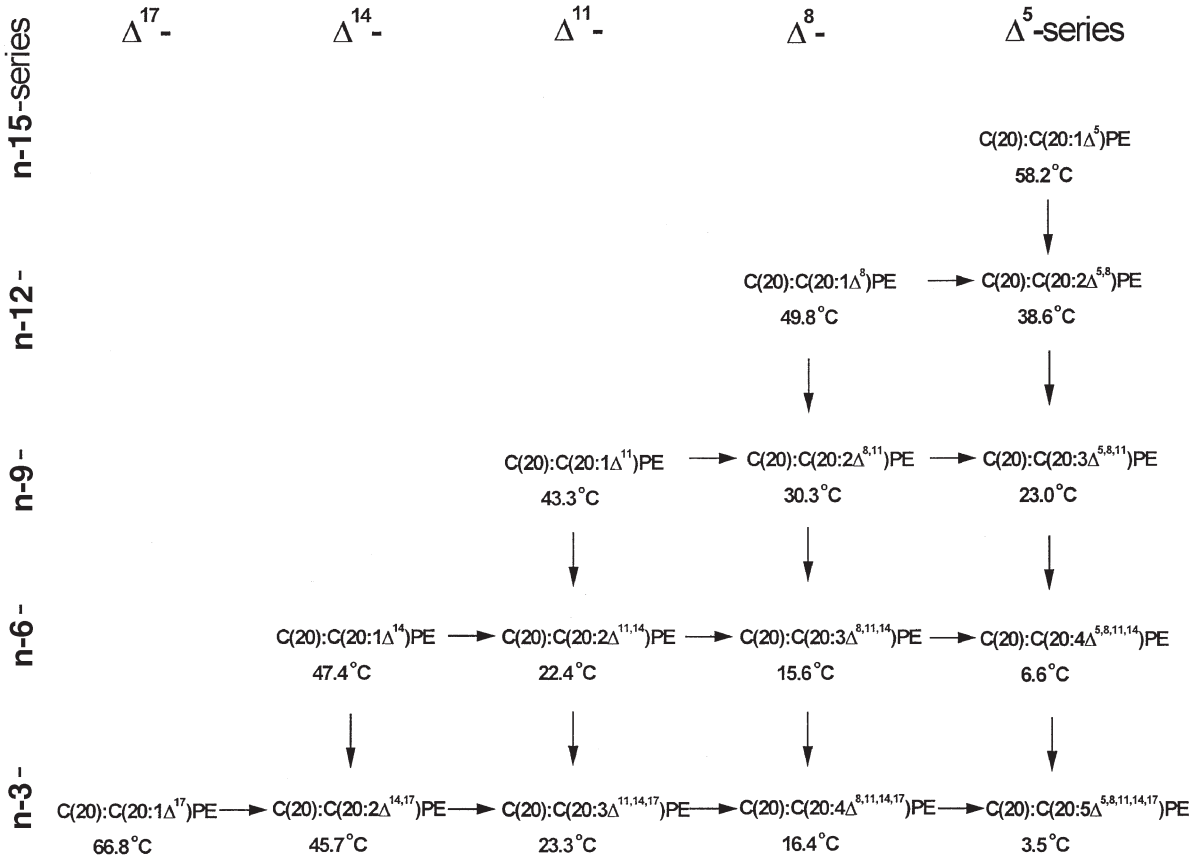


**FIG. 16.** The $T_m$-diagram of *sn*-1 $C_{20}$-saturated/*sn*-2 $C_{20}$-unsaturated PtdEth. Lipids in each row share a common n-carbon, where the n-carbon is defined as the first olefinic carbon atom in the lipid's *sn*-2 acyl chain when counting from the chain terminal methyl end. The five parallel rows of unsaturated lipids from top to bottom are n-15PtdEth, n-12PtdEth, n-9PtdEth, n-6PtdEth, and n-3PtdEth series as indicated. Lipids in each column share a common $\Delta^n$-bond, and lipids in each column thus belong to a $\Delta^n$PtdEth series, where the superscript *n* denotes the position of the common *cis* carbon-carbon double bond in the *sn*-2 acyl chain when counting from the carbonyl end. In this case, the carbonyl carbon is designated as the first carbon of the acyl chain. The five columns of unsaturated lipids from left to right are $\Delta^{17}$PtdEth, $\Delta^{14}$PtdEth, $\Delta^{11}$PtdEth, $\Delta^8$PtdEth, and $\Delta^5$PtdEth series, respectively, as indicated. Altogether, there are 15 molecular species of unsaturated mixed-chain PtdEth in this $T_m$ diagram, and their $T_m$ values are given under the abbreviated names of the corresponding mixed-chain PtdEth.

C(16):C(18:1$\Delta^9$)PtdEth (Fig. 17). Furthermore, the single-crystal structure of linoleic acid, determined by Ernst *et al.* (27), is characterized by a crankshaft-like motif with a kink sequence of $s^-\Delta s^+s^+\Delta s^-$ (–119, –2.3, 123, 124, –3.3, –121°). The most stable structure of dienoic PtdEth in the crystalline-state bilayer can thus be expected to have a kinked conformation. As expected, a rotamer with a kink sequence of $s^-\Delta s^+s^+\Delta s^-$ is indeed found by MM calculations to be energetically the most stable conformation for the s*n*-2 acyl chain of C(16):C(18:2$\Delta^{9,12}$)PtdEth (Fig. 17; 28). MM simulations, in fact, further show that the roughly crankshaft-like conformation is a common motif underlying the energy-minimized structure of other *sn*-2 acyl chains of mixed-chain PtdEth containing three or more methylene-interrupted Δ-bonds (Fig. 18).

The energy-minimized structures of C(20):C(20)PtdEth and its five unsaturated n-3 derivatives are illustrated in Figure 19 (25). These structures, to a first approximation, correspond to the optimal and static structures of PtdEth molecules packed in the crystalline-state bilayer. Here, the zigzag plane of the all-*trans* *sn*-1 acyl chain in each lipid species lies perpendicular to the paper plane, whereas the *sn*-2 acyl chain projects in front of the *sn*-1 acyl chain. For C(20):C(20)-PtdEth, the *sn*-2 acyl chain has an all-*trans* segment extending from C(3) to C(19) with 17 methylene units. This segment is designated as the all-*trans* segment, abbreviated as ATS. For C(20):C(20:1$\Delta^{17}$)PtdEth, the *sn*-2 acyl chain has a crankshaft-like topology with a kink sequence given under the energy-minimized structure in Figure 19. As a result, the *sn*-2 acyl chain can be viewed to consist of two chain segments linked by the kink sequence. The all-*trans* segment in the long-chain segment of the kinked *sn*-2 acyl chain is defined as the ATS. For C(20):C(20:1$\Delta^{17}$)PtdEth, the ATS has 14 consecutive methylene units extending from C(3) to C(16) along the kinked *sn*-2 acyl chain. Note that the ATS is one C–C bond length shorter than that of the long chain segment due to the fact that the C–C single bond preceding the $\Delta^{17}$-bond has an $s^-$ conformation. For the energy-minimized structures of polyunsaturated n-3PtdEth shown in Figure 19, the *sn*-2 acyl chains are seen to adopt roughly an overall kinked motif. The ATS is observed invariably to locate in the upper chain segment in each kinked *sn*-2 acyl chain. Moreover, the length of the ATS is shortened progressively by three methylene

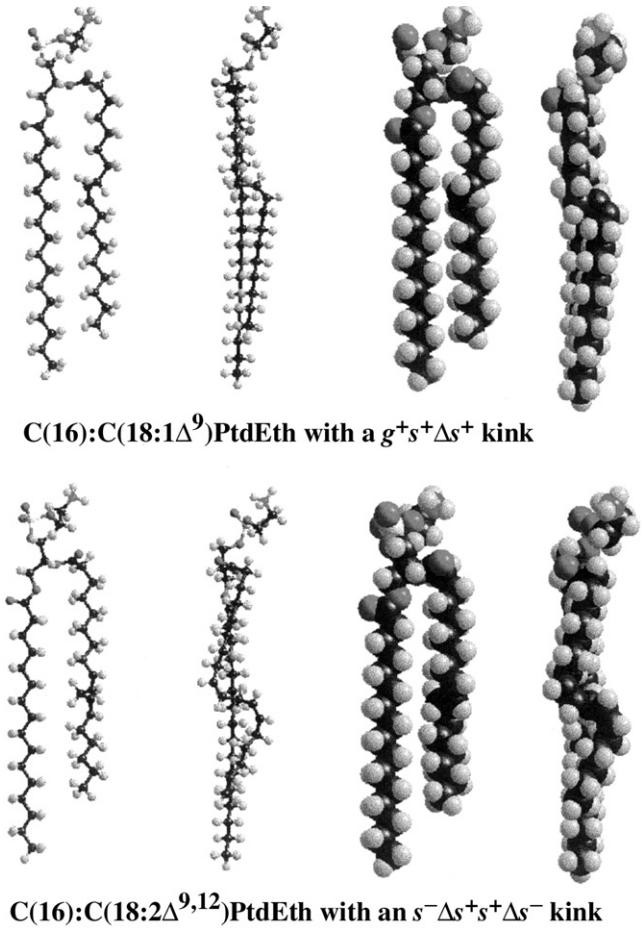


**FIG. 17.** The energy-minimized structures of C(16):C(18:1$\Delta^{9}$)PtdEth and C(16):C(18:2$\Delta^{9,12}$)PtdEth; each is characterized by a kinked crankshaft-like motif.

units as a Δ-bond is added successively on the carbonyl side of the existing Δ-bonds at the methylene-interrupted position. In contrast, the short chain segment succeeding the $\Delta^{17}$-bond is identical in length (Fig. 19). It should be emphasized that the structural features of polyunsaturated n-3PtdEth mentioned above are simulated results obtained with MM computations. These simulated structures do not explicitly provide information about the dynamic nature of lipid molecules in the gel-state bilayer. For instance, the *sn*-2 acyl chains of all unsaturated n-3PtdEth shown in Figure 19 share a common chain terminal segment of C(16)-C(17)=C(18)-C(19)-C(20), in which all C–C single bonds are most likely dynamic in the gel-state bilayer. The assumed flexible nature at $T < T_m$ can be attributed mainly to the large degree of rotational freedom of two single C–C bonds adjacent to the Δ-bond as well as the C–C single bond containing the methyl terminal carbon atom. Hence, it is reasonable to suppose that this disordered methyl terminal segment does not undergo the thermally induced *trans* → *gauche* isomerizations at $T_m$, and it thus makes no contributions to the chain disordering process at $T_m$. The dynamic nature of this short terminal segment, however, is not revealed by any of the MM simulated structures illustrated in Figure 19. On the other hand, as the number of Δ-bonds in this series of n-3PtdEth increases from zero to five, the length of ATS is shortened in each step by a constant length of three methylene units. This variation in ATS, shown clearly in Figure 19, is considered to be a structural feature that persists in the gel-state bilayer.

Similar to lipids in the n-3PtdEth series, the five lipids in the $\Delta^{5}$PtdEth series seen in the $T_m$ diagram also contain up to five methylene-interrupted Δ-bonds in the *sn*-2 acyl chain. The energy-minimized structures of these lipids have also been determined by MM calculations, and a crankshaft-like topology characterizes them all. In these lipids, the short chain segments in the kinked *sn*-2 chains are located near the $H_2O$/hydrocarbon interface, extending from C(3) to C(5). All ATS, however, are located in the lower chain segments; the length of each ATS decreases progressively by three methylene units as a methylene-interrupted Δ-bond is added successively on the methyl side of the existing Δ-bond(s). This decreasing trend in ATS is, in essence, identical to that observed in Figure 19 for the n-3PtdEth series.

In Figure 20, the minimum-energy structures of C(20):C(20:1$\Delta^{11}$)PtdEth, C(20):C(20:2$\Delta^{11,14}$)PtdEth, and C(20):C(20:3$\Delta^{11,14,17}$)PtdEth in the $\Delta^{11}$PtdEth series are illustrated. For C(20):C(20:1$\Delta^{11}$)PtdEth, the $\Delta^{11}$-double bond is located in the middle of the *sn*-2 acyl chain, and the additional Δ-bonds in C(20):C(20:2$\Delta^{11,14}$)PtdEth and (20):C(20:3$\Delta^{11,14,17}$)PtdEth are on the methyl side of the $\Delta^{11}$-bond. Unlike the variable length of ATS shown in Figure 19, all three lipids in this series of $\Delta^{11}$PtdEth share a constant chain length of ATS extending from C(3) to C(10) in the *sn*-2 acyl chain (Fig. 20). It is evident that as the C(20):C(20:2$\Delta^{11,14}$)-PtdEth (an n-6PtdEth) → C(20):C(20:3$\Delta^{11,14,17}$)PtdEth (an n-3PtdEth) conversion occurs, the added $\Delta^{17}$-bond is introduced on the methyl side of the existing Δ-bonds, and the length of the ATS is not affected by the n-6PtdEth → n-3PtdEth conversion. The last two $\Delta^{8}$PtdEth species shown in the $T_m$-diagram are C(20):C(20:3$\Delta^{8,11,14}$)PtdEth and C(20):C(20:4$\Delta^{8,11,14,17}$)-PtdEth, which are n-6PtdEth and n-3PtdEth, respectively. Interestingly, they share a constant length of ATS that is located in the upper chain segment of the kinked *sn*-2 acyl (25). The fourth Δ-bond is added on the methyl side of the existing Δ-bonds as the n-6PtdEth → n-3PtdEth conversion takes place. Hence, this n-6PtdEth → n-3PtdEth conversion is structurally similar to the C(20):C(20:2$\Delta^{11,14}$)PtdEth (an n-6PtdEth) → C(20):C(20:3$\Delta^{11,14,17}$)PtdEth (an n-3PtdEth) conversion seen in Figure 20.

Let me summarize what is known about the structural features of *sn*-1 saturated/*sn*-2 polyunsaturated phospholipids from MM simulations: (i) the *sn*-1 acyl chain has an all-*trans* conformation; (ii) the *sn*-2 acyl chain extending from C(3) to the methyl terminus has a crankshaft-like conformation; hence, it can be considered to consist of two chain-segments linked by a kink sequence; (iii) the relative length of the two chain-segments in the kinked *sn*-2 acyl chain depends on the position of the multiple Δ-bonds; (iv) the consecutive all-*trans* segment in the long chain segment of the kinked *sn*-2 chain can be defined as ATS, and the length of ATS is

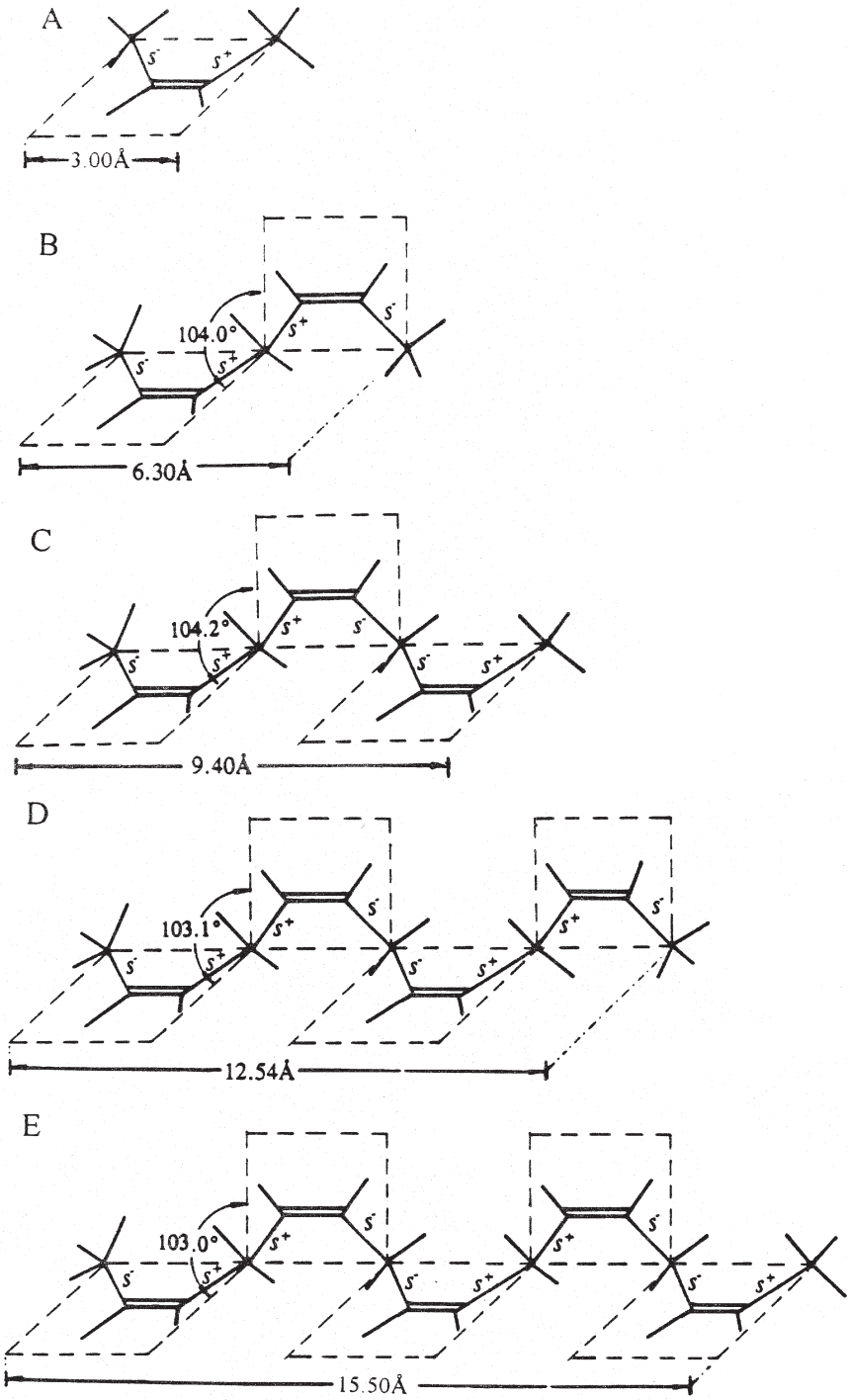


**FIG. 18.** The energy-minimized structure of hydrocarbon chains containing 1–5 methylene-interrupted *cis*-double bonds.

shortened by three methylene units if a methylene-interrupted Δ-bond is incorporated into the ATS; (v) the kink sequence in the dienoic *sn*-2 acyl chain is $s^{-}\Delta s^{+}s^{+}\Delta s^{-}$ or $s^{+}\Delta s^{-}s^{-}\Delta s^{+}$, depending on whether the position of *n* in $\Delta^{n,n+3}$-bond is an odd- or even-carbon atom in the *sn*-2 acyl chain.

Earlier, we presented a simple molecular model that can interpret adequately the large $T_m$-lowering effect of acyl chain monounsaturation and the V-shaped characteristic dependency of $T_m$ on the position of the single Δ-bond along the *sn*-2 acyl chain. We can extend this simple model to interpret the different effects of the *sn*-2 acyl chain polyunsaturation on the phase transition behavior as seen in the $T_m$-diagram (Fig. 16). In this extended molecular model, three basic assumptions are made. (i) The *sn*-2 acyl chain in the mixed-chain PtdEth (or PtdCho) with *sn*-1 saturated/*sn*-2 unsaturated acyl chains is assumed to adopt, at $T < T_m$, an energy-minimized crankshaft-like kink motif. Hence, it consists of a longer and a shorter chain-segment separated by the Δ-containing kink. (ii) At $T < T_m$, the

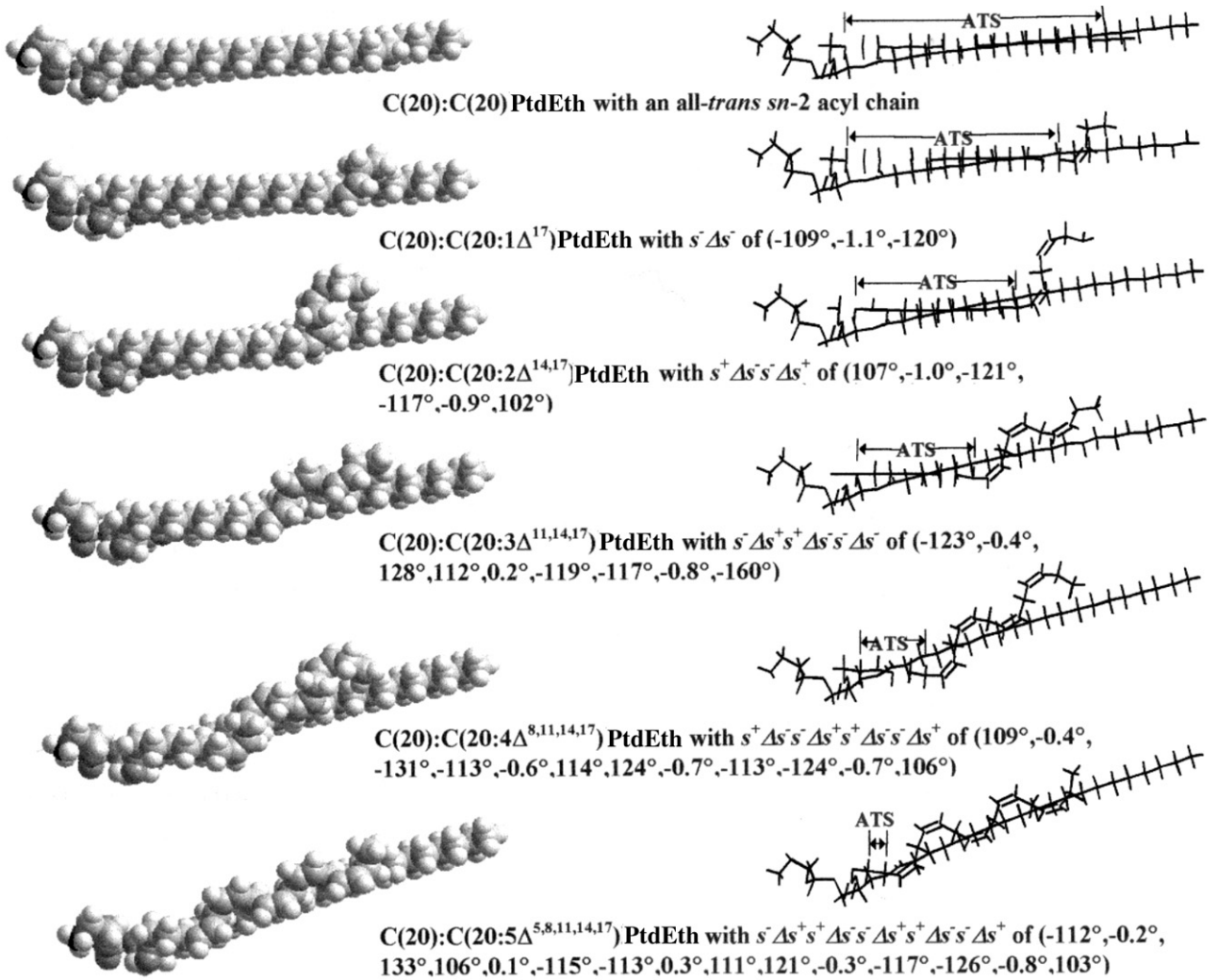


**FIG. 19.** The energy-minimized structures of C(20):C(20)PtdEth and its five unsaturated n-3 derivatives as shown molecular-graphically by space-filling and wire models. These unsaturated n-3PtdEth molecules contain 1–5 *cis*-Δ-bonds. The ATS shown in the wire model denotes the all-*trans* segment (ATS) of the hydrocarbon chain in the *sn*-2 acyl chain. The kink sequence in the *sn*-2 unsaturated acyl chain and a set of optimal torsion angles associated with the kink sequence are presented under each unsaturated lipid species. The length of ATS is shortened progressively by three methylene units as the new methylene-interrupted *cis*-double bond is added successively on the carbonyl side of the $\Delta^{17}$-bond in the *sn*-2 acyl chain of C(20):C(20:1$\Delta^{17}$)PtdEth. In contrast, the short chain segment succeeding the $\Delta^{17}$-bond is identical in length.

ATS in the long chain segment of the kinked *sn*-2 acyl chain and the neighboring all-*trans sn*-1 acyl chain are proposed to undergo attractive van der Waals interaction. In contrast, the shorter chain-segment of the kinked *sn*-2 acyl chain is assumed to be partially disordered. However, if the length of the shorter chain-segment differs from that of the longer one by 1–3 C–C bond lengths, the shorter chain-segment may contribute somewhat to the chain-melting process at $T_m$. (iii) When the number of Δ-bonds in the *sn*-2 acyl chain is larger than two, the methylene-interrupted Δ-bonds act together as a rigid structural unit, which prevents the rotational isomerizations of C–C bonds in the neighboring chains. Hence, the *sn*-2 acyl chain with three or more methylene-interrupted Δ-bonds can facilitate somewhat the lateral chain-chain interactions in the gel-state bilayer.

According to the extended molecular model presented above, the variations of $T_m$ upon successive chain unsaturation in the *sn*-2 acyl chain reflect mainly the changes of the following two opposing effects: (i) the $T_m$-lowering effect caused by the progressive shortening of ATS, and (ii) the $T_m$-elevating effect exerted by the increased rigidity of three to five Δ-bonds. These two opposing effects are brought about, paradoxically, by the same structural change, by the increasing degree of acyl chain unsaturation in the *sn*-2 position. We can now apply this model to explain why two distinct types of $T_m$ profile are observed for lipids upon the successive addition of methylene-interrupted Δ-bonds in the *sn*-2 acyl chain. One type is characterized by a nonlinearly decreased $T_m$-curve and the other is characterized by a down-and-up $T_m$-profile in the plot of $T_m$ vs. the number of Δ-bonds in the *sn*-2 acyl chain.

We begin with the discussion of the $T_m$ profile exhibited by lipids in the n-3PtdEth series derived from C(20):C(20)-PtdEth. This series can be taken as an example to represent all series of PtdEth characterized by a nonlinearly decreased $T_m$ profile. As shown in Figure 21, the length of ATS is short-

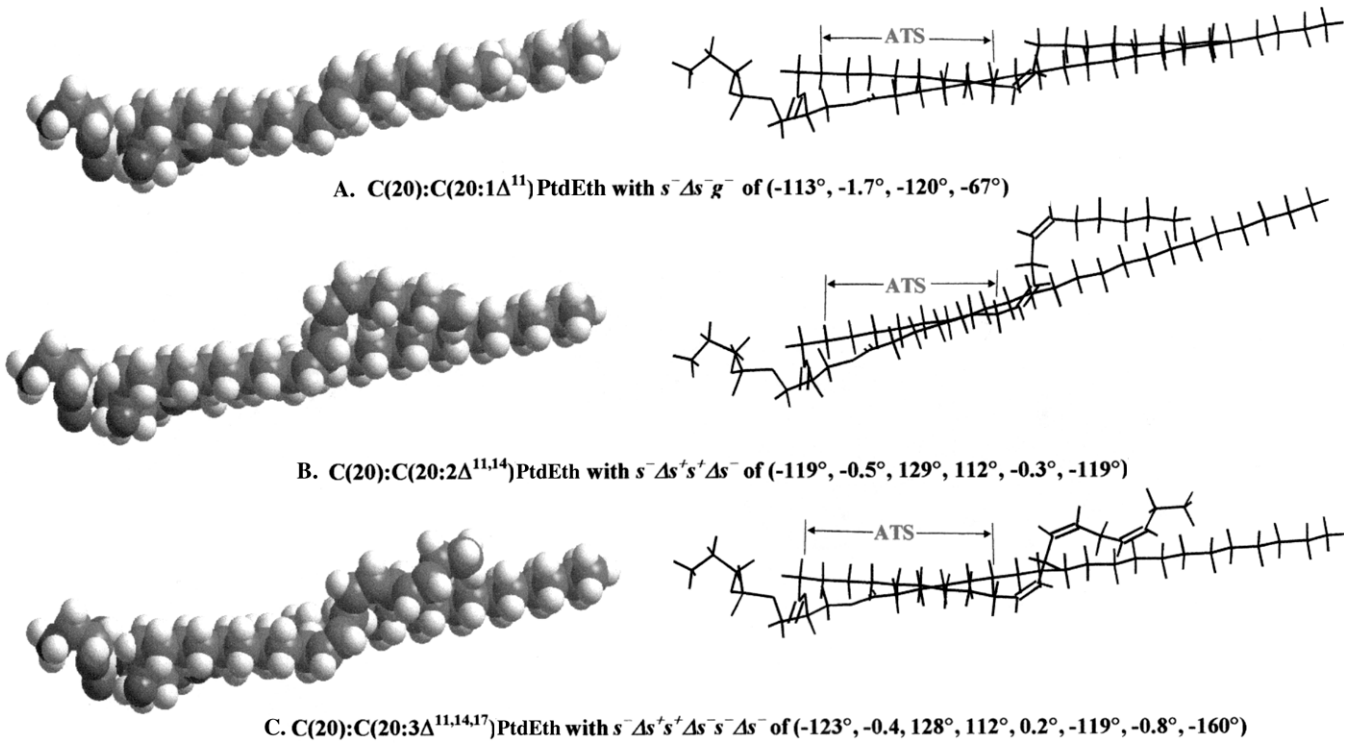


**FIG. 20.** Molecular graphics representations of the energy-minimized structures of C(20):C(20:1$\Delta^{11}$)PtdEth, C(20):C(20:2$\Delta^{11,14}$)PtdEth, and C(20):C(20:3$\Delta^{11,14,17}$)PtdEth in the $\Delta^{11}$PtdEth series as depicted by ball-and-stick and space-filling models. ATS in each lipid species is located in the upper chain-segment of the kinked *sn*-2 acyl chain; moreover, a constant length of ATS is shared by all three lipid species. The added Δ-bond is introduced on the methyl side of the existing Δ-bond. The kink sequence and associated torsion angles for each unsaturated acyl chain are presented under the energy-minimized structure of the corresponding lipid. For abbreviations see Figures 1 and 19.

ened progressively by a constant length of three methylene units as a methylene-interrupted Δ-bond is added successively into the *sn*-2 acyl chain. Based on the observed constant decrease in ATS alone, the $T_m$ values of lipids in the n-3PtdEth series can be expected to fall on a straight line with a negative slope. On the other hand, when the total number of Δ-bonds in the *sn*-2 acyl chain is three or more, the multiple Δ-bonds are proposed to act as a rigid structural unit, thus leading to a gradual increase in $T_m$. However, we assume that this $T_m$-elevating effect is less than the $T_m$-lowering effect exerted by the shortening of the ATS in the *sn*-2 acyl chain. On balance, the absolute value of the $T_m$ increment will be decreased progressively as the third and subsequent Δ-bonds are introduced stepwise into the *sn*-2 acyl chain, with the largest decrease in the $T_m$ increment occurring at the highest number of Δ-bonds. Consequently, the $T_m$ values of lipids in the n-3PtdEth series in the plot of $T_m$ vs. the number of Δ-bonds are expected to fall on a nonlinearly decreased curve; furthermore, the $T_m$ from the lipid species with the highest number of Δ-bonds is expected to deviate most from the straight line. These expectations are indeed borne out by experimental data (Fig. 21). Therefore, we may conclude that the presence of *cis*-double bonds in the *sn*-2 acyl chain contributes to the positive deviation of $T_m$ from the linear $T_m$-profile in the plot of $T_m$ vs. the number of *cis*-double bonds, although its effect vanishes when the total number of Δ-bonds in the *sn*-2 acyl chain is equal to or less than two.

Our proposed model can also explain the down-and-up $T_m$-profile exhibited by lipids in the $\Delta^8$PtdEth- and $\Delta^{11}$PtdEth-series shown in the $T_m$ diagram. Here, the $\Delta^{11}$PtdEth-series is chosen as an example to illustrate our points. The energy-minimized structures of the three lipid species in the $\Delta^{11}$PtdEth-series are shown in Figure 22, in which the ATS is seen to locate in the upper chain-segment. Moreover, the length of ATS remains unchanged among the three lipid species. It should be noted that the lower chain-segment in the C(20):C(20:1$\Delta^{11}$)PtdEth has a length that is only one C–C bond length shorter than the upper chain-segment. According to our proposed molecular model, this short chain-segment may contribute somewhat to the chain-melting process at $T_m$. Consequently, the $T_m$ value for the lipid bilayer composed of C(20):C(20:1$\Delta^{11}$)PtdEth can be expected to be higher than that of C(20):C20:2$\Delta^{11,14}$)PtdEth, although these two unsaturated lipids share a common length of ATS. The introduction of a third *cis*-double bond into the *sn*-2 acyl chain of C(20):C(20:2$\Delta^{11,14}$)PtdEth, an n-6PtdEth, converts the lipid into an n-3PtdEth, or C(20):C(20:3$\Delta^{11,14,17}$)-PtdEth. Based on the constant length of ATS alone, the $T_m$ increment between the n-6PtdEth and the n-3PtdEth would be zero. However, the n-3PtdEth has an additional Δ-bond in comparison to the n-6PtdEth; based on our proposed molecular model, a positive $T_m$ increment is thus expected to associate with the n-6PtdEth → n-3PtdEth conversion in this $\Delta^{11}$PtdEth series. On the basis of our proposed molecular

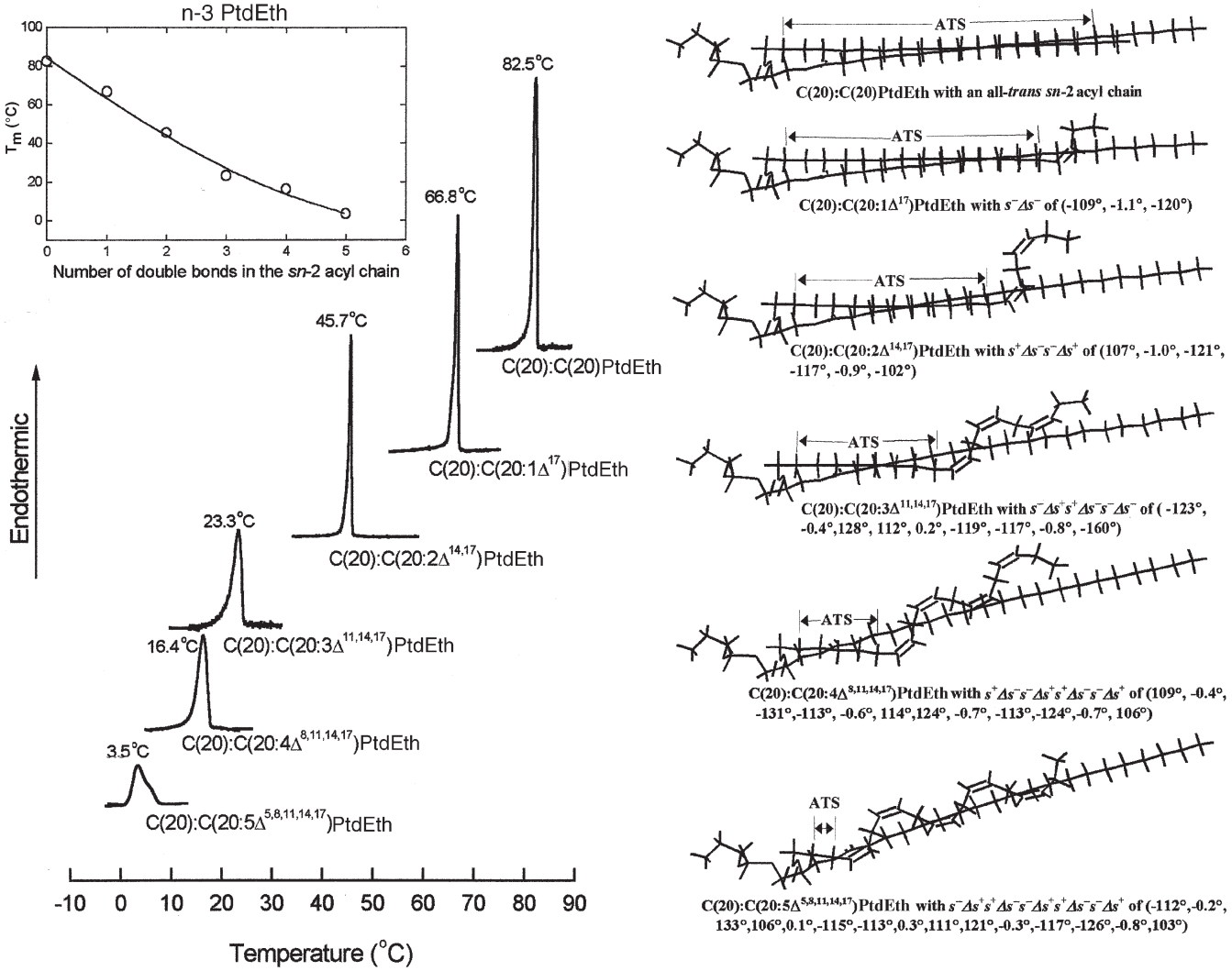


**FIG. 21.** The energy-minimized structures of C(20):C(20)PtdEth and its five unsaturated n-3 derivatives are presented in the presence of the thermograms exhibited by these six lipid species. The $T_m$-profile shown in the inset can be roughly correlated with the variation of the ATS length in the *sn*-2 acyl chains of these six lipid species.

model, the consecutive C(20):C(20:1$\Delta^{11}$)PtdEth $\rightarrow$ C(20):C(20:2$\Delta^{11,14}$)PtdEth $\rightarrow$ C(20):C(20:3$\Delta^{11,14,17}$)-PtdEth conversion should be accompanied by a down-and-up $T_m$-profile. And this is indeed observed calorimetrically as shown in Figure 22. Moreover, such a down-and-up $T_m$-profile has also been observed calorimetrically for PtdCho in two different $\Delta^{11}$PtdCho series (29).

I would now like to conclude by considering once more the $T_m$-diagram (Fig. 16) constructed for mixed-chain phospholipids with saturated and unsaturated *sn*-1 and *sn*-2 acyl chains, respectively, that are originated from a common precursor, C(20):C(20)PtdEth. All unsaturated lipids with two or more *cis*-double bonds shown in this $T_m$-diagram have methylene-interrupted *cis*-double bonds, which are the hallmark of the naturally occurring phospholipids found in animal cells. Moreover, the energy-minimized structures of all the unsaturated *sn*-2 acyl chains of lipids shown in this $T_m$-diagram are characterized by a kinked crankshaft-like motif as obtained by MM calculations. A novel virtue of this $T_m$-diagram is that the $T_m$-profiles exhibited by various series of lipids arranged along the horizontal, vertical, and diagonal lines can be compared simply and simultaneously. In this sense, the $T_m$-diagram is somewhat analogous to the periodic table, in which all chemical elements are arranged simultaneously in a simple and systematic manner. Finally, my emphasis is that a simple molecular model can explain adequately the various shapes of the $T_m$-profiles exhibited by different series of mixed-chain phospholipids shown in this $T_m$-diagram. It is hoped that the experimental and computational results obtained with pure lipids containing well-defined numbers and positions of *cis*-double bonds as summarized in this presentation may lead us to many informative new experiments to be carried out with biological membranes in the future.

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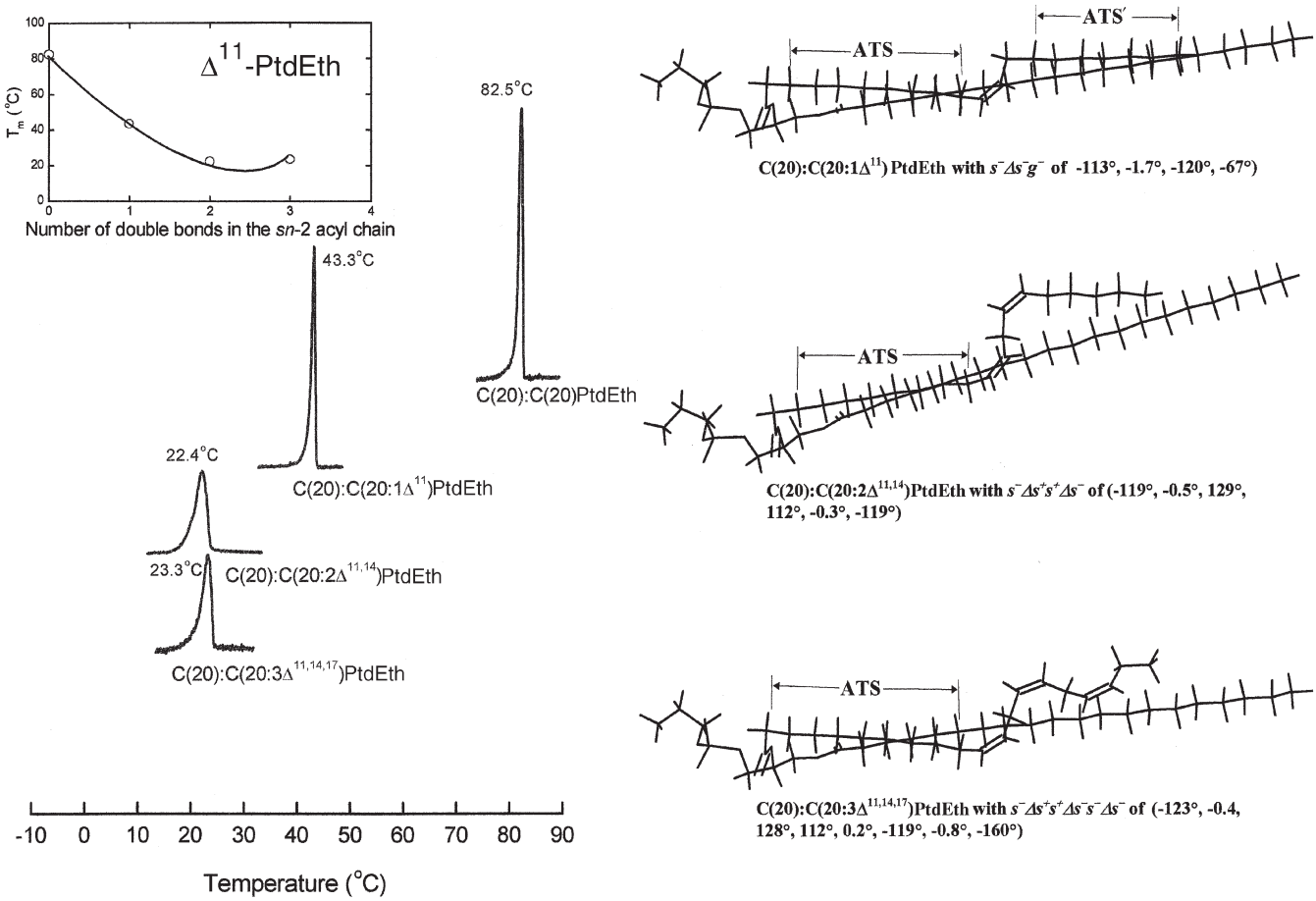


**FIG. 22.** The energy-minimized structures of the three lipids in the $\Delta^{11}$PtdEth-series are shown in the presence of the down-and-up $T_m$-profile exhibited by these lipids.

is expressed to all the students, research associates, and collaborators whose work I have cited in this presentation. I am extremely grateful to Drs. Thomas E. Thompson, Robert G. Langdon, and James W. Ogilvie, who introduced me to the fertile field of membrane lipids while I was a graduate student as well as a junior faculty member. I would also like to express my deep appreciation to Drs. Rodney Biltonen, Joseph Larner, Ronald Taylor, and Thomas E. Thompson for their encouragement and appreciation. Finally, I thank my wife, Laura, and children, Tien-tsin and Tien-wei, for their continuous support during the long period of my research career.

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# Inhibitory Effect of Conjugated Linoleic Acid on Linoleic Acid Elongation in Transformed Yeast with Human Elongase

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**ABSTRACT:** Conjugated linoleic acid (CLA; 18:2) refers to a group of positional and geometric isomers derived from linoleic acid (LA; Δ9,12-18:2). Using a growing baker's yeast (*Saccharomyces cerevisiae*) transformed with human elongase gene, we examined the inhibitory effect of CLA at various concentrations (10, 25, 50, and 100 μM) on elongation of LA (25 μM) to eicosadienoic acid (EDA; Δ11,14-20:2). Among four available individual CLA isomers, only *c*9,*t*11- and *t*10,*c*12-isomers inhibited elongation of LA to EDA. The extent of inhibition (ranging from 20 to 60%) was related to the concentration of CLA added to the medium. In the meantime, only these two isomers, when added at 50 μM to the media, were elongated to conjugated EDA (*c*11,*t*13- and *t*12,*c*14-20:2) by the same recombinant elongase at the rate of 28 and 24%, respectively. The inhibitory effect of CLA on LA elongation is possibly due to competition between CLA isomers and LA for the recombinant elongase. Thus, results from this study and a previous study suggest that the biological effect of CLA is exerted through its inhibitory effect on Δ6-desaturation as well as elongation of LA which results in a decrease in long-chain n-6 fatty acids and consequently the eicosanoid synthesis.

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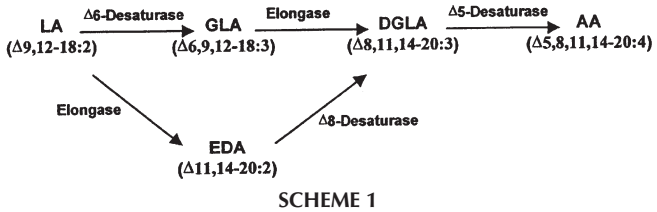
Conjugated linoleic acid (CLA), a mixture of positional and geometric dienoic isomers derived from linoleic acid (LA, Δ9,12-18:2), has many beneficial effects in animals. It can decrease the development of atherosclerosis in rabbits and hamsters (1,2) and modulate the immune function in rats (3). CLA can also decrease breast cancer cell proliferation and inhibit mammary gland, skin, and stomach tumorigenesis in experimental animals (4–9).

The mechanism by which CLA exerts its biological function is still not fully understood. CLA may modulate the immune function through a modification of eicosanoid synthesis (10–12). Results from our previous study demonstrated that CLA significantly inhibited Δ6-desaturation (13), the rate-limiting step for the production of polyunsaturated fatty acid (PUFA) and eicosanoids in mammalian cells. In these cells, when Δ6 desaturation is suppressed, an alternate metabolic pathway for LA to form PUFA could also take place (see Scheme 1). Some LA could be elongated to form eicosadienoic acid (EDA; Δ11,14-20:2) (14), followed by Δ8-desaturation to form dihomo-γ-linolenic acid (DGLA; Δ8,11,14-20:3) and subsequently to arachidonic acid (AA, Δ5,8,11,14-20:4) by the action of Δ5-desaturase (15,16). Since there was a significant decrease of AA in the CLA-fed animals (11,17), it is reasonable to postulate that CLA can inhibit not only the Δ6-desaturation but also the elongation of LA.

The objective of this study was to examine whether CLA could inhibit the elongation of LA by directly competing with the enzyme elongase, the first step of the alternate pathway. This study was performed in a simple system of an established transformed yeast containing the human PUFA-specific elongase gene without the presence of other enzymes in the metabolic pathway of PUFA synthesis.

## MATERIALS AND METHODS

*Chemicals.* Triheptadecanoin (a synthetic triacylglycerol containing three molecules of heptadecanoic acid, 17:0) LA, EDA, and a mixture of CLA isomers (free fatty acid form, containing 41% of *c*9,*t*11-isomer, 44% of *t*10,*c*12-isomer, and others) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Four individual isomers of CLA (*c*9,*t*11; *t*9,*t*11; *c*9,*c*11; and *t*10,*c*12) and a mixture of conjugated eicosadienoic acid (CEDA) isomers (free fatty acid form, containing 53% of *c*11,*t*13 isomer, 29% of *c*11,*c*13 isomer, and others) were obtained from Matreya Inc. (Pleasant Gap, PA). Yeast minimal medium (YMM) was prepared by mixing 26.7 g Dropout base (DOB) medium and 0.69 g complete supplement mixture




SCHEME 1

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Abbreviations: AA, arachidonic acid (Δ5,8,11,14-20:4); CEDA, conjugated eicosadienoic acid; CLA, conjugated linoleic acid; CSM-LEU, complete supplement mixture minus leucine; DGLA, dihomo-γ-linolenic acid (Δ8,11,14-20:3); DOB, Dropout base; EDA. eicosadienoic acid (Δ11,14-20:2); GC, gas chromatography; GLA, γ-linolenic acid (Δ6,9,12-18:3); LA, linoleic acid (Δ9,12-18:2); MS, mass spectrometry; PUFA, polyunsaturated fatty acid; YMM, yeast minimal medium.

minus leucine (CSM-LEU). Both DOB medium and CSM-LEU were from Bio 101, Inc. (Vista, CA). YPD medium containing yeast extract, peptone, and dextrose was from Difco Laboratories (Detroit, MI). Hexane was ultraviolet grade and other solvents were distilled-in-glass quality.

*Plasmids and yeast strain.* The pYX242 expression vector was chosen for the construction of the clone pRAE-58. This expression vector has a strong, constitutive triose phosphate isomerase promoter, leucine selection marker, and ampicillin resistance marker. The pYX242 is a 2μ plasmid, which allows it to replicate autonomously in yeast and be stably maintained at 25–100 copies per cell. In this study, two plasmids, pYX242 (vector only) and pRAE-58 (with human elongase cDNA), were constructed and transformed into a host strain of *Saccharomyces cerevisiae*, SC334 (18). The transformation protocol and growth conditions followed the procedures described previously (18).

*Incubation conditions and experimental design.* Colonies of the transformed yeasts were grown overnight in YPD medium at 30°C. Cultures ($1 \times 10^8$ cells) were then inoculated into 50 mL YMM. Cell numbers were maintained at the same level in all studies. The cultures were grown at 30°C for 48 h. The culture temperature (30°C) has previously been shown to be optimal for expression of the elongase activity (18). Cells were harvested by centrifugation, and cell pellets were washed once with sterile distilled-deionized $H_2O$. The yeast transformed with only vector (pYX242) was used as the negative control.

To confirm whether the activity of elongase (conversion of LA to EDA) was expressed in the transformed yeast, LA (100 μM) was provided as the exogenous substrate in the YMM. To study if CLA could affect the conversion rate of LA to EDA, CLA (as a mixture of four isomers or individual isomers) was supplemented to the medium at different levels (10, 25, 50, and 100 μM) while LA was maintained at 25 μM. To examine whether CLA could be elongated to CEDA, 100 μM of four individual isomers were added to the medium separately. To determine the uptake of substrates (i.e., LA and CLA isomers) and conversion rates of LA to EDA and CLA to CEDA by elongase, LA and two CLA isomers (*c*9,*t*11 and *t*10,*c*12) were supplemented at 50 μM.

*Lipid extraction and fatty acid analysis.* The extraction of yeast lipids was performed according to the procedure described previously (13). Briefly, the rinsed cell pellet was extracted with 20 mL of chloroform/methanol (2:1, vol/vol) containing 16 μg triheptadecanoin (used as the internal standard). After extraction, the yeast lipids were saponified and methylated as described by Yamasaki *et al.* (19). Fatty acid methyl esters were then analyzed by gas chromatography (GC) using a flame-ionization detector and a fused-silica capillary column (Omegawax; 30 m × 0.32 mm, i.d., Supelco, Bellefonte, PA). The identity of CEDA was confirmed by GC–mass spectrometry (MS), using a Hewlett-Packard mass selective detector (model 5972) operating at an ionization voltage of 70 eV with a scan range of 20–500 Da. The mass spectrum of any new peak obtained was compared with that of standard in the database NBS75K.L (National Bureau of Standards). In this study, the conversion of substrates to products was determined based on the ratio of [product]/[product + substrate] × 100%. The amount of LA and CLA isomers taken up by the yeast was calculated from the percentage of LA or CLA isomers in total yeast lipids.

*Statistical analyses.* Data were analyzed by analysis of variance and Fisher's protected least significant difference to determine differences between means of the uptake rates and between means of the conversion rates. Means differences were considered significant at the $P \leq 0.05$ level.

## RESULTS

When the recombinant human elongase, expressed in yeast strain 334(pRAE-58), was incubated with 100 μM linoleic acid (LA) for 48 h, a substantial portion (13%) of LA was elongated to form EDA (Fig. 1B). There were also increases in the levels (relative concentration as well as absolute amount) of Δ11-18:1, the elongation product of palmitoleic acid (Δ9-16:1), and Δ13-20:1, the elongation product of Δ11-18:1. No increases in these elongation products (Δ11-18:1, Δ13-20:1, and EDA) were observed in the control 334(pYX242) yeast (Fig. 1A).

To examine the effect of CLA on elongation of LA to EDA in the transformed yeast, various concentrations of CLA mixture (10, 25, 50, or 100 μM) were added to the growth medium containing 25 μM of LA. Results in Figure 2A show that addition of the CLA mixture inhibited the conversion of LA to EDA in the transformed yeast. Elongation of LA to EDA was inhibited by 50% when equal concentrations of

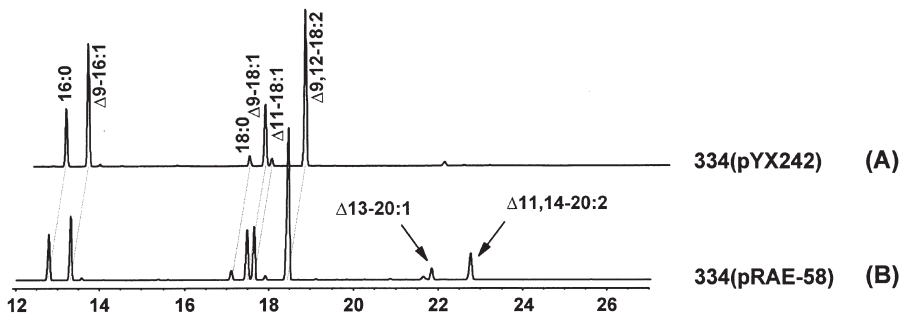


**FIG. 1.** Gas chromatographic analysis of fatty acid methyl esters (FAME) from the total lipids of the transformed yeast with (A) only the vector [334(pYX242)] or (B) the elongase gene [334(pRAE-58)]. All yeast cells were incubated in the medium containing 100 μM linoleic acid (LA). Arrows indicate the appearance of Δ13-20:1, the elongation product of Δ11-18:1, and eicosadienoic acid (Δ11,14-20:2, EDA), the elongation product of LA.

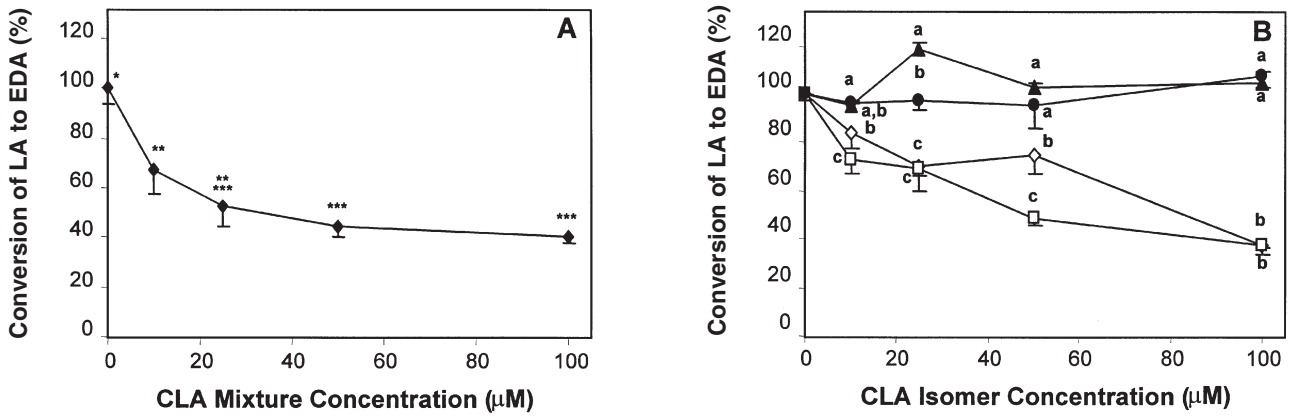


**FIG. 2.** Effect of different concentrations of conjugated linoleic acid (CLA) isomers in mixture (A) or individually (B) on elongation of LA to EDA in yeast transformed with human elongase gene. LA was maintained at 25 μM in the medium. Conversion of LA to EDA was calculated as [product/(product + substrate)] × 100%. Yeasts incubated with medium containing only 25 μM LA were designed as the control (100%). Each value point represents the mean of three incubations. At same substrate concentration, values with different letters (a,b,c) or superscripts (*,**,***) indicate a significant difference ($P < 0.05$) in the decrease in elongation of LA to EDA. *c*9,*t*11-CLA isomer (◇); *t*10,*c*12-CLA isomer (□); *c*9,*c*11-CLA isomer (▲); and *t*9,*t*11-CLA isomer (●).

CLA (25 μM) and LA (25 μM) were added to the medium. No additional inhibitory effect was observed when the concentration of CLA was greater than 25 μM.

The effect of individual CLA isomer on the elongation of LA to EDA was also examined. Figure 2B depicts that only *c*9,*t*11- and *t*10,*c*12-CLA isomers inhibited the conversion of LA to EDA, whereas the other two isomers (*c*9,*c*11- and *t*9,*t*11-CLA) exerted no such effect.

To examine whether CLA itself could be elongated by the same recombinant elongase, the transformed 334(pRAE-58) yeast were incubated with four individual CLA isomers separately for 48 h. Results in Figure 3 show that only two isomers, *c*9,*t*11- and *t*10,*c*12-CLA, could be elongated to form *c*11,*t*13-20:2 and *t*12,*c*14-20:2, respectively (Figs. 3A, 3B). No detectable elongation metabolites were observed in the 334(pRAE-58) yeasts incubated with either *c*9,*c*11- or *t*9,*t*11-CLA isomers (Figs. 3C, 3D).

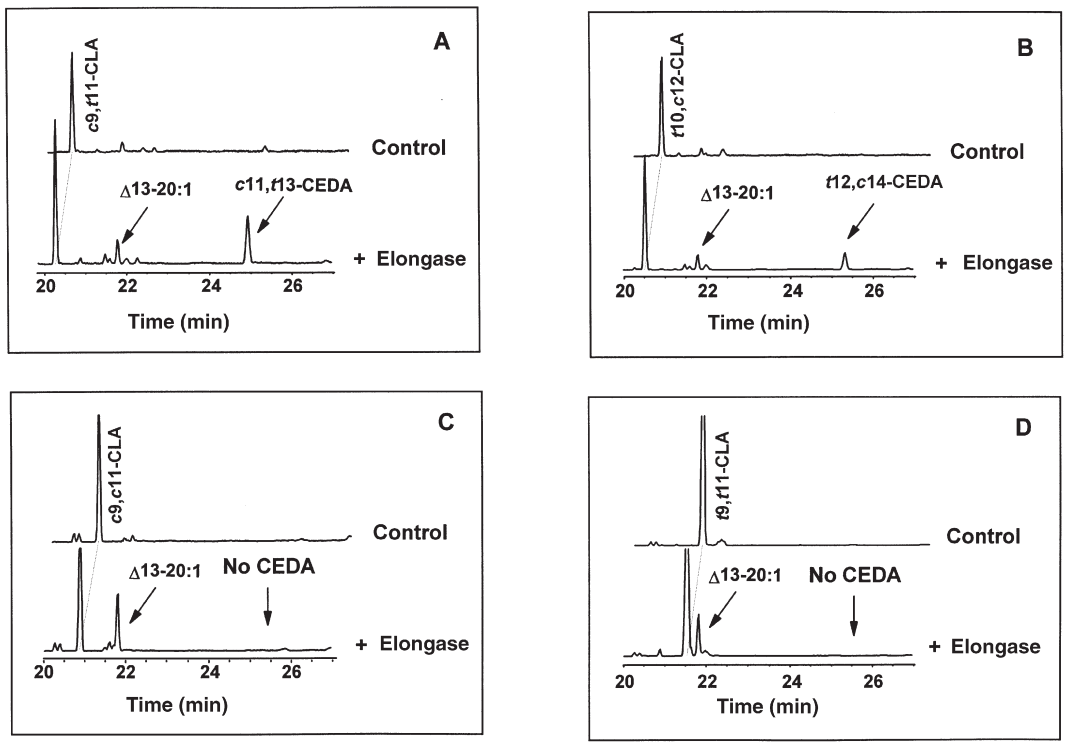


**FIG. 3.** Gas chromatographic analysis of FAME from the total lipids of yeasts transformed with only the vector (Control) or the elongase gene. Yeast cells were incubated in their respective meda containing *c*9,*t*11-CLA (A); *t*10,*c*12-CLA (B); *c*9,*c*11-CLA (C); and *t*9,*t*11-CLA (D). Arrows indicate the appearance Δ13-20:1 and conjugated eicosadienoic acid (CEDA) isomer. For other abbreviations see Figures 1 and 2.

The identity of *c*11,*t*13-20:2, the elongation product of the *c*9,*t*11-18:2, was confirmed by its retention time (25 min) in GC; and the mass peak (*m/z* = 322) and fragmentation pattern in the GC–MS spectrum were identical to the authentic *c*11,*t*13-CEDA standard (data not shown). The elongation product of *t*10,*c*12-CLA also had the same mass peak (*m/z* = 322) and a similar fragmentation pattern, but it was different in intensity from *c*11,*t*13-CEDA (data not shown). Although no authentic standard was available for comparison, evidence from later in this report suggests that the peak was *t*12,*c*14-CEDA.

The uptake of the individual CLA isomers (100 μM) and the elongation of individual CLA isomer were also examined. Figure 4 illustrates that *c*9,*t*11- and *t*10,*c*12-CLA were the two active isomers. Approximately 25% of these isomers were taken up by the transformed yeast, and significant amounts of these isomers were elongated to their respective CEDA. In contrast, approximately 40% of *c*9,*c*11- and 70% of *t*9,*t*11-isomers were taken up, but no detectable amounts of elongation products were observed.

To examine the substrate specificity of the recombinant elongase, equal amounts (50 μM) of LA and the two active forms of isomers (*c*9,*t*11- and *t*10,*c*12-CLA) were added separately into the medium, and the percentage of elongation of each substrate was determined. Results in Figure 5 show that the amounts of LA and CLA taken up by the yeast were similar. Approximately 12% of LA was elongated to EDA, but more than 25% of the two active isomers were elongated to CEDA.

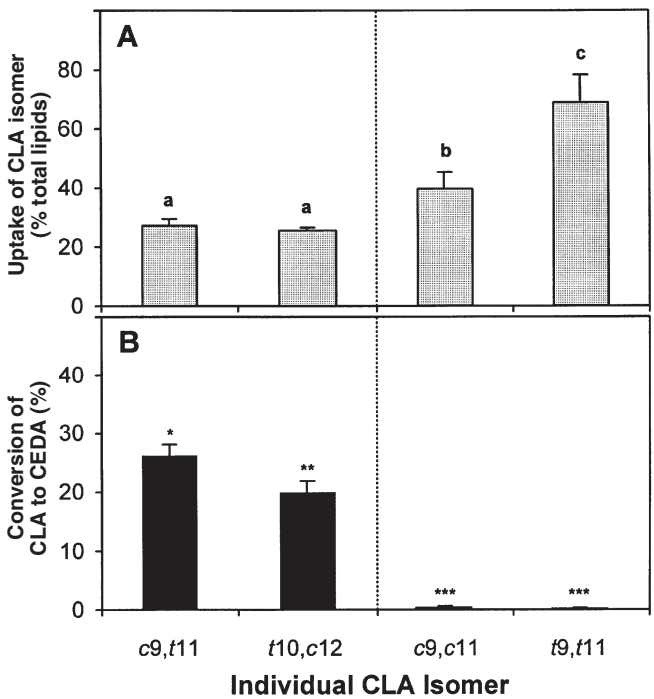


**FIG. 4.** Uptake of CLA (A) and conversion of CLA to CEDA (B) in yeasts transformed with human elongase gene. The yeast cells were cultured in 100 μM of four individual CLA isomers, respectively. The conversion of substrates to products was defined as the same as the legend of Figure 2. The uptake of CLA isomers was calculated based on the sum of CLA and CEDA isomers (% total lipids) in yeast lipids. All results are mean ± SE of three incubations. Values with different symbols or letters significantly differ from each other at $P < 0.05$. For abbreviations see Figures 2 and 3.

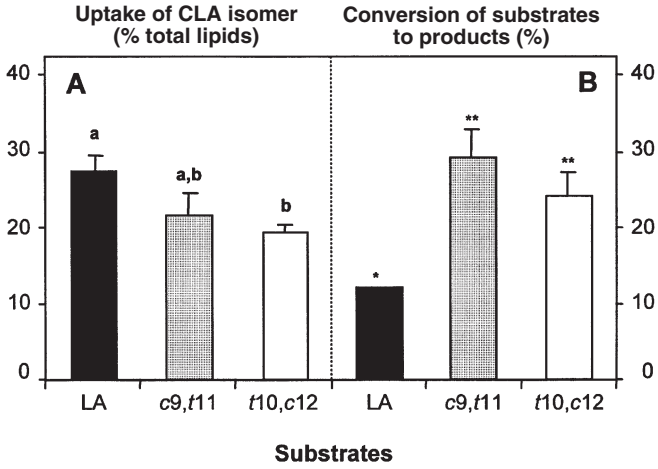


**FIG. 5.** Comparison of uptake of LA and CLA isomers (A) and conversion of LA to EDA and CLA to CEDA (B) in yeasts transformed with human elongase gene. The concentration of LA or CLA in the medium was 50 μM. All results are mean ± SE of three incubations. Values with different symbols or letters indicate a significant difference ($P < 0.05$). For abbreviations see Figures 1–3.

## DISCUSSION

Using a yeast transformed with human PUFA-specific elongase gene, we demonstrated that CLA significantly inhibited the alternate pathway of LA metabolism, i.e., elongation of LA to EDA (Fig. 2A). We also demonstrated that only *c*9,*t*11- and *t*10,*c*12-CLA isomers, among the four available isomers, could inhibit LA elongation and then themselves be elongated (Figs. 3A, 3B). There were no elongation metabolites found from the other two isomers (*c*9,*c*11 and *t*9,*t*11) (Figs. 3C, 3D), despite these two CLA isomers being taken up more readily and incorporated into the 334(pRAE-58) yeast than the two active CLA isomers. Thus, the inability to elongate these two CLA isomers was due to the substrate specificity of the recombinant elongase. It is possible that the recombinant elongase recognize only *cis*-, *trans*- or *trans*-,*cis*- configuration, but not *cis*-,*cis*- or *trans*-, *trans*- configuration of CLA. These findings suggest that the inhibitory effect of CLA on LA elongation was a result of competition between the two *c*9,*t*11- and *t*10,*c*12-CLA isomers and LA as substrate for the recombinant elongase in the transformed yeasts. Interestingly, these two isomers have been previously identified as the most biologically active CLA isomers (9,13, 20,21).

CLA plays an important role in modulating immune functions (10). The decrease of eicosanoid synthesis is attributed to the competitive role of CLA in n-6 PUFA metabolism (10–12). Another hypothesis suggests that incorporation of CLA into the cell membrane might change membrane fluidity which, in turn, influences the mobility of receptors and membrane proteins and affects signal transduction, antigen recognition, receptor-ligand interactions, and cell cycle induction (22). Unfortunately, no direct evidence to support this interesting hypothesis is yet

available. On the other hand, results from the present study and from our previous study (13), demonstrate that the two active forms of CLA (*c*9,*t*11- and *t*10,*c*12-) can inhibit the two metabolic pathways of LA (Δ6-desaturation and elongation) and hence decrease PUFA (e.g., AA) synthesis. This in turn could decrease the amount of AA entering the cyclooxygenase/lipoxygenase pathways for eicosanoid synthesis in mammalian cells.

In conclusion, we demonstrated in this study that CLA can inhibit the elongation of LA to EDA, and thus repress the alternate metabolic pathway of LA. The inhibition is likely due to the competition between LA and two active CLA isomers (*c*9,*t*11- and *t*10,*c*12-) for the enzyme elongase. Only *c*9,*t*11- and *t*10,*c*12-CLA isomers could be metabolized by the transformed yeast with recombinant elongase, and only these two isomers exerted the suppressive effect on conversion of LA to EDA. Together with a previous study, we suggest that the *c*9,*t*11- and *t*10,*c*12-isomers are the two active forms of CLA, and their ability to modulate LA metabolism may be responsible at least in part for their biological effects.

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# A Unique Antioxidant Activity of Phosphatidylserine on Iron-Induced Lipid Peroxidation of Phospholipid Bilayers

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**ABSTRACT:** The relationship between the antioxidant effect of acidic phospholipids, phosphatidic acid (PA), phosphatidylglycerol (PG) and phosphatidylserine (PS), on iron-induced lipid peroxidation of phospholipid bilayers and their abilities to bind iron ion was examined in egg yolk phosphatidylcholine large unilamellar vesicles (EYPC LUV). The effect of each acidic phospholipid added to the vesicles at 10 mol% was assessed by measuring phosphatidylcholine hydroperoxides (PC-OOH) and thiobarbituric acid-reactive substances. The addition of dipalmitoyl PS (DPPS) showed a significant inhibitory effect, although the other two acidic phospholipids, dipalmitoyl PA (DPPA) and dipalmitoyl PG (DPPG), did not exert the inhibition. Neither dipalmitoyl PC (DPPC) nor dipalmitoyl phophatidylethanolamine (DPPE) showed any remarkable inhibition on this system. None of the tested phospholipids affected the lipid peroxidation rate remarkably when the vesicles were exposed to a water-soluble radical generator. The iron-binding ability of each phospholipid was estimated on the basis of the amounts of iron recovered in the chloroform/methanol phase after separation of the vesicle solution to water/methanol and chloroform/methanol phases. EYPC LUV containing DPPS, DPPA, and DPPG had higher amounts of bound iron than those containing DPPC and DPPE, indicating that these three acidic phospholipids possess an iron-binding ability at a similar level. Nevertheless, only DPPS suppressed iron-dependent decomposition of PC-OOH significantly. Therefore, it is likely that these three acidic phospholipids possess a significant iron-binding ability, although this ability *per se* does not warrant them antioxidative activities. The ability to suppress the iron-dependent decomposition of PC-OOH may explain the unique antioxidant activity of PS.

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The peroxidation of phospholipid bilayers composing biomembranes has been recognized to be one of the primary events leading eventually to the damage of cellular and subcellular membranes. Moreover, the occurrence of phospholipid peroxidation has been observed in degenerative diseases (1,2). Use of liposomal vesicles as a model membrane in the study of antioxidants against the destruction of biomembranes has brought out several important features of their action mechanism (3–5). Because of its availability and convenience, lamellar vesicles prepared from egg yolk phosphatidylcholine (EYPC) frequently have been allowed to oxidize with various prooxidants such as heme compounds (6,7), transition metal ions (8,9), and so on.

The antioxidant activity of phospholipids has been reported to be a consequence of the binding of prooxidant species, often transition metal ions, by the anionic group in their structures (3). Yoshida *et al.* (8) reported the inhibitory effect of phosphatidylserine (PS), and Tadolini *et al.* (10) claimed the inhibitory effect of phosphatidic acid (PA) using EYPC lamellar vesicles as a model system to study the iron ion-induced lipid peroxidation. In both cases, the inhibitory effect was reported to be associated with the anionic charge of the polar head group. However, the mechanism of the interaction of the anionic head group of each phospholipid with iron ion and its efficiency on iron ion-induced lipid peroxidation is still unclear. Therefore, the mechanism of inhibition or promotion of lipid peroxidation by phospholipids is still a subject of argument. The purpose of this study is to clarify whether the ability of binding iron ions by acidic phospholipids can fully explain their antioxidant activity on iron ion-induced lipid peroxidation. To better understand the antioxidant mechanism of acidic phospholipids, their ability to prevent the iron ion-dependent decomposition of PC hydroperoxides (PC-OOH) was also evaluated.

## MATERIAL AND METHODS

*Chemicals.* Dipalmitoyl phosphatidic acid (DPPA), dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylcholine (DMPC), egg yolk phosphatidylcholine (EYPC), dipalmitoyl phosphatidylethanolamine (DPPE), dipalmitoyl phosphatidylglycerol (DPPG), dipalmitoyl phosphatidylserine (DPPS), and desferal were purchased from Sigma Chemical Co. (St. Louis, MO). The purchased EYPC was purified to remove preformed PC-OOH following the method described by Terao *et al.* (11). The PC-OOH content in purified EYPC was lower than 0.14 mol% of the preparation. Iron(III) nitrate and 2,2′-azobis(2-amidinopropane)-dihydrochloride (AAPH) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were of reagent grade and were used without

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Abbreviations: AAPH, 2,2′-(2-amidinopropane)-dihydrochloride; BBPS, bovine brain phosphatidylserine; DMPC, dimyristoyl phosphatidylcholine; DPPA, dipalmitoyl phosphatidic acid; DPPC, dipalmitoyl phosphatidylcholine; DPPE, dipalmitoyl phosphatidylethanolamine; DPPG, dipalmitoyl phosphatidylglycerol; DPPS, dipalmitoyl phosphatidylserine; EYPC, egg yolk phosphatidylcholine; HPLC, high-performance liquid chromatography; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PC-OOH, phosphatidylcholine hydroperoxides; TBARS, thiobarbituric acid reactive-substances.

purification. PC-OOH was prepared from EYPC using soybean lipoxygenase (12) and purified by reverse-phase column chromatography by the method described previously (11).

*Preparation of unilamellar vesicles.* A purified EYPC in hexane solution and each phospholipid in chloroform/methanol (95:5, vol/vol) solution added at 10 mol% to EYPC were placed into test tubes, and the solvent was removed completely under a stream of nitrogen gas followed by further removal using a vacuum pump. Then, 0.7 mL of Tris-HCl buffer (10 mM, pH 7.4) was added to the thin lipid film on the glass wall and mixed by vortex for 1 min followed by ultrasonic irradiation for 30 s (Astrason Sonicator W-380, Heat Systems-Ultrasonics, Inc., New York) to obtain the suspension of multilamellar vesicles (MLV). Large unilamellar vesicles (LUV) were prepared from MLV by an extrusion method (13). The suspension was extruded (21 times) through a polycarbonate filter (100 nm pore size diameter) mounted in an extrusion apparatus (Liposofast; Avestin Inc., Ottawa, Canada).

*Oxidation of LUV*. After 5 min of preincubation of EYPC LUV (0.5 mL) with each added phospholipid in the dark at 37°C with continuous shaking, lipid peroxidation was initiated by the addition of 0.1 mL of $Fe(NO_3)_3$ and ascorbate solution or 0.1 mL of AAPH. The final concentrations of the components in the system were as follows: EYPC, 5 mM; phospholipids, 0.5 mM; $Fe(NO_3)_3$/ascorbic acid, 50 μM/500 μM, respectively; and AAPH, 10 mM. The reaction mixture was incubated in the same conditions of preincubation.

*Determination of PC-OOH.* The quantification of PC-OOH was carried out according to Terao *et al.* (11) using a high-performance liquid chromatography (HPLC) methodology. Aliquots of reaction mixture were withdrawn at specific time intervals and injected into an HPLC apparatus (Shimadzu SPD-10 A, Kyoto, Japan) equipped with a column Tosoh TSK gel RP-8 (150 × 4.6 mm) with a flow rate of 1.0 mL/min and detection at 235 nm. The concentration of PC-OOH was calculated from the standard curve of PC-OOH.

*Measurement of thiobarbituric acid-reactive substances (TBARS).* Thiobarbituric acid assay was performed by the method of Uchiyama and Mihara (14), and the amount of TBARS was expressed as (μM malonaldehyde equivalents using an equation obtained from a standard curve of tetraethoxypropane at the appropriate concentration.

*Iron-binding of phospholipids*. EYPC LUV (0.5 mL) containing each phospholipid at 10 mol% were prepared as described. Then, 0.1 mL of $Fe(NO_3)_3$ and ascorbate solution was added to the unilamellar vesicles. The final concentrations of the components in the reaction system were as follows: EYPC, 5.0 mM; phospholipids, 0.5 mM; and $Fe(NO_3)_3$/ascorbic acid, 50 μM/500 μM, respectively. The phase separation was accomplished by adding 2.0 mL of chloroform/methanol (1:1, vol/vol) to the mixture, followed by vortex mixing for 1 min. Afterward, the mixture was left to stand for 5 min to obtain a separation into two phases. The iron contents in aqueous and organic phases were measured using a flame atomic absorption spectrophotometer (Hitachi, Z-6100 Polarized Zeeman, Tokyo, Japan).

*The effect of phospholipids on iron-dependent decomposition of PC-OOH.* PC-OOH (0.1 μmol) and each phospholipid or desferal (0.1 μmol) in 0.8 mL of Tris-HCl buffer (10 mM, pH 7.4) were mixed by vortex and ultrasonicated for 30 s, followed by the addition of 0.2 mL of $Fe(NO_3)_3$ and ascorbate solution in Tris-HCl buffer (10 mM, pH 7.4). The final concentration of the components in the reaction systems was as follows: PC-OOH, 100 μM; phospholipids or desferal, 100 μM; $Fe(NO_3)_3$/ascorbic acid, 10 μM/100 μM, respectively. After 10 min of incubation of the reaction mixture at 37°C, the extent of PC-OOH decomposition was quantified by the measurement of PC-OOH using HPLC as described.

*Ultrafiltration of DMPC LUV.* DMPC LUV containing each phospholipid at 10 mol% to DMPC were prepared by the same procedure described above. Then, DMPC LUV (0.5 mL) was preincubated for 5 min in the dark at 37°C with continuous shaking, followed by the addition of 0.1 mL of $Fe(NO_3)_3$ and ascorbate solution. The final concentration of DMPC, phospholipids, and initiators were the same as of those described in the oxidation of EYPC LUV. Then, the mixture was incubated for 10 min under the same conditions of preincubation. At the end of incubation, DMPC LUV (0.3 mL) was submitted to centrifugal separation at 12,000 rpm for 40 min using ultrafiltrate membrane UFC3TGC00 with pore size of 10 nm (Millipore Co, Tokyo, Japan). The lower filtrate and upper layers were collected for the measurement of volumes.

*Statistical analysis.* Assays were carried out using triplicate samples. Statistical analysis was performed using the one-way analysis of variance followed by the Bonferroni/Dunn *post hoc* multiple comparison test. The level of significant difference was set at $P < 0.05$.

## RESULTS

*Antioxidant activity of phospholipids on iron-induced lipid peroxidation in EYPC LUV.* The antioxidant effect of phospholipids on iron-induced lipid peroxidation in EYPC LUV was estimated either by their ability to inhibit the accumulation of PC-OOH (Fig. 1A) or TBARS (Fig. 1B). During 5 h of incubation, PC-OOH scarcely accumulated in the vesicles containing DPPS. EYPC LUV containing DPPG, DPPC, DPPA, and DPPE showed a fast accumulation of PC-OOH during the first 3 h of incubation. After 5 h of incubation, EYPC LUV containing DPPS provided significantly lower amounts of TBARS (27.8–38.2%) than the other four vesicle solutions.

*Antioxidant activity of phospholipids on AAPH-induced lipid peroxidation in EYPC LUV.* The antioxidant activity of each phospholipid was evaluated on aqueous radical-induced lipid peroxidation, using AAPH (Fig. 2). EYPC LUV containing each added phospholipid showed a rapid increase in the amounts of PC-OOH during the first 3–4 h. During the fifth hour of incubation, PS and the other phospholipids somewhat inhibited the accumulation of PC-OOH. After 5 h of incubation, the amounts of TBARS were similar in all EYPC LUV tested.

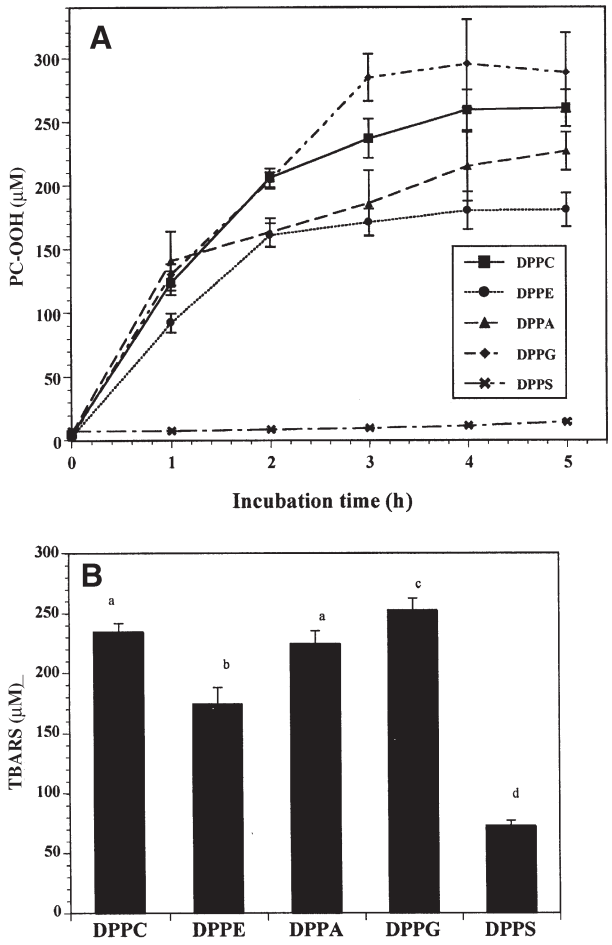


**FIG. 1.** Effect of added phospholipids in iron-induced lipid peroxidation of EYPC LUV. (A) PC-OOH formation; (B) TBARS accumulation after 5 h of incubation. EYPC LUV (0.5 mL) containing each phospholipid at 10 mol% was mixed with 0.1 mL of $Fe(NO_3)_3$ and ascorbic acid. The final concentration of components in the system was EYPC (5 mM), phospholipids (0.5 mM), $Fe(NO_3)_3$/ascorbic acid (50 μM/500 μM, respectively). The reaction was carried out in the dark at 37°C with continuous shaking. Results are means ± standard deviation of three independent measurements. Means not sharing a common letter are significantly different ($P < 0.05$). EYPC LUV, egg yolk phosphatidylcholine large unilamellar vesicles; PC-OOH, phosphatidylcholine hydroperoxide; TBARS, thiobarbituric acid-reactive substances; DPPC, dipalmitoyl phosphatidylcholine; DPPE, dipalmitoyl phosphatidylethanolamine; DPPA, dipalmitoyl phosphatidic acid; DPPG, dipalmitoyl phosphatidylglycerol; DPPS, dipalmitoyl phosphatidylserine.

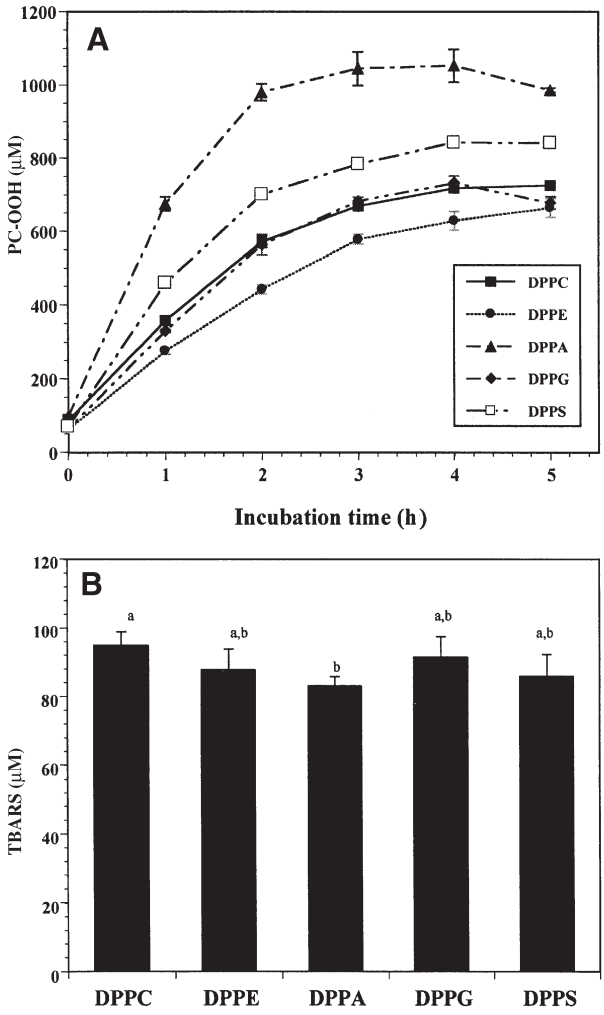


**FIG. 2.** Effect of added phospholipids on AAPH-induced lipid peroxidation in EYPC LUV. (A) PC-OOH accumulation; (B) TBARS formation after 5 h of incubation. Lipid peroxidation in EYPC LUV (0.5 mL) containing added phospholipids at 10 mol% was mixed with 0.1 mL of AAPH solution. The final concentration of EYPC and the phospholipids was the same as those in Figure 1, and the final concentration was AAPH, 10 mM. The reaction was carried out in the dark at 37°C with continuous shaking. Results are means ± standard deviation of three independent measurements. Means not sharing a common letter are significantly different ($P < 0.05$). AAPH, 2,2′-(2-amidinopropane)-dihydrochloride. For other abbreviations, see Fig. 1.

*Iron-binding activity of phospholipids.* The phosphorus assay (15) confirmed that all phospholipids moved to the organic phase (chloroform/methanol phase) after centrifugation of the mixture of LUV solution and chloroform/methanol. Consequently, iron bound to phospholipids should be present in the organic phase and free iron in the aqueous phase (water/methanol phase), respectively. The amount of iron recovered in the chloroform/methanol phase from EYPC LUV containing PS, PA, and PG was higher by far than that from EYPC LUV containing PC and PE (Fig. 3). Meanwhile, EYPC LUV with neutral (PC and PE) phospholipids showed a higher amount of iron in the aqueous phase than the three acidic phospholipids.

*Ability of phospholipids to prevent the iron/ascorbic acid–dependent decomposition of PC-OOH.* The ability of phospholipids to prevent the iron-dependent PC-OOH decomposition was estimated on the basis of the decrease of PC-OOH after the reaction with $Fe(NO_3)_3$/ascorbic acid (Fig. 4). In the absence of any added phospholipids (control), approximately 74% of PC-OOH was decomposed. Phospholipids other than DPPS had little preventive effect on PC-OOH decomposition. However, DPPS significantly prevented the decomposition of PC-OOH and its effect was comparable to that of desferal, a well-known iron ion chelator.

*Water-holding ability and perturbation of DMPC LUV.* The effect of incorporation of each phospholipid into the vesicles on water-holding ability and further perturbation of the

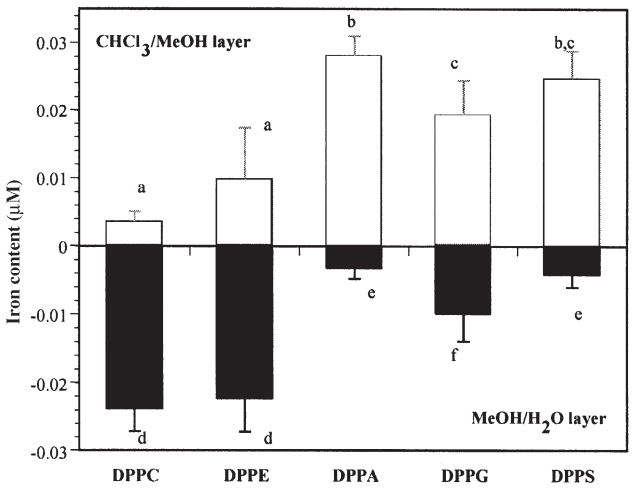


**FIG. 3.** Iron-binding activity of phospholipids. $Fe(NO_3)_3$ and ascorbic acid (0.1 mL) were added to the EYPC LUV (0.5 mL) containing phospholipids at 10 mol%. The final concentration of components in the system was the same as described in Figure 1. The components were separated in two phases by the addition of 2 mL of chloroform/methanol (1:1, vol/vol). Results are means ± standard deviations of three independent measurements. Means not sharing a common letter are significantly different ($P < 0.05$). For abbreviations see Figure 1.

lipid core of LUV was evaluated by submitting the DMPC LUV contents to separation by ultrafiltration. Because of its saturated acyl chain, DMPC was chosen to avoid complication with the formation of lipid peroxidation products during the analysis. After the filtration, the phosphorus assay (15) ensured that phospholipids were present only in the upper phase fraction (data not shown). Therefore the upper phase represented the LUV lipid core. Meanwhile, DMPC LUV containing anionic phospholipids (DPPS, DPPA, and DPPG) produced filtrate volumes higher than those containing neutral phospholipids (DPPC and DPPE) (Table 1). The amounts of collected filtrate volumes increased in the following order: DPPC = DPPE < DPPA < DPPS < DPPG.

**TABLE 1**
**Ultrafiltration of DMPC LUV[a]**

| | Volumes (μL) | | | | |
|---|---|---|---|---|---|
| | DPPC | DPPE | DPPA | DPPG | DPPS |
| Upper phase | 175 ± 10[a] | 165 ± 5[a] | 135 ± 15[b] | 60 ± 5[c] | 100 ± 5[d] |
| Filtrate phase | 125 ± 5[e] | 135 ± 5[e] | 165 ± 10[f] | 240 ± 10[g] | 200 ± 15[h] |

[a]DMPC LUV containing phospholipids at 10 mol% were prepared by the procedure described in the Materials and Methods section. The final concentrations of components in the reaction system were as follows: DMPC (5 mM), phospholipids (0.5 mM), $Fe(NO_3)_3$/ascorbic acid (50 μM/500 μM). At the end of incubation, DMPC LUV (0.3 mL) was submitted to centrifugal separation at 12,000 rpm for 40 min using ultrafiltrate membrane UFC3TGC00 (pore size 10 nm). The filtrate and upper phases were collected for the measurement of volumes. Results are means ± standard deviation of three independent measurements. Means with different roman letter superscripts are significantly different ($P < 0.05$). DMPC LUV, dimyristoyl phosphatidylcholine large unilamellar vesicles; DPPC, dipalmitoyl phosphatidylcholine; DPPE, dipalmitoyl phosphatidylethanolamine; DPPA, dipalmitoyl phosphatidic acid; DPPG, dipalmitoyl phosphatidylglycerol; DPPS, dipalmitoyl phosphatidylserine.

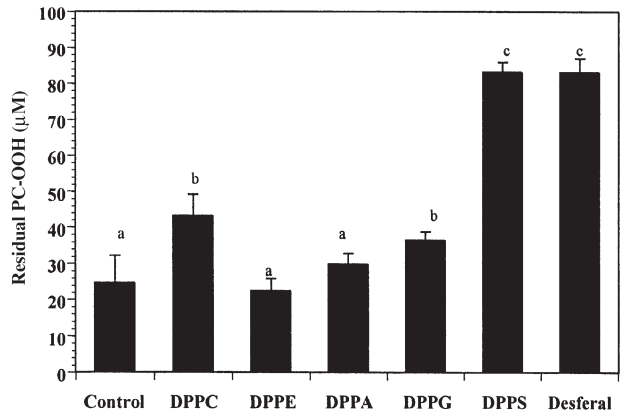


**FIG. 4.** Effect of phospholipids on iron-dependent decomposition of PC-OOH. $Fe(NO_3)_3$ and ascorbic acid (0.2 mL) were added to PC-OOH (0.1 μmol) and phospholipids or desferal (0.1 μmol) in 0.8 mL of 10 mM Tris-HCl buffer (pH 7.4). The final concentration of components in the reaction systems were as follows: PC-OOH, 100 μM; phospholipids or desferal, 100 μM; $Fe(NO_3)_3$ and ascorbic acid, 10 and 100 μM, respectively. The reaction was carried at 37°C for 10 min. Results are means ± standard deviations of three independent measurements. Means not sharing a common letter are significantly different ($P < 0.05$). For abbreviations see Figure 1.

## DISCUSSION

EYPC LUV used in this study were prepared and allowed to oxidize at pH 7.4 in the presence of iron. The increase in PC-OOH amount observed during the incubation of EYPC LUV confirmed the prooxidant role of iron ion/ascorbic acid, as earlier demonstrated by Kunimoto *et al.* (16), Fukuzawa *et al.* (17) and Tadolini *et al.* (18). Moreover, TBARS measurements also confirmed the extent of lipid peroxidation in the vesicles. Among all added phospholipids to EYPC LUV, only the addition of DPPS resulted in a remarkable inhibition of lipid peroxidation (Fig. 1). In contrast, neither DPPS nor other phospholipids tested avoided the accumulation of PC-OOH and TBARS when AAPH was used as a radical generator (Fig. 2). This result indicates that DPPS and other phospholipids tested do not act as radical scavengers.

When the EYPC LUV contents were separated into two phases (organic and aqueous phases), phospholipids were detected only in the organic phase. Thus, phospholipid-bound iron is likely to be dragged into the organic phase whereas free iron seems to remain in the aqueous phase. All EYPC LUV containing added phospholipids with anionic polar head groups (DPPA, DPPG, and DPPS) showed a relatively higher amount of iron in the organic phase, as compared with those with neutral polar head groups (DPPC and DPPE) (Fig. 3). Therefore, it is apparent that the anionic groups of phospholipids are equally responsible for the binding of iron ions. Because the incorporation of phospholipids with anionic groups

in EYPC LUV resulted in a significantly higher amount of trapped irons (DPPA, 92.2%; DPPG, 67.3%; and DPPS, 82.7%) than the incorporation of those containing neutral polar head groups (DPPC, 12.3%; and DPPE, 32.6%), it is expected that all acidic phospholipids contribute to the enhancement of the stability of vesicles against the attack of iron ion. However, this was not the case because two phospholipids with anionic polar head groups, DPPA and DPPG, did not enhance the oxidative stability of LUV (Fig. 1). Earlier, Yoshida *et al.* (8) claimed that the inhibitory effect of PS on iron ion-dependent lipid peroxidation was due to its negatively charged polar head group, which neutralizes the prooxidant action of iron. Present findings suggest that the binding of iron ion by the polar head group of PS does not explain fully the unique antioxidant effect of PS on iron-induced lipid peroxidation.

Prior to this study, we purified the EYPC to eliminate the preformed PC-OOH. However, small amounts of PC-OOH (less than 10 μM) were detected at the start of incubation (Fig. 1A). The iron-induced lipid peroxidation in EYPC LUV is believed to start with the decomposition of preformed PC-OOH by the metal iron, producing unstable species such as peroxyl and alkoxyl radicals, which are later responsible for the propagation of chain reaction (19). Hence, the inhibition of lipid peroxidation rate should be reciprocally associated with the protection of iron-dependent PC-OOH decomposition (Fig. 4). The present study showed the unique ability of DPPS to protect PC-OOH against the attack of iron ion. This protective effect was comparable to that of desferal, a well-known iron ion chelator. The protective mechanism of lipid peroxidation in LUV containing DPPS appears to occur as shown in Scheme 1.

$$Fe^{3+} + AsA^{-} \rightarrow Fe^{2+} + AsA\bullet$$
$$PC\text{-}OOH + Fe^{2+} \rightarrow PC\text{-}O\bullet + OH^{-} + Fe^{3+}$$
$$PC\text{-}O\bullet + PC \rightarrow \text{Radical chain reaction}$$
$$PC\text{-}OOH + Fe^{2+}\text{-DPPS} \rightarrow \text{no decomposition}$$

**SCHEME 1**

$AsA^{-}$: ascorbic acid; PC-O•: alkoxy radical

Other acidic phospholipids (DPPA and DPPG) and neutral phospholipids (DPPC and DPPE) did little to prevent PC-OOH decomposition. Thus, the differences in the reactivity of the phospholipid-bound iron ion toward the PC-OOH preformed in the bilayers should be an essential factor to explain the different behaviors of added phospholipids on liposomal lipid peroxidation. Nevertheless, the mechanisms through which chelators influence the lipid peroxidation reactions are not fully understood (9,20).

While none is bound to the phosphate group in the structure of PA, PG and PS have glycerol and serine moieties, respectively, attached to the phosphate group. The incorporation of charged molecules into phospholipid bilayers may generate an electrostatic charge that is related to the formation of an electrostatic surface potential responsible for a redistribution of cations and anions, including protons and hydroxyl ions at the bilayer–water interface (21). The electrostatic surface at the interface of the membrane may alter the intensity of interaction of iron ion with lipid core. Thus, a stronger barrier at the interface appears to favor the enhancement of the oxidative stability of EYPC LUV. This could also affect the water-holding activity of the bilayers. As shown by changes in water-holding activity (Table 1), the incorporation of acidic phospholipids, DPPA, DPPG and DPPS, seems to cause changes in the size of EYPC LUV and disturb their rigidity. However, this observation does not explain the unique antioxidant activity of PS, because the water-holding ability was nonspecific and not correlated with the suppression of lipid peroxidation.

The role of phosphoserine on lipid peroxidation of EYPC LUV was also examined (data not shown) in order to clarify whether the inhibitory effect of PS depends only on the polar head group. The inhibitory effect of DPPS did not depend only on the action of phosphoserine but may depend on the whole structure of PS as one compound. The acyl chain of PS may play a key role in localization and distribution of phosphoserine in the interface of membrane where the inhibition of iron ion-dependent lipid peroxidation seems to take place.

PS is an essential component of human cellular and subcellular membranes. Several studies have suggested that treatment with PS is effective on the age-related mental decline (22,23). PS is also postulated to function as a regulator of immune and inflammatory responses (24,25). The antioxidant effect of PS shown in this study may be related to some pharmacological activities or physiological activities. Further studies are required to clarify its role in oxidative stress in cellular and subcellular systems.

In conclusion, PS possesses a unique antioxidant activity on iron-induced lipid peroxidation in phospholipid membranes. This effect may originate from its effective prevention of preformed hydroperoxide decomposition.

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# Antioxidative Activity of 3,4-Dihydroxyphenylacetic Acid and Caffeic Acid in Rat Plasma

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**ABSTRACT:** The purpose of the present paper is to study and compare *in vitro* the inhibitory effect of 3,4-dihydroxyphenylacetic acid (DOPAC) and caffeic acid (CA) on lipid peroxidation in rat plasma. Rat plasma was oxidized at 37°C by the radical initiators 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) or 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN). The consumption of endogenous α-tocopherol (α-TOH) and the accumulation of conjugated diene hydroperoxides were measured by high-performance liquid chromatography and by ultraviolet spectroscopy, respectively. α-TOH was consumed at the same rate in the presence of 20 mM AAPH or 2 mM MeO-AMVN. DOPAC and CA suppressed the α-TOH consumption in a dose-dependent manner. A concentration of 50 μM of both phenolic acids was sufficient to induce a lag phase and to delay the rate of α-TOH consumption. The effect was more pronounced in rat plasma oxidation by AAPH than by MeO-AMVN. CA spared vitamin E more effectively than DOPAC in both oxidations. DOPAC and CA suppressed the formation of conjugated diene hydroperoxides. DOPAC and CA at concentration 50 μM suppressed α-TOH consumption during oxidation of soybean phosphatidylcholine (2.8 mM) multilamellar vesicles containing 15 μM α-TOH, in which the lipophilic initiator 2,2′-azobis (2,4-dimethylvaleronitrile) (6 mM) was incorporated. In conclusion, we demonstrated that DOPAC and CA in micromolar concentrations have antioxidant activity in rat plasma, a medium very close to the conditions *in vivo*, suggesting that supplementation with the phenolic acids will provide significant antioxidant protection.

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Hydroxybenzoic and hydroxycinnamic acids and their derivatives have been studied recently for their antioxidant activity *in vitro* and *in vivo* in humans and in rats (1–19). Among them, caffeic acid (CA) shows the highest antioxidant activity and the greatest radical scavenging activity (1,2,4–8, 15–17) despite the different experimental conditions. The daily uptake in humans of CA is 206 mg (20), and the major sources are coffee (20–22), black and green teas (23,24), fruits and fruit juices (25–29), tomatoes and tomato juices (25,26), carrots (30), honey (31), red wine (10,32), and olive oil (1,2,33).

3,4-Dihydroxyphenylacetic acid (DOPAC), whose structure is analogous to that of CA but lacks a double bond in the aliphatic chain, also exerts antioxidant activity in lipids (7,34). The daily uptake of DOPAC with food is 7.27 mg, and it is present in nanomolar concentrations in human plasma (35,36), DOPAC has been studied in relation to sympathetic nervous activity (35–37) but not as an antioxidant *in vitro* in plasma or low density lipoproteins (LDL) or *in vivo*. DOPAC has a structure analogous to hydroxytyrosol, the most effective antioxidant in olive oil (38–40), and is used as an antioxidant instead of hydroxytyrosol (34) because it is not commercially available.

Phenolic antioxidants from olive oil protect LDL against oxidative modifications (38–42), and have a beneficial effect against atherosclerosis. They are active in the defense of the cardiovascular, immune, and respiratory systems in humans (38); lower the risk of gastrointestinal diseases (39); and show anticancer potential (1). The antimicrobial properties of honey are attributed to CA and other phenolic compounds contained in it (31).

To elucidate the antioxidant and the biological significance of the phenolic acids, we studied *in vitro* the inhibitory effect of DOPAC and CA on lipid peroxidation in rat plasma. For the purpose, rat plasma was oxidized in the presence of DOPAC or CA at 37°C by the radical initiators 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) or 2,2′-azobis (4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN). The inhibitory effect of DOPAC and CA on the consumption of α-tocopherol (α-TOH) in soybean phosphatidylcholine (PC) multilamellar vesicles (MLV), containing α-TOH, and oxidized by incorporated 2,2′-azobis (2,4-dimethylvaleronitrile) (AMVN), was also studied in order to distinguish the point where the phenolic antioxidants act.

## MATERIALS AND METHODS

*Materials.* Ascorbic acid (AA), AAPH, AMVN, MeO-AMVN, 2,2,5,7,8-pentamethyl-6-chromanol (PMC), and α-TOH were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). DOPAC and CA were obtained from

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Abbreviations: AA, ascorbic acid; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); CA, caffeic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; HPLC, high-performance liquid chromatography; LDL, low density lipoproteins; MeO-AMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); MLV, multilamellar vesicles; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PMC, 2,2,5,7,8-pentamethyl-6-chromanol; α-TOH, α-tocopherol.

Sigma Chemical Co. (St. Louis, MO). Soybean PC was purchased from Nichiyu Liposome Co., Inc. (Tokyo, Japan). Ethanol and 2-propanol were from Wako Pure Chemical Industries Ltd. and methanol and *n*-hexane from Kanto Chemical Co, Inc. (Tokyo, Japan). The *n*-hexane and 2-propanol were of high-performance liquid chromatography (HPLC) grades. All other reagents were of analytical grade.

*Animals and diets*. All experimental procedures were conducted in compliance with Teikyo University's policy on animal care and use. Seven male rats, Sprague-Dawley strain, 5 wk old, were purchased from Saitama Experimental Animals Supply Co., Ltd. (Saitama, Japan). They were fed Certified-1 Diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) for 30 wk. During this period, blood from the retro-ocular plexus of each animal was taken at certain intervals. The α-TOH content in the plasma, obtained by centrifugation of rat blood, was in the range 14–17 μM.

*Rat plasma oxidation and its inhibition by antioxidants.* Rat plasma was obtained by mixing the plasma from several rat blood samples centrifuged twice at 2500 rpm for 5 min. Fresh rat plasma (2 mL), without or with the antioxidants DOPAC, CA in ethanol solution, or a water solution of AA added beforehand at appropriate concentration, was incubated for 2–3 min at 37°C in a waterbath shaker before the oxidants AAPH (20 mM) in 1 mM EDTA solution or MeO-AMVN (2 mM) in methanol solution were added to start the oxidation. The final concentration of methanol and ethanol was 1 and 0.5 vol%, respectively. A freshly prepared water solution of AA in 1 mM EDTA was used. Aliquots of 0.2 mL of oxidized rat plasma were withdrawn at measured time intervals. The oxidation was stopped by the addition of EDTA (100 μM) and freezing (43).

*MLV preparation, oxidation, and inhibition of MLV oxidation.* Soybean PC (2.8 mM), α-TOH (15 μM), and AMVN (6 mM) were dissolved in chloroform, mixed, and the solvent was evaporated completely under nitrogen ($N_2$). Phosphate buffered saline (PBS; 10 mM, pH 7.4) containing EDTA (0.1 mM) was added and mixed on a vortex mixer to form an MLV suspension. The MLV suspension was divided into three tubes. DOPAC and CA at concentrations of 50 μM were added to two of them. The MLV suspensions with DOPAC or CA and without antioxidant were placed in a waterbath shaker (37°C, 150 oscillations per minute) to start the oxidation. Aliquots of 0.2 mL were withdrawn at measured time intervals and handled as already described.

*Extraction of α-TOH from rat plasma or MLV and its detection by HPLC with fluorometric detector.* α-TOH was extracted from rat plasma or MLV suspension by the modified method of Abe and Katsui (44), and Abe *et al.* (45), as described by Shabit *et al.* (46). A volume of 15 μL ethanol containing 3 μg PMC as an internal standard was added to oxidized rat plasma or MLV suspension (0.2 mL). The sample was diluted with distilled water (2.0 mL) and ethanol (2.0 mL) after consecutive mixings for 1 min with a vortex mixer. The α-TOH was extracted with *n*-hexane (5.0 mL). A 0.9% NaCl water solution (2.0 mL) was added and mixed 2–3 min with a vortex mixer at which time rat plasma was used (for precipitation of the proteins). The mixture was centrifuged at 3200 rpm for 10 min at 4°C. The upper layer, containing α-TOH, the internal standard, and the lipids, was pipetted. All procedures were performed at 4°C in an ice bath. The *n*-hexane was evaporated under $N_2$. The residue was then dissolved in *n*-hexane (1.6 mL). A volume of 20 μL of this solution was injected into the column of the HPLC system.

The HPLC system contained an injector (Rheodyne Incorporated, Cotati, CA), DGU-4A Shimadzu degasser (Shimadzu Corp., Kyoto, Japan), $NH_2$-1251-N, 4.6 × 250 mm, Senshu Pak column (Senshu Scientific Co., Ltd., Tokyo, Japan), LC –10 AD Shimadzu liquid chromatograph, RF-10 A Shimadzu spectrofluorometric detector, and C-R6A Shimadzu Chromatopac recorder. The α-TOH was detected at an excitation wavelength of 298 nm and an emission wavelength of 325 nm. The mobile phase was *n*-hexane/2-propanol (98:2, vol/vol) at flow rate 1.0 mL/min.

*Ultraviolet (UV) analysis of conjugated diene hydroperoxides formation.* Diluted oxidized rat plasma (plasma/distilled water = 1:80, vol/vol) without or with antioxidants was analyzed by U-3310 Hitachi spectrophotometer (Tokyo, Japan) by scanning from 300 to 200 nm at a rate of 60 nm/min. Eighty times diluted unoxidized rat plasma was used as a reference.

## RESULTS AND DISCUSSION

*Rat plasma oxidation.* Azo compounds generate free radicals by their spontaneous thermal decomposition. They have been used successfully in studies, both *in vitro* and *in vivo*, on the actions of free radicals upon biological molecules and on the protective effect of antioxidants (47).

We found that 20 mM AAPH in 1 mM EDTA water solution or 2 mM MeO-AMVN in methanol induced the oxidation of rat plasma at 37°C. In Figure 1 the kinetic curves of the consumption of endogenous α-TOH (A) and the accumulation of conjugated diene hydroperoxides, measured at 233 nm (B), in oxidized rat plasma are shown. α-TOH was consumed at the same rate in the presence of the initiators AAPH (20 mM) and MeO-AMVN (2 mM). For example, the change in absorbance of 80 times diluted rat plasma, oxidized by AAPH (20 mM), as determined by scanning from 300 to 200 nm at a speed of 60 nm/min, is shown at Figure 2.

*Inhibition of rat plasma and MLV oxidation by DOPAC and CA*. DOPAC and CA in the concentration range of 10–100 μM (Figs. 3A and 3B, respectively) suppressed α-TOH consumption in rat plasma, oxidized by AAPH (20 mM). Nardini *et al.* (15) also showed a dose-response effect of CA on $Cu^{2+}$-mediated and AAPH-induced oxidative modifications of LDL, as measured by formation of conjugated dienes at 234 nm. A concentration of 50 μM of either DOPAC (Fig. 3A) or CA (Fig. 3B) was sufficient to induce a remarkable lag phase and to delay the rate of α-TOH consumption in rat plasma.

DOPAC and CA exogenously added at a concentration of 100 μM delayed the formation of conjugated diene hydroper-

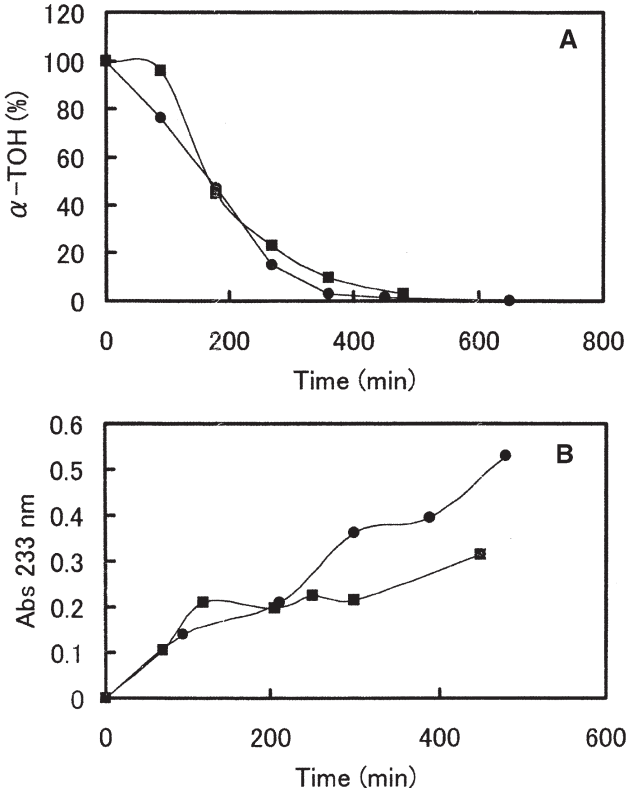


**FIG. 1.** α-Tocopherol (α-TOH) consumption (A) and change in absorbance at 233 nm (B) with time in the oxidation of rat plasma, oxidized by 20 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) (●) and by 2 mM 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile (MeO-AMVN) (■) under air at 37°C.

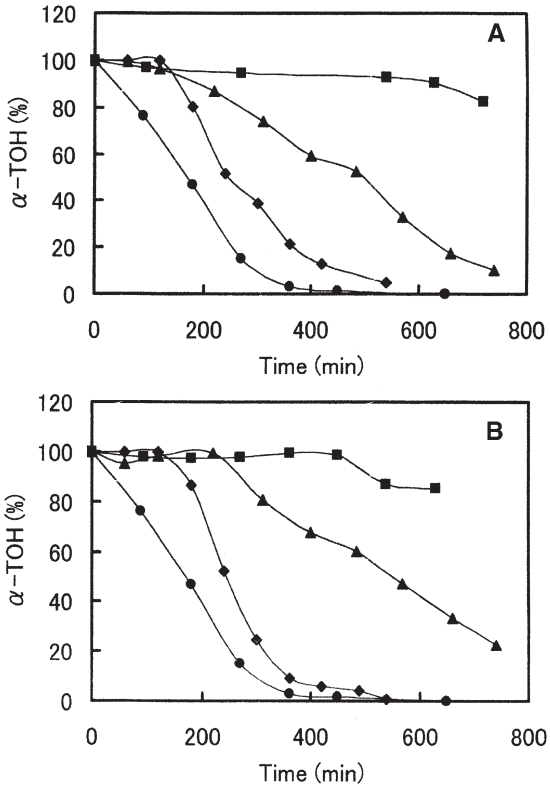


**FIG. 3.** Dose-dependent effect of the antioxidants 3,4-dihydroxyphenylacetic acid (DOPAC) (A) and caffeic acid (CA) (B) on α-TOH consumption in the oxidation of rat plasma induced by AAPH. The antioxidants, at concentrations of 10 (◆), 50 (▲), and 100 μM (■), were added to rat plasma (2 mL) before AAPH (20 mM) was finally added to start the oxidation under air at 37°C. (●) α-TOH consumption without the addition of antioxidant. For abbreviations see Figure 1.

oxides, as shown in Figure 4. AA added at the same concentration also delayed the formation of conjugated diene hydroperoxides but had a smaller effect than the two phenolic acids (Fig. 4).

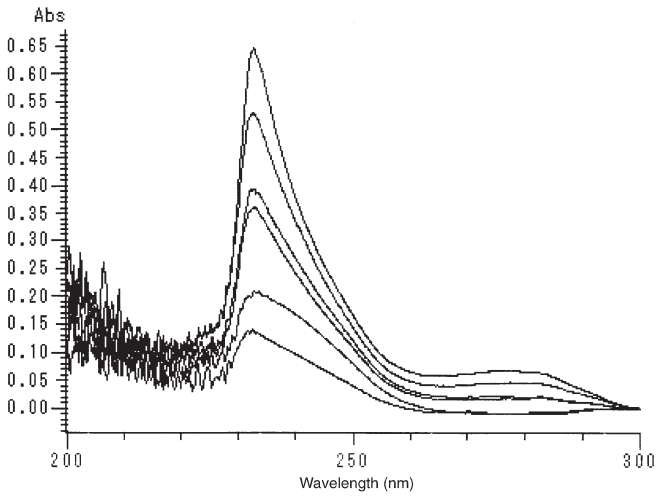


**FIG. 2.** Increase in absorbance of rat plasma, oxidized by AAPH (20 mM). Samples of 0.05 mL of oxidized rat plasma were withdrawn at 95, 210, 300, 390, 480, and 570 min of oxidation time from the reaction mixture and were dissolved in 4 mL of distilled water. The spectrum was scanned from 300 to 200 nm at a speed 60 nm/min. For abbreviation see Figure 1.

Our data showed that DOPAC and CA in micromolar concentrations were inhibitory to lipid peroxidation in rat plasma. This result is relevant because DOPAC and CA are present in human plasma in nanomolar concentrations (32,35,36) and CA is present in rat and rabbit plasma in micromolar concentrations (9,33), indicating antioxidant activity among the other biological activities of both phenolic acids in plasma. We have demonstrated here that DOPAC has antioxidant activity in a natural substrate such as rat plasma, not just in purified food lipids (7,13,34). CA is known to be active against oxidative modifications in LDL at micromolar concentrations (3,5,8,10,14,15,17), but its effect has not been investigated in plasma.

The inhibitory effects of DOPAC, CA, and AA on α-TOH consumption were compared in AAPH- and MeO-AMVN-induced rat plasma oxidations (Figs. 5A and 5B, respectively). DOPAC and CA spared α-TOH more efficiently in AAPH-induced oxidation, where the hydrophilic initiator produced radicals in the aqueous phase, than in MeO-AMVN-induced oxidation, where the initiator produced radicals in the lipid environment and in the aqueous phase. There were a notable lag-phase and delay of the rate of α-TOH consumption in AAPH-induced oxidation (Fig. 5A) compared with the MeO-AMVN-induced oxidation (Fig. 5B).

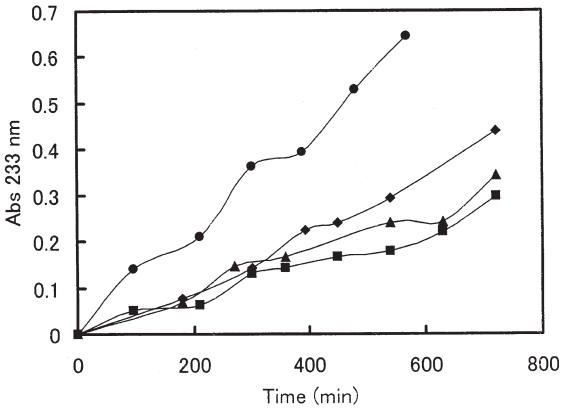


**FIG. 4.** Inhibitory effect of the antioxidants ascorbic acid (AA) (◆), DOPAC (▲), and CA (■) on the formation of conjugated diene hydroperoxides in the oxidation of rat plasma induced by AAPH. The antioxidants, at concentrations of 100 μM, were added to rat plasma (2 mL) before AAPH (20 mM) was finally added to start the oxidation under air at 37°C. (●) Change in absorbance at 233 nm without the addition of antioxidant. For abbreviations see Figures 1 and 3.

To distinguish where the phenolic antioxidants DOPAC and CA act, α-TOH consumption was followed during oxidation at 37°C of soybean PC (2.8 mM) MLV where α-TOH (15 μM) and AMVN (6 mM) were incorporated (Fig. 6). DOPAC and CA at concentrations of 50 μM spared 45% of the α-TOH. α-TOH was consumed almost completely in an MLV suspension without the addition of phenolic antioxidant. In an MLV suspension DOPAC and CA showed identical antioxidant activity: The curve of consumption of α-TOH in the course of oxidation was the same for both phenolic acids, as seen from Figure 6. For comparison, in rat plasma CA was more effective than DOPAC in sparing α-TOH, as shown in Figure 5.

DOPAC and CA spared α-TOH consumption more effectively in rat plasma oxidation induced by a hydrophilic initiator than in rat plasma oxidation induced by a lipophilic initiator and at the same time showed inhibition activity in an MLV system, oxidized by the lipophilic initiator AMVN. It can be concluded that DOPAC and CA most probably acted as antioxidants at the interface of the biological membranes. Laranjinha and Cadenas (3) suggested that the antioxidant activity of CA is largely related to its localization at the bilayer surface where it can encompass the reduction of the α-TOH radical rather than intercept directly any secondary lipid peroxyl radicals. Carbonneau *et al.* (8), investigating the antioxidant effect of CA, sinapic and ferulic acids on LDL oxidation, proposed that phenolic acids are loosely associated with LDL, possibly remaining at least partially at the surface of the particle by virtue of their mainly hydrophilic properties. The antioxidant potential of ferulic acid (11) was associated with its distribution. The major portion of the ferulic acid was found in the albumin-rich fraction of the plasma, and a portion was found to partition between the LDL and the aqueous phase. However, ferulic acid does not associate with the lipid portion of the LDL particle, suggesting that it exerts its antioxidant properties from the aqueous phase.

Our results showed that CA spared α-TOH more efficiently than DOPAC in both AAPH- and MeO-AMVN-induced rat plasma oxidations. Previous results indicated that CA had a greater antioxidant activity than DOPAC during oxidation of lard triacylglycerols at 100°C because the DOPAC radical participated in chain propagation reactions (7). CA has the highest antioxidant activity among the phenolic acids

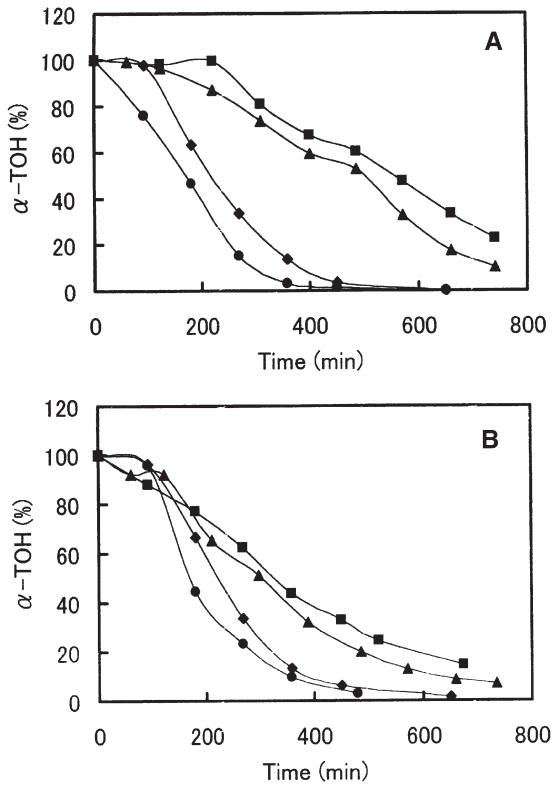


**FIG. 5.** Comparison of the effect of the antioxidants AA (◆), DOPAC (▲), and CA (■) on α-TOH consumption in the oxidation of rat plasma induced by AAPH (A) or by MeO-AMVN (B). The antioxidants, at concentrations of 50 μM, were added to rat plasma (2 mL) before AAPH (20 mM) or MeO-AMVN (2 mM) was added to start the oxidation under air at 37°C. (●) α-TOH consumption without the addition of antioxidant. For abbreviations see Figures 1 and 3.

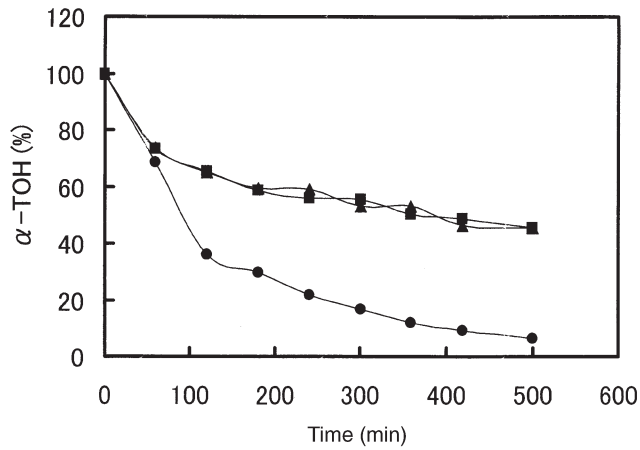


**FIG. 6.** Inhibitory effect of the antioxidants DOPAC (▲) and CA (■) at concentrations of 50 μM on the oxidation of soybean phosphatidylcholine (2.8 mM) multilamellar vesicles where α-TOH (15 μM) and AMVN (6 mM) were incorporated. (●) α-TOH consumption without the addition of antioxidant. For abbreviations see Figures 1 and 3.

(6,12,16) and displays one of the highest reactivities toward peroxyl radicals (19) owing to the presence of two hydroxyl groups in the ortho-position and to the stabilization of the caffeic acid radical by resonance in the aliphatic double bond.

Exogenously added AA in AAPH or MeO-AMVN oxidations also delayed the rate of α-TOH consumption and induced a lag-phase, but its effect was smaller than DOPAC or CA (Fig. 5). It also delayed the formation of conjugated diene hydroperoxides to a lesser extent than the two phenolic acids (Fig. 4). CA and *p*-coumaric acid (3,5) showed a greater antioxidant effect than AA in LDL oxidation mediated by ferrylmyoglobin. Ferulic acid (11) was a more effective antioxidant against LDL oxidation than the hydrophilic AA.

We considered the antioxidant activity of DOPAC and CA in rat plasma, where other natural antioxidants such as α-TOH (14–20 μM), AA (50–90 μM) (9,48–53), ubiquinone (0.5–1 μM) (48,53,54), glutathione (1.2 μM) (49), and uric acid (0.6–20 mM) (9,49) are contained. We evaluated their synergistic action with the antioxidants present in rat plasma. The inhibitory activity of DOPAC and CA in the MLV system must be considered a consequence of their synergistic action with α-TOH. Laranjinha *et al.* (3) demonstrated a synergistic action between CA, α-TOH, and AA and between CA or *p*-coumaric acid and AA (5) in LDL, oxidized by ferrylmyoglobin. Carbonneau *et al.* (8) assumed that flavonoids such as CA, sinapic and ferulic acids played a role similar to that of vitamin C at the surface of LDL particles by regenerating the reduced form of vitamin E from its oxidized form chromanoxyl and by generating phenoxyl radicals. Nardini *et al.* showed that CA spared α-TOH *in vivo* (9) and preserved α-TOH and β-carotene *in vitro* in $Cu^{2+}$ and AAPH LDL oxidations (15).

In conclusion, we demonstrated that DOPAC and CA in micromolar concentrations have antioxidant activity in rat plasma, a medium having conditions very close to those *in vivo*, suggesting that supplementation with the phenolic acids will provide significant antioxidant protection.

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# Postprandial Triglyceride-Rich Lipoprotein Metabolism and Insulin Sensitivity in Nonalcoholic Steatohepatitis Patients

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**ABSTRACT:** Nonalcoholic steatohepatitis (NASH) is a syndrome frequently associated with obesity, diabetes mellitus, and dyslipidemia. Increased fasting insulinemia and blood glucose levels may trigger a reduced catabolism of lipoproteins rich in triglycerides by lipoprotein lipase (LPL) and an increase in their fasting and postprandial levels. An association between postprandial lipemia and coronary heart disease has been observed, and many studies now support this concept. The most important result of our study is the increase in triglyceride-rich lipoproteins response after a fat load in NASH patients, the increase of incremental area under the postprandial curve, and the duration of the hypertriglyceridemic peaks. The persisting postprandial plasma triglyceride elevation in NASH patients was mostly due to the elevated plasma level of large triglyceride-rich particles. These data are coupled with lower plasma HDL2-cholesterol levels. As for lipoprotein analyses, the number of apolipoprotein B100 (ApoB100) particles is not significantly different between the two groups, and the higher content of triglycerides in NASH very low density lipoproteins (VLDL) increases the triglyceride-to-ApoB ratio and the particle size. A decreased enzymatic activity of LPL or a defective assembly and secretion of VLDL from hepatocytes due to a moderate reduction in microsomal triglyceride transfer protein could be involved in the overloading of VLDL. Moreover, the undetectable levels of ApoB48 in triglyceride-rich lipoproteins fraction A could be related to the synthesis of smaller and denser chylomicrons. NASH patients not only are insulin resistant but also tend to present alterations in fatty meal delivery, suggesting that an increase in fasting plasma insulin and glucose, with insulin resistance, joins with depressed metabolism of triglyceride-rich lipoproteins. An increase in postprandial triglyceride levels with production of large VLDL suggests an atherogenic behavior of lipid metabolism, in accordance with the high prevalence of the metabolic syndrome in NASH patients. This paper suggests that a fat load may be useful in early detection of atherogenic risk in the presence of otherwise normal fasting plasma lipids.

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Hyperlipidemia (hypertriglyceridemia, hypercholesterolemia, or both) is a common abnormality in 20 to 81% of patients with nonalcoholic steatohepatitis (NASH) (1), an increasingly recognized chronic liver disease characterized by the histological pictures of alcoholic disease in the absence of significant ethanol consumption (2). Other conditions commonly associated with NASH are obesity and non-insulin-dependent diabetes mellitus (1,3). An increase in fasting blood glucose with reduced glucose tolerance is coupled with depressed metabolism of triglyceride-rich lipoproteins (TRL) and an increase in their fasting and postprandial levels (4,5). The links between glucose and lipoprotein metabolism have received particular attention in the context of the metabolic syndrome (6). Plasma insulin concentration, an indirect index of insulin sensitivity, was shown to be related to the production of large very low density lipoproteins (VLDL) particles (Svedberg flotation rate, Sf, of 60 to 400) in experimental studies with humans (7).

The postprandial phase is regarded as potentially atherogenic (6–8). Investigation of dynamic metabolism after a mixed or a fatty meal provides more complete data about the behavior of other lipoprotein classes. TRL are a heterogeneous population of lipoprotein particles. Those containing apolipoprotein (Apo) B100 are secreted from the liver (VLDL), whereas those with ApoB48 are secreted from the intestine as chylomicrons after fat intake. Several reports have concluded that postprandial alterations in healthy men induce transient compositional alterations of VLDL that link these lipoprotein species to the formation of lipid deposits on arterial walls (4,6,7,9,10). TRL can also trigger an impaired fibrinolysis and endothelial dysfunction (11), which may be ultimately responsible for cardiovascular disease (12). Moreover, the fasting lipid profile may be in the normal range and fail to reflect the atherogenic risk of subjects with impaired postprandial triglycerides (Tg) removal (12,13).

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Abbreviations: Apo, apolipoprotein; AUC, area under the postprandial triglyceride curve; BMI, body mass index; Chol, cholesterol; FFA, free fatty acid; FPG, fasting plasma glucose; FSIGT, frequently sampled intravenous glucose tolerance test; HDL, high density lipoproteins; LDL, low density lipoproteins; LPL, lipoprotein lipase; MTP, microsomal triglyceride transfer protein; NASH, nonalcoholic steatohepatitis; OFL, oral fat load; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Sf, Svedberg flotation rate; SI, sensitivity index; Tg, triglyceride; TRL, triglyceride-rich lipoproteins; VLDL, very low density lipoproteins.

The aim of the study was to examine the dynamic postprandial metabolism of TRL and assess its relationship to insulin sensitivity in 11 nonobese, nondiabetic NASH patients and 16 healthy subjects with a normal fasting lipid profile after a standardized oral fat-loading test.

## MATERIALS AND METHODS

*Study subjects.* Lipoproteins from 11 NASH patients and 16 healthy subjects were studied. None of the participants was obese [(body mass index (BMI) 25.9 ± 2.0 kg/m$^2$ for patients and 25.0 ± 2.5 kg/m$^2$ for controls] or diabetic. Fasting plasma lipoprotein lipid concentrations are shown in Table 1. There was no clinical or laboratory evidence of thyroid dysfunction or other conditions leading to secondary hyperlipoproteinemia in either group. Inclusion criteria for the 11 NASH patients (35 ± 9 yr, 10 male/1 female) were as follows: histological feature of alcoholic steatohepatitis with chronically elevated aminotransferases and/or γ-glutamyl transpeptidase; negative history of alcohol consumption (>40 g/wk); negative markers for hepatitis B virus/hepatitis C virus; no recent use of known steatogenic drugs; absence of autoantibodies related to autoimmune liver disease; normal serum iron and copper metabolism profile; absence of serum immunoglobulin M antibodies, anti-Epstein-Barr virus, herpes virus, and cytomegalovirus. The controls were 16 healthy subjects matched for age, sex (33 ± 9 yr, 15 male/1 female), and the main anthropometric parameters. All subjects provided their informed consent. Participants received a questionnaire about fat intake, alcohol intake, and physical activity. Dietary habits were similar between young and elderly subjects (20% of calories of protein, 50% from carbohydrates, and 30% from fat, with a polyunsaturated to saturated fat ratio of 1.50). Mean daily cholesterol (Chol) intake was 200–250 mg.

**TABLE 1**
**Baseline Characteristics, Frequently Sampled Intravenous Glucose Tolerance Test (FSIGT), and Minimal Model Parameters for NASH Patients and Controls[a]**

| | NASH patients ($n$ = 11) | Controls ($n$ = 16) | $P$ |
|---|---|---|---|
| Age (yr) | 35 ± 9 | 33 ± 9 | NS |
| Sex (M/F) | 10/1 | 15/1 | NS |
| BMI (kg/m$^2$) | 25.9 ± 2.0 | 25 ± 2.5 | 0.330 |
| Waist (cm) | 91 ± 6 | 83 ± 5 | 0.002 |
| FPG (mg/dL) | 97 ± 12 | 89 ± 14 | 0.135 |
| IRI (μU/mL) | 13.9 ± 4.5 | 6.9 ± 2.8 | 0.0001 |
| Insulin sensitivity index (min$^{-1}$/μU/mL) | 3.66 ± 2.86 | 9.15 ± 2.94 | 0.000 |
| Triglycerides (mg/dL) | 97 ± 67 | 75 ± 27 | 0.246 |
| Total cholesterol (mg/dL) | 183 ± 40 | 185 ± 22 | 0.868 |
| LDL-cholesterol (mg/dL) | 108 ± 34 | 106 ± 25 | 0.861 |
| HDL-cholesterol (mg/dL) | 52 ± 7 | 59 ± 16 | 0.187 |
| HDL2-cholesterol (mg/dL) | 13.0 ± 5.0 | 18.4 ± 7.5 | 0.047 |
| HDL3-cholesterol (mg/dL) | 39.4 ± 4.1 | 40.5 ± 7.1 | 0.648 |
| Apo AI (mg/dL) | 119 ± 24 | 121 ± 26 | 0.841 |
| Apo B (mg/DL) | 83 ± 30 | 85 ± 21 | 0.840 |

[a]Data are presented as mean ± standard deviation. NASH, nonalcoholic steatohepatitis; BMI, body mass index; FPG, fasting plasma glucose; IRI, immuno-reactive insulin; LDL, low density lipoprotein; HDL, high density lipoprotein; Apo, apolipoprotein; NS, not significant.

*Determination of lipid parameters at baselines.* A hospital medical examination was carried out at 9 A.M. after a fast of 12 h or more. Blood samples drawn from the antecubital vein were used to determine the Tg, total Chol, low density lipoprotein (LDL) Chol, high density lipoprotein (HDL) Chol, HDL2-Chol, and HDL3-Chol, Apo AI, Apo B, glucose, creatinine, albumin, and thyroid-stimulating hormone baselines. The subjects were then asked to return on different mornings for the oral fat load (OFL) test after a fast of not less than 12 h. Blood (1 mg/mL EDTA-$Na_2$) was centrifuged for 30 min at 2500 rpm and 4°C in a J6B centrifuge (Beckman Instruments, Palo Alto, CA) and stored at –20°C until processed. Total Chol and Tg were measured enzymatically (Poli Diagnostici, Milan, Italy). Plasma HDL-Chol was determined after precipitation of ApoB-containing lipoproteins with heparin and manganese chloride (14). Plasma HDL2- and HDL3-Chol levels were determined according to Gidez *et al.* (15): HDL2 and HDL3 were separated after precipitation of ApoB-containing lipoproteins with heparin and manganese chloride, and HDL2 were further precipitated with dextran sulfate. The formula of Friedewald *et al.*(16) was used to calculate LDL-Chol. Plasma ApoAI and ApoB were determined by an automated immunoturbidimetric method (Poli Diagnostici) with a Shimadzu CL-7000 (Shimadzu Instruments, Kyoto, Japan).

*DNA analysis.* APOE (apolipoprotein E) genotypes were determined by polymerase chain reaction (PCR) amplification of genomic DNA using specific oligonucleotide primers (17). Genomic DNA was isolated from frozen EDTA whole blood through the QIAamp Blood Kit (Qiagen-Genenco M-Medical s.r.l., Florence, Italy). Genomic DNA (0.5 μg) was amplified in 25 μL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3); 50 mmol/L KCl; 200 μmol each of dATP, dCTP, dGTP, and dTTP; 1.5 mmol/L $MgCl_2$; 200 pmol of each primer, and 1.8 U Taq DNA polymerase (Roche Diagnostics S.p.A., Milan, Italy). The amplification cycle was performed in a Gene Cycler (Bio-Rad, Milan, Italy). Five minutes of denaturation at 94°C was followed by 30 cycles of 1 min at 90°C, 1 min at 60°C, and 2 min at 70°C. PCR products were cleaved with 4 U of HhaI restriction endonuclease (New England Biolabs, Ipswich, MA), as recommended by the manufacturer. Subsequently, the samples were electrophoresed through a 10% nondenaturing polyacrylamide gel and visualized by silver staining (Bio-Rad, Milan, Italy). The sizes of HhaI fragments were estimated by comparison with known size markers (MspI-digested pUC18 DNA).

*Frequently sampled intravenous glucose tolerance test (FSIGT).* Insulin sensitivity was assessed by performing an FSIGT. The data were analyzed with the minimal modeling technique to estimate the insulin sensitivity index (SI) as described in detail elsewhere (18).

*Oral fat load.* Five minutes after drawing a blood sample (t0), the subjects consumed a liquid OFL consisting of a mixture of dairy cream (35% fat) and egg yolk. The total amount

of fat was based on the subject's body surface area (77.5 g fat, 0.5 g Chol per $m^2$). The OFL was consumed during a period of 15 min, and the subjects then remained without food for 10 h. A catheter (Venflon Viggo AB, Helsingborg, Sweden) inserted in the antecubital vein and kept patent during the test was used to draw samples after 2, 4, 6, 8, and 10 h (t2, t4, t6, t8, and t10) for the determination of plasma TRL. Strenuous activity was forbidden during the test, because exercise can reduce postprandial lipemia.

*Laboratory analyses.* Plasma total Chol and Tg were measured by means of automated enzymatic methods. The area under the postprandial triglyceride curve (AUC) was evaluated using the trapezoid rule to estimate the overall response of plasma Tg during the entire 10-h postprandial period.

*Separation of VLDL and TRL subfractions.* VLDL were isolated through preparative ultracentrifugation and subsequently assayed for their Tg and total Chol content from all the subjects' blood samples. Briefly, one aliquot of plasma was brought to densities of 1006 g/L by addition of a KBr solution ($d$ = 1330 g/L) and centrifuged at 105,000 × $g$ in a type 50 rotor for 21 h at 10°C in a Beckman L8-70M ultracentrifuge. Concentrations of Tg and total Chol were then determined in lipoprotein fractions enzymatically. The first amount of blood (10 mL) was drawn for subfractionating TRL by ultracentrifugation on a discontinuous density gradient (19). Plasma separated as already described was brought to a density of 1.10 g/mL by the addition of solid KBr. The density gradient was prepared manually by adding to 4 mL of this plasma 3 mL of a 1.065 g/mL solution containing 0.05% KBr/NaCl plus EDTA (pH 7.4); 3 mL of a similar solution at 1.020 g/mL; and 3 mL of physiological saline at 1.006 g/mL. The sample was ultracentrifuged in a SW4O rotor in a Beckman L8-70M centrifuge at 20°C in stages allowing the separation of four VLDL fractions with decreasing Sf values: A > 400; B = 175–400; C = 100–175; D = 20–100. The first centrifugation (28,300 rpm for 43 min) isolated fraction A in a floating volume of 0.5 mL. The volume removed was replaced with physiological saline, and the sample was centrifuged at 40,000 rpm for 67 min to isolate fraction B. This procedure was then repeated at 40,000 rpm for 71 min and at 37,000 rpm for 18 h to isolate fractions C and D, respectively. The automated methods mentioned above were used to determine Chol and Tg on the four fractions.

*ApoB48 and ApoB100 analysis.* ApoB48 and ApoB100 analysis were performed on five subjects with NASH and on five controls matched for age, sex, and the main anthropometric parameters.

VLDL ApoB48 and ApoB100 were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 3.9% gel according to Battula *et al.* (20). Nondelipidated lipoprotein samples were reduced in SDS sample buffer for 4 min at 96°C. Samples were applied to the gel and run at 40 mA in 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. Gel was stained with Silver Stain (Bio-Rad). Since the chromogenicity of ApoB48 is similar to that of ApoB100 (21), a protein standard was prepared from LDL isolated by sequential ultracentrifugation and used to quantify ApoB100 and ApoB48. The bands were quantified by densitometry using Gel Doc equipment (Bio-Rad). Density values were assigned to the ApoB100 bands of the standard LDL and a standard curve was constructed. The values were recalculated by linear regression.

*Statistics.* Means and standard deviations (SD) were calculated for the lipid baseline parameters, and differences between the patients and controls were determined with the $t$ test, or the Mann-Whitney test when comparing postprandial area. Tg levels were log-transformed before testing. Differences in the postprandial responses (plasma Tg and VLDL fraction Tg values) were assessed by connecting Tg plasma levels vs. time with segments. The AUC was computed by the trapezoid method on the area exceeding baseline (incremental area). Spearman's coefficient was used to assess the correlation between parameters concerning insulin sensitivity and basal and postprandial lipid profile.

## RESULTS

*Patients' clinical characteristics and fasting plasma lipids and lipoproteins.* Mean ± SD of clinical anthropometric and basal laboratory determinations of glucose and lipid metabolism in NASH patients and controls are reported in Table 1. Although cases and controls showed similar BMI, there was a tendency to visceral fat accumulation in NASH patients (waist: 91 ± 6 vs. 83 ± 5 cm, $P$ = 0.002). Fasting total plasma Chol (183 ± 40 vs. 185 ± 22 mg/dL, $P$ = 0.618) and Tg (97 ± 67 vs. 75 ± 27 mg/dL, $P$ = 0.246) were not significantly different. HDL2-Chol levels were significantly lower in the patients (13.0 ± 5.0 vs. 18.4 ± 7.5 mg/dL, $P$ = 0.047). The other Chol lipoprotein fractions had similar levels in the two groups as well as ApoAI and ApoB (Table 1). Fasting plasma insulin (13.9 ± 4.5 vs. 6.9 ± 2.8 μU/mL, $P$ = 0.0001) and fasting plasma glucose (FPG) levels (97 ± 12 vs. 89 ± 14 mg/dL, $P$ = 0.135) were increased in the group of NASH patients. There was no difference in ApoE allelic frequency: 12 NASH patients were E3/E3, 2 were 4/3, and 2 were 3/2; 8 controls were E3/E3, 2 were 4/3, and 1 was 3/2. There was no difference in percentage of smokers: 3 patients and 2 controls.

*Insulin sensitivity.* NASH patients had significantly lower insulin sensitivity (SI 3.66 ± 2.86 vs. 9.15 ± 2.94 $min^{-1}$/μU/mL, $P$ = 0.000)

*Postprandial response of plasma Tg.* The total OFL ingested by the NASH group was 390 ± 5.2 g (mean ± SD), and by the control group was 385 ± 4.8 g. It was well tolerated, and there were no instances of gastrointestinal disturbance or steatorrhea during or after the test. The postprandial plasma Tg and VLDL-Tg curves are reported in Figure 1 (right-hand panel). Plasma Tg peaked 4 h after the meal and returned to fasting levels after 8 h in both groups (Fig. 1). Total Chol and LDL-Chol displayed a flat pattern in both groups, and the HDL-Chol levels were unchanged. Plasma Tg were higher in the patients at all time points with a significant difference at 4 h ($p$ = 0.006). The response of VLDL-Tg is illustrated in Figure 1. VLDL-Tg are higher in the

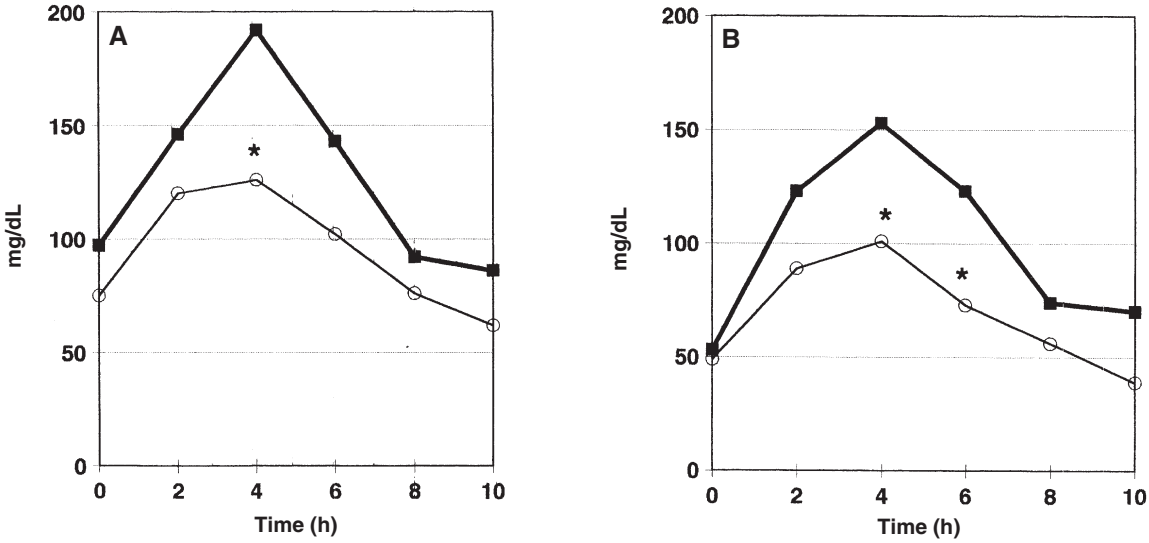


**FIG. 1.** Change in (A) plasma triglyceride (Tg) and in (B) very low density lipoprotein (VLDL) Tg concentrations after the fat load in 11 nonalcoholic steatohepatitis (NASH) subjects (■) patients and 16 controls (○). Results are expressed as mean ± standard error of the mean. *$P < 0.05$. **$P < 0.01$.

NASH group at all time points with significant differences at 4 and 6 h ($P = 0.043$ and $P = 0.019$). There was no significant difference in the incremental AUC for Tg: patients, 1329 ± 596.2 mg/dL × 10 h, $P = 0.063$; controls, 984.6 ± 322 mg/dL × 10 h. The NASH group SI was significantly correlated with the fasting total Tg concentration ($r_s = –0.67$, $P = 0.042$), fasting VLDL-Tg ($r = –0.61$, $P = 0.048$), VLDL-Tg (fraction B) ($r_s = –0.67$, $P = 0.033$), and VLDL-Tg (fraction C) ($r_s = –0.64$, $P = 0.044$), but not with any other basal or postprandial parameter. A correlation between fasting plasma Tg and the AUC was observed in both groups: NASH, $r = 0.835$, $P = 0.0013$ ($r_s = 0.763$, $P = 0.0062$); controls, $r = 0.487$, $P = 0.0551$ ($r_s = 0.633$, $P = 0.0083$). The slope for the regression line between fasting Tg and AUC was less steep in the control group (AUC = 574.67 + 5.46 Tg) than that in the NASH group (AUC = 584.26 + 7.66 Tg).

*Postprandial Tg levels in TRL subfractions.* The lipid content of large and small TRL particles isolated by ultracentrifugation after the OFL is shown in Figure 2. The lipid composition of fraction A showed a transient Tg increase after 4 and 6 h in the NASH group ($P = 0.03$ and 0.04, respectively). The difference was no longer significant during the last postprandial hours. The Tg content of VLDL subfractions B, C, and D displayed similar curves in both groups throughout the postprandial load period. There were no significant differences between the two groups in the total Chol variations at any time.

*ApoB48 and ApoB100 in TRL subfractions.* Absolute postprandial changes of ApoB48 and ApoB100 concentrations in VLDL fractions are shown in Figures 3 and 4, respectively. ApoB48 in VLDL fraction A was detected only in the control group. In VLDL fractions B, C, and D, ApoB48 was at lower levels in NASH patients. In the control group, the ApoB48 concentrations increased significantly in the VLDL fraction D at the baseline and at 2 and 10 h. The ApoB100 concentration in VLDL fraction A, B, and C responded to the OFL in a similar way in both control and NASH individuals. The ApoB100 increased and peaked at 4 h in VLDL fraction D in the control group, but this difference was not significant.

## DISCUSSION

The present study was designed to highlight some mechanisms that potentially link atherosclerosis to an abnormal fat tolerance in NASH patients. NASH is a syndrome frequently associated with obesity, diabetes mellitus, and dyslipidemia (1,3). Elevated levels of free fatty acids (FFA) in the liver are supposed to be responsible for the development of steatohepatitis as a part of the metabolic syndrome (6). Increased fasting blood glucose levels may trigger a depressed metabolism of lipoproteins rich in Tg and an increase in their fasting and postprandial levels (4,5).

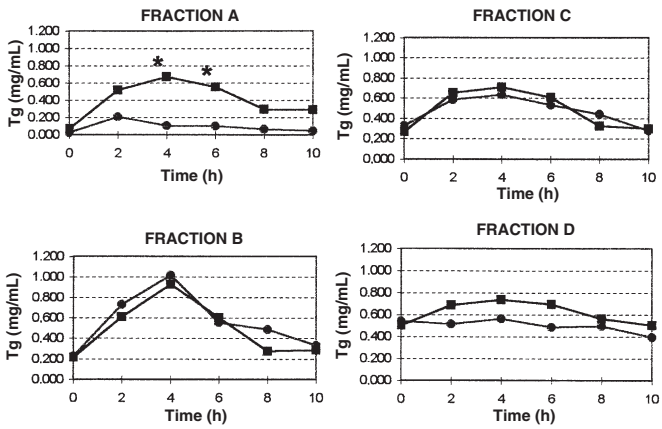


**FIG. 2.** Tg concentrations during the fat load test in the Sf A > 400, Sf B = 175–400, Sf C = 100–175, Sf D = 20–100 lipoprotein fractions (for method see Materials and Methods section) in 11 NASH patients (■) and 16 controls (●). Results are expressed as means. *$P < 0.05$. For abbreviations see Figure 1.

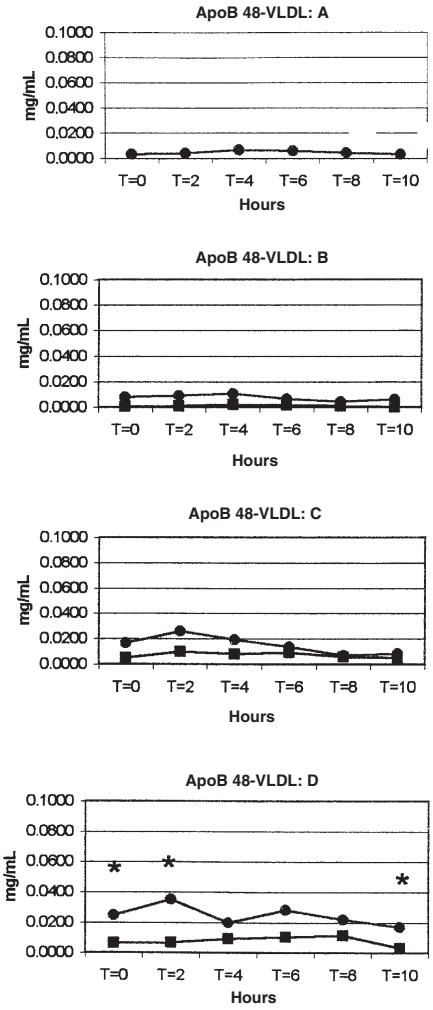


**FIG. 3.** Line plots of changes in plasma apolipoprotein B48 (ApoB48) concentrations during the fat load test in Sf A > 400, Sf B = 175–400, Sf C = 100–175, Sf D = 20–100 lipoprotein fractions (for method see Materials and Methods section) in 5 NASH patients (■) and 5 controls (●). Results are expressed as means. *$P < 0.05$. VLDL, very low density lipoprotein; for other abbreviations see Figure 1.

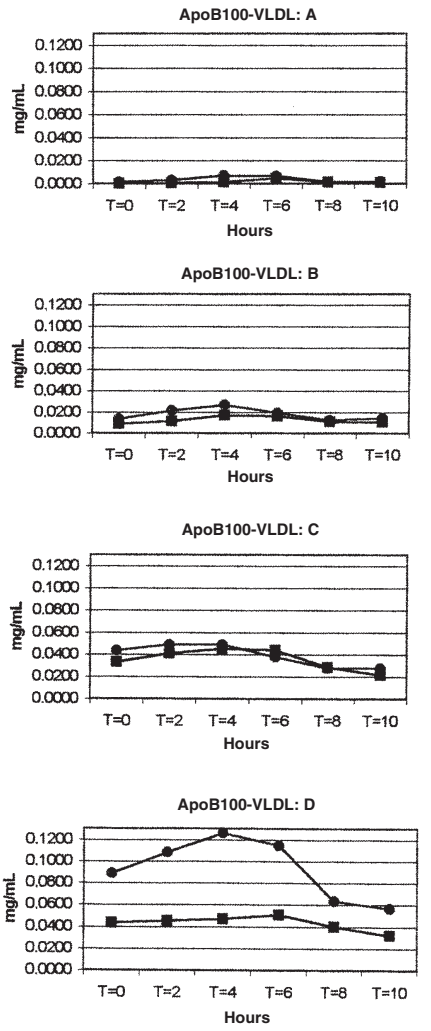


**FIG. 4.** Line plots of changes in plasma ApoB100 concentrations during the fat load test in Sf A > 400, Sf B = 175–400, Sf C = 100–175, Sf D = 20–100 lipoprotein fractions (for method see Materials and Methods section) in 5 NASH patients (■) and 5 controls (●). Results are expressed as means. For abbreviations see Figures 1 and 3.

In our patients, fasting plasma insulin was significantly increased and HDL2-Chol levels were significantly decreased. SI was negatively correlated to fasting total Tg concentration, fasting VLDL-Tg, VLDL-Tg (fraction B), and VLDL-Tg (fraction C) but not to any other basal or postprandial parameter. The most important results of our study are the increase of TRL response after a fat load in the NASH patients, the increase of the incremental area under the postprandial curve, and the duration of the hypertriglyceridemic peaks. Furthermore, we observed a significant correlation between plasma Tg and the Tg AUC in the NASH group. The slope of the regression line between fasting and the Tg AUC was steeper in the NASH group. If the fasting Tg level mirrors the postprandial Tg level, then NASH patients have a higher postprandial Tg level than the control for the same level of fasting Tg.

NASH patients seem to have a disturbance in the metabolism of TRL particles, since they displayed a rapid increase of Tg fraction A levels of lipoprotein and a sustained elevation in the postprandial phase. The curves of both groups for the other VLDL classes were superimposed. The persistent postprandial plasma Tg elevation in NASH patients was mostly due to the elevated level of large TRL particles (fraction A). The production of large VLDL particles (Sf rate of 60 to 400) seems to be regulated by plasma insulin concentrations in experimental studies in humans (7). Some authors have shown that this persistent elevation is ascribable to reduced clearance of postprandial large VLDL, which could also be related to increased competition with chylomicrons for the same lipolytic pathway (22). NASH patients present an impaired metabolism in controlling plasma Tg levels after fat intake, and this may depend on their insulin-resistant state. The role of postprandial insulin in the regulation of postprandial lipid metabolism is still poorly understood. The roles of hyperinsulinemia and insulin resistance in the alteration of lipid metabolism are not clear either (23). Nevertheless, some authors have reported that postprandial lipemia is related to the degree of insulin resistance

even in nondiabetic subjects (24). In the plurimetabolic syndrome the simple fasting hyperinsulinemia is associated with the increased postprandial lipemia even if hypertriglyceridemia and diabetes mellitus are absent (25).

Our data may show that there is no significant difference in the content of ApoB100 in VLDL fraction A between NASH and control subjects. These results mean that the number of ApoB100 particles is not significantly different between the two groups. Because postprandial Tg are higher in NASH subjects, the net effect is to increase the Tg-to-ApoB ratio and to increase particle size. In our study, VLDL fraction A of NASH subjects carry more Tg than VLDL isolated from control subjects, suggesting a decreased enzymatic activity of lipoprotein lipase (LPL), a common abnormality in insulin resistance, or a defective assembly and secretion of VLDL from hepatocytes due to a moderate reduction in microsomal Tg transfer protein (MTP) as a consequence of different gene polymorphism (26).

The most striking result of our study is that ApoB48 in TRL fraction A in NASH subjects is below the sensitivity of the silver stain. ApoB48 levels are extremely low in fraction A, and this fact could be related to an altered functioning of the MTP. Authors have reported that MTP play a key role in the first steps of chylomicron assembly (27). Even if MTP has no effect on ApoB48 synthesis, in the intestine MTP appears to be important in regulating the Tg content of the chylomicron particle (28). Thus, an impaired functioning of MTP in NASH subjects could give rise to smaller and denser chylomicrons, and it could explain why ApoB48 is absent in fraction A and present in the other fractions.

The reduction in ApoB48 and ApoB100 in VLDL fraction D in NASH subjects could be explained with a flaw of lipolysis of larger and less dense lipoproteins.

Since LDL are produced during VLDL catabolism, augmented secretion and availability of the Tg bound to the VLDL and the events subsequent to these metabolic changes may account for the production of altered particles (29). Exaggerated postprandial lipemia has consequences for the particle composition of plasma lipoprotein: Tg-enriched LDL are good substrates for hepatic lipase and the result is smaller and denser LDL ($1.040 < d < 1.063$ g/mL) constituting an atherogenic lipoprotein phenotype (19,29,30). Because the smaller, denser LDL are more susceptible to oxidation (31), the degree of oxidation may also vary in function of the main changes in the metabolism of the Tg bound to the VLDL. Moreover, the greater the degree of LDL oxidation, the greater the atherogenic potential of the particle (32).

These results may suggest that impaired postprandial Tg metabolism in NASH patients is associated with their increased visceral obesity. Reduced SI, FFA elevation (33), and reduced TRL catabolism by LPL are common in the metabolic syndrome (34,35).

An association between postprandial lipemia and coronary heart disease had been observed since the early 1950s (36), and many studies now support this concept (8–10,37).

According to some workers, chylomicrons and their remnants deliver dietetic Chol to the arterial wall, thus contributing to atherogenesis (8); other investigators maintain that the postprandial phase is a critical moment, marked by an abnormal metabolism of Tg transportation, its carriers (38), and other related enzymes or lipoprotein classes (39,40). It has also been suggested that a prolonged postprandial hypertriglyceridemic response alters the coagulation-fibrinolytic balance and favors thrombosis (13).

High plasma Tg is also closely correlated with low HDL-Chol (41–43) and may influence the composition of the HDL; an inverse relationship between HDL2-Chol levels and the postprandial Tg levels is also evident (44). This shows that the substantial alterations in TRL metabolism upstream from the lipolytic and maturative cascade of the lipoproteins have a profound effect on the variations in the other fractions. These observations lead to the conclusion that HDL levels and composition reflect the efficiency of TRL catabolism (38,42,43), and that high HDL-Chol indicates a low production of atherogenic remnants according to a mechanism that also involves the cholesteryl ester transfer protein (45).

It seems appropriate to stress that NASH patients not only are insulin-resistant but also tend to present alterations in fatty meal delivery, suggesting that an increase in fasting plasma insulin and FPG, with insulin resistance, is coupled with lower plasma HDL2-Chol and depressed metabolism of TRL. An increase in fasting and postprandial Tg levels with production of large VLDL suggests an atherogenic behavior of lipid metabolism, in accordance with the high prevalence of the metabolic syndrome in these patients (6,46). Longitudinal studies are needed to confirm this finding.

These findings indicate that the presence of NASH should be systematically sought both in patients with unexplained increase of liver enzymes in the absence of other known mechanisms of liver injury and in subjects with two or more elements of the metabolic syndrome, irrespective of the presence of obesity or visceral adiposity. Owing to the frequent association of the two syndromes, an oral glucose tolerance test and plasma insulin assay together with an OFL should be systematically performed in NASH patients for the early detection of atherogenic risk in the presence of quite normal fasting plasma lipids.

## ACKNOWLEDGMENTS

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# Absorption and Effects of 3-(*N*-phenylamino)-1,2-Propanediol Esters in Relation to Toxic Oil Syndrome

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**ABSTRACT:** Toxic Oil Syndrome (TOS) was an epidemic disease related to the consumption of rapeseed oil denatured with aniline that made its sudden appearance in Spain in 1981. The fatty acid esters of 3-(*N*-phenylamino)-1,2-propanediol (PAP), which is a chemical class of by-products resulting from the reaction of aniline with oil components, have shown a strong association with TOS-related oils. These compounds also show some structural similarities to platelet-activating factor (PAF). In search of a toxic agent that could explain the widespread systemic effects observed in TOS patients, we investigated the intestinal absorption and biotransformation of the different PAP esters found in TOS-related oil samples and the possible pathophysiological effect of these mediators and their metabolic products if acting as PAF analogs. Results indicate that PAP esters are absorbed in the gastrointestinal tract and are distributed and stored in different organs, particularly in the liver and brown adipose tissue. PAP in these organs showed different patterns of fatty acids, indicating the ability of the gastrointestinal tract to modify the fatty acid composition of the parent PAP. Thus, the fatty acid profile of the PAP esters found in intestine appears to be related to the type of oil used as vehicle. Some of these PAP esters, when a long acyl chain was present in the *sn*-1 position of the molecule, showed an inhibitory effect on the PAF synthesis. This is an important observation in line with the systemic nature of the disease.

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Toxic Oil Syndrome (TOS) was a toxic epidemic disease that made its sudden appearance in Spain in 1981 and affected more than 20,000 people. Epidemiological data revealed that the disease was related to the consumption of rapeseed oil denatured with aniline, illegally refined, mixed with other oils, and sold as edible olive oil (1). Further investigations showed that aniline and fatty acid anilides were present in TOS-related oils, and these compounds became the first markers for toxic oil. More recently, the fatty acid esters of 3-(*N*-phenylamino)-1,2-propanediol (PAP) have shown a strong association with TOS (2). However, no etiological agent has been unequivocally identified for this disease. The main barrier to the identification of the agent(s) has been the absence of an experimental model or bioassay system that fully duplicates TOS.

As for PAP, more than 20 related compounds in TOS-associated oils have been identified. These compounds include monoesters (mPAP) and diesters (dPAP) of PAP (3) (Scheme 1). The toxicity of these compounds is yet to be demonstrated. However, a possible mechanism of toxicity is suggested by the structural similarities of PAP esters to platelet-activating factor (PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Scheme 1). This bioactive mediator leads to cell damage by several mechanisms. An excess of PAF has been implicated in a variety of pathologies (4), including ischemia-reperfusion, necrotizing enterocolitis, and asthma.

Significant quantities of PAF-related phospholipids are formed in various cells (6). This fact can be explained on the basis of the relatively low substrate specificity of the enzymes responsible for PAF biosynthesis. Thus, some different enzymes do not discriminate between the alkyl and acyl chains in the *sn*-1 position (5). Keeping this in mind, the structural similarities between PAF (with an alkyl chain at *sn*-1) and PAP esters (with an acyl chain at *sn*-1) and the possible metabolism of the latter into functional PAF analogs provides the incentive for the study of the pathophysiology of these compounds within the TOS context.

In consideration of all these facts, the objectives of this work were (i) to investigate the absorption and distribution of different PAP esters found in TOS-related oil samples, and (ii) to evaluate the possible biological effect of these molecules and their metabolic products as possible PAF analogs.

The availability of these data could provide an insight into the uptake, acyl modifications, and disposition of PAP derivatives. It may also allow us to understand the well-known systemic effects that the toxic oil produced on TOS patients.

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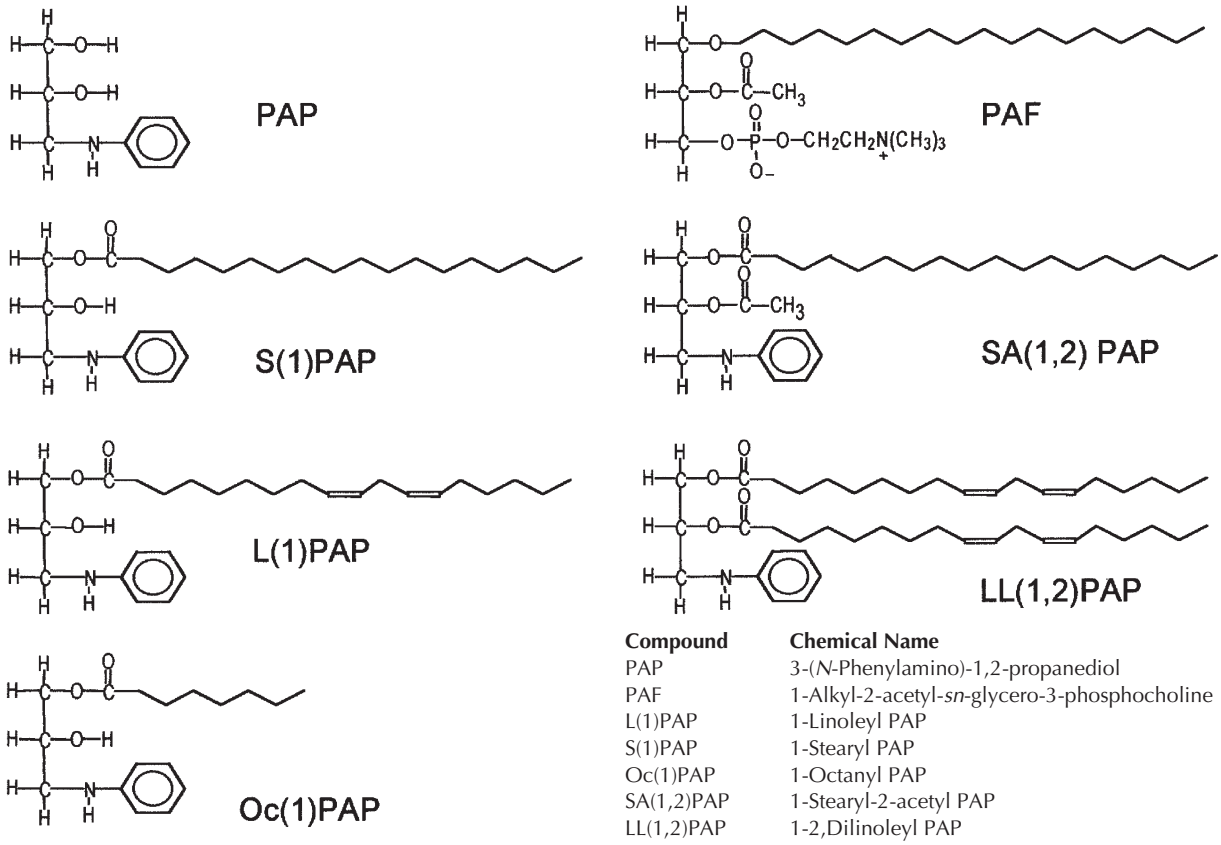
Abbreviations: APCI, atmospheric pressure chemical ionization; BAL, bronchoalveolar lavage; BAT, brown adipose tissue; DMSO, dimethylsulfoxide; dPAP, diester of 3-(*N*-phenylamino)-1,2-propanediol; EMS, eosinophilia myalgia syndrome; HPLC, high-performance liquid chromatography; L(1)PAP, 1-linoleyl PAP; LL(1,2)PAP, 1,2-dilinoleyl PAP; LO(1,2)PAP, 1-linoleyl, 2-oleyl PAP; LPS, lipopolysaccharide; mPAP, monoester of 3-(*N*-phenylamino)-1,2-propanediol; O(1)PAP, 1-oleoyl PAP; Oc(1)PAP, 1-octanyl PAP; PAF, platelet-activating factor; PAP, 3-(*N*-phenylamino)-1,2-propanediol; S(1)PAP, 1-stearyl PAP; SA(1,2) PAP, 1-stearyl-2-acetyl PAP; TOS, toxic oil syndrome.




| Compound | Chemical Name |
|---|---|
| PAP | 3-(*N*-Phenylamino)-1,2-propanediol |
| PAF | 1-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine |
| L(1)PAP | 1-Linoleyl PAP |
| S(1)PAP | 1-Stearyl PAP |
| Oc(1)PAP | 1-Octanyl PAP |
| SA(1,2)PAP | 1-Stearyl-2-acetyl PAP |
| LL(1,2)PAP | 1-2,Dilinoleyl PAP |

**SCHEME 1**

## MATERIAL AND METHODS

*Samples and standards.* The structures of the standards for 1-linoleyl PAP [L(1)PAP], 1,2-dilinoleyl PAP [LL(1,2)PAP], 1-stearyl PAP [S(1)PAP], 1-octanyl PAP [Oc(1)PAP], and 1-stearyl-2-acetyl PAP [SA(1,2)PAP] are shown in Scheme 1. L(1)PAP and LL(1,2)PAP were also labeled with $^{14}C$ in the phenylamino group and with $^{3}H$ in the acyl chain: 3-([U-$^{14}C$]phenyl)amino-1,2-propanediyl di[9,10,12,13(n) $^{3}H$]linoleate. All these compounds were synthesized by Guardiola and Messeguer (7). Chemical purity was higher than 93% for all compounds as verified by high-performance liquid chromatography (HPLC) analysis.

*Absorption and distribution.* Radiolabeled L(1)PAP or LL(1,2)PAP, dissolved in 1 mL of olive oil, was administered orally to male Wistar rats (250–300 g body wt) purchased from Iffa Credo (L'Arbreste, France). The dose administered (60 mg/kg) contained 2μ Ci of $^{3}H$ and 1.5 μCi of $^{14}C$. At 4 ($n$ = 4) or 18 ($n$ = 4) h after administration, samples of rat tissues, plasma, and urine were obtained, weighed, dissolved with Soluene-350 (Packard, Canberra, Australia)/$H_2O_2$ and counted in a β-counter for $^{3}H$ and $^{14}C$ channels.

In some samples, the lipidic fraction was extracted by the Bligh and Dyer method (8), and the content of radioactivity in the organic and aqueous fractions was counted in a β-counter for $^{3}H$ and $^{14}C$ channels.

*Biotransformation.* To investigate both the metabolic modifications of mPAP during gastrointestinal absorption and the influence of the vehicle in the biotransformation of PAP, 60 mg/kg of unlabeled L(1)PAP was orally administered to three groups of rats ($n$ = 8 for each group). The vehicle used (1 mL) was olive oil in the first group, coconut oil in the second, and palm oil in the third. Four hours later, the animals were sacrificed, and samples of the intestine (jejunum), liver, brown adipose tissue (BAT), and lung were obtained. Control animals received oil free of PAP esters. The lipid fraction of these samples was extracted by the method of Bligh and Dyer (8). The extracts were evaporated to dryness under a helium stream and stored at –40°C until analysis.

Mass spectrometric analyses were conducted by using a Finnigan TSQ 700 triple quadrupole mass spectrometer. This instrument was provided with an atmospheric pressure ionization source for atmospheric pressure chemical ionization (APCI) from Finnigan (San Jose, CA). Instrument tuning, calibration, and fragmentation studies were performed by continuous infusion of the standards into the spectrometer.

HPLC chromatography was carried out using a Hewlett-Packard model 1100 with a binary pump and an ultraviolet detector (245 nm). Samples were injected with a Triathlon automatic injector (Spark Holland B.V., Emmen, Holland) provided with a 50-μL loop.

Target compounds were separated under gradient conditions using a reversed-phase column (Partisil ODS-3, 5 μm particle size, 2 × 150 mm) from Technokroma (Barcelona,

Spain). The analytical column was preceded by a 2 × 10 mm ODS column (Upchurch Scientific, Oak Harbor, WA). The injection volume was 10 µL, and the flow rate 300 µL/min. The mobile phase was composed of water/methanol (20:80 + 0.1% acetic acid) as solvent A and isopropanol/methanol (20:80 + 0.1% acetic acid + 0.5% hexane) as solvent B. A solvent gradient was used for chromatography. The gradient started at 20% B and increased to 80% in 0.1 min, then increased again to 100% of B in 3.5 min where it was held for 5 min.

Quantification of PAP utilized the precursor ion mode on the TSQ 700 mass spectrometer and selected ion monitoring. The $[M + H]^+$ ions of the PAP targeted for analysis were monitored in the first quadrupole, and the common product ion at *m/z* 132 was monitored in the second analyzer (third quadrupole). The collision energy was set at –25 eV for monoacyl-PAPs and –35 for diacylPAP. The collision gas (argon) pressure was 1.5 mTorr, and the multiplier was set at 1800 V. Ion spray source parameters were: capillary voltage, 4.5 kV; capillary temperature, 250°C; sheath gas (nitrogen) pressure, 70 psi; auxiliary gas (nitrogen) flow, 40 mL/min. APCI source parameters were: corona discharge, 1 kV; vaporizer temperature, 500°C; sheath gas (nitrogen) pressure, 20 psi; no auxiliary gas.

Quantification was carried out using 1-heptadecanoyl-2-oleyl-PAP and 1-heptadecanoyl-PAP as internal standards for diacyl and monoacyl PAP, respectively (9). Total fatty acid levels were determined in palm, olive, and coconut oils by standard gas chromatographic methods [IUPAC 2301 (fatty acid methylation) and IUPAC 2302 (gas–liquid chromatographic analysis): Ref. 10].

*Biological (PAF-like) activity of PAP esters: Changes in vascular permeability. (i) Animal preparation.* Male Wistar rats (200–250 g body wt) were anesthetized with 1 mL/kg of 10% urethane and placed in a supine position. A midline abdominal incision was made to allow a section of mesentery from the midjejunum to be exteriorized. All exposed tissue was covered with saline-soaked gauze to minimize tissue dehydration. The mesentery was superfused at 1 mL/min for 30 min (Harvard "22" infusion pump; Harvard apparatus, Edenbridge, United Kingdom) with phosphate-buffered saline (pH 7.4) containing the different mediators administered (11).

*(ii) Vascular permeability.* Evans blue 1% was used as a marker of vascular permeability (12). In all series of animals, Evans blue (1.8 mL/kg) was injected intravenously into the inferior vena cava 15 min before the start of superfusion. At the end of superfusion, samples of mesentery were obtained, weighed, and put in 1 mL of formamide for 48 h. The amount of Evans blue extracted in formamide was determined by spectrophotometry at a 620 nm wavelength using a Labsystems (Franklin, MA) iEMS plate reader. Results were plotted on a standard curve of Evans blue (1.5–50 µg/mL). The Evans blue content of each sample was expressed as µg/g tissue.

*(iii) Treatments.* Animals were superfused with Lyso-PAF, PAF, S(1)PAP, O(1)PAP, L(1)PAP, and SA(1,2)PAP. Some animals were treated with a PAF antagonist BN-52021 (i.v. 10 mg/kg) (IHB, LePlessise Robinson, France) prior to the superfusion. In addition, some animals were superfused simultaneously with PAF and S(1)PAP at different concentrations in order to evaluate a possible blocking effect of the PAP.

*Effect of PAP on PAF synthesis. (i) Alveolar macrophage isolation.* Alveolar macrophages were obtained by bronchoalveolar lavage (BAL). Lungs were dissected free of the thoracic cavity and a small length of tubing was inserted into the trachea and ligated. BAL was carried out with 10 mL of ice-cold Hank's saline solution instilled four times and withdrawn from the lungs. BAL from 2 or 3 animals was pooled, the cell suspension was centrifuged at 400 × *g*, and the pellet was resuspended in RPMI 1640 medium in presence of penicillin (100 units/mL) and streptomycin (100 mg/mL). Cells were counted, cultured in 24-well plates ($10^6$ cells/well), incubated for 1 h at 37°C under 5% $CO_2$ in air, and then washed twice with warm (37°C) medium to remove nonadherent cells. Supernatants of cultured alveolar macrophages were obtained after 24 h of cell culture at 37°C under 5% $CO_2$ in air (13).

*(ii) Cell treatment.* Synthesis of PAF was stimulated by incubation of alveolar macrophages with lipopolysaccharide (LPS) (10 ng/mL) during 45 min. In addition, mono- or diesters of PAP (100 nM, final concentration), dissolved in dimethylsulfoxide (DMSO), were added simultaneously with LPS in some wells ($n = 4$). Final concentration of DMSO was <0.5%. The same amount of DMSO was added at the start of the procedure in control and LPS wells. At the end of the incubation, 1 mL of cold methanol was added to the wells.

*(iii) PAF extraction.* Supernatants were transferred to 10-mL tubes and methanol, chloroform, and water were added to achieve a final composition of 2:2:2 (by vol). Samples were strongly vortexed and centrifuged (3000 × *g*; 15 min). The lower chloroform phase was collected, and the extraction of the aqueous phase was repeated. The two lipid-containing chloroform phases were pooled, evaporated to dryness, and resuspended in a mixture of methanol/water 15:85 to be processed through C18 Sep Pak cartridges (Waters, Milford, MA). Cartridges were activated with 10 mL of methanol and 10 mL of water, and washed with 10 mL of methanol/water 1:9, 10 mL of water, and 20 mL of petroleum ether. Finally, cartridges were eluted with 4.5 mL of methanol/chloroform (2:1) with 2% of acetic acid. Eluates were vacuum-evaporated to dryness and resuspended in assay buffer (14).

*(iv) PAF assay.* The amount of PAF was quantified with a "Platelet Activating Factor $^3H$ scintillation proximity assay" commercial kit, from Amersham (Buckinghamshire, United Kingdom), according to the supplier's instructions.

## RESULTS

*Studies on absorption, distribution, and excretion of PAP esters.* The distribution of $^3H$ and $^{14}C$ radioactivity found in different organs at 4 and 18 h after administration of radiol-labeled mono- and diesters of linoleyl PAP is shown in Figure 1. These two time periods were selected in order to distinguish

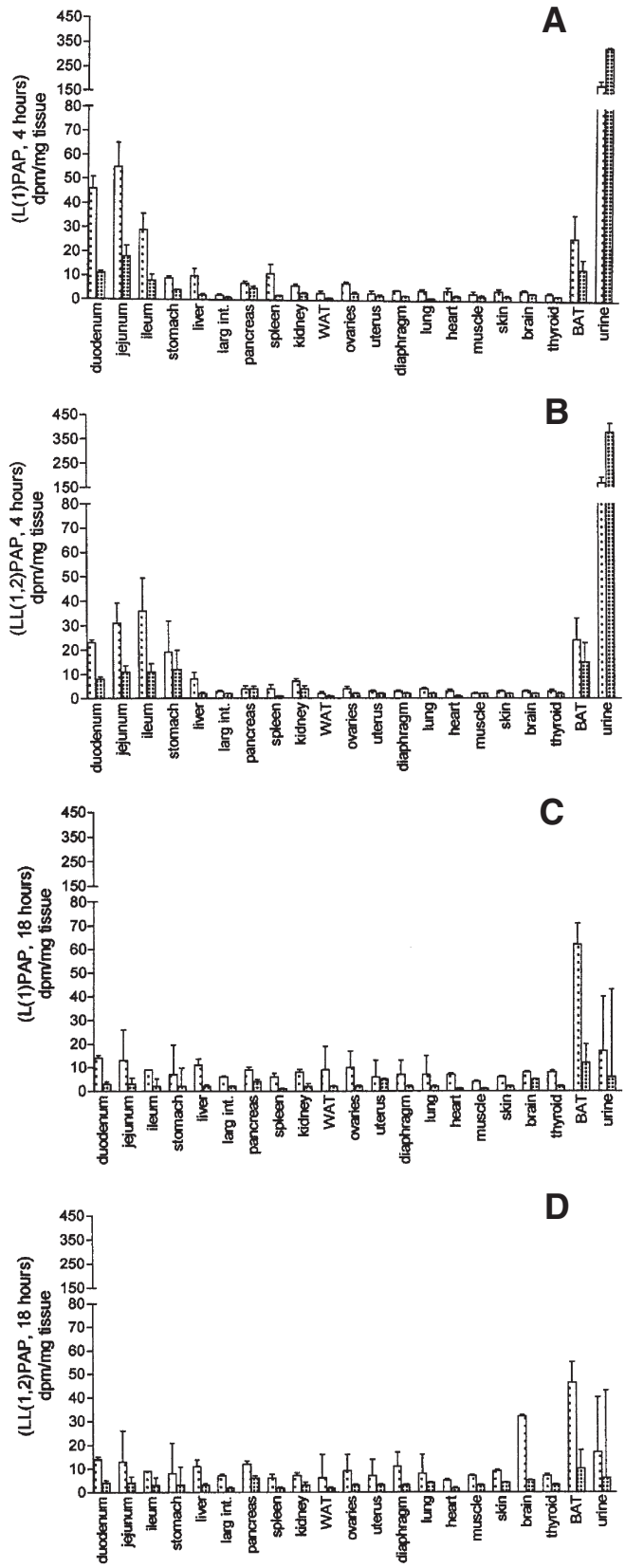



**FIG. 1.** Distribution of radioactivity (expressed as dpm/mg) in different organs 4 and 18 h after oral administration of 1-linoleyl-3-(*N*-phenylamino)-1,2-propanediol [L(1)PAP] or 1,2-dilinoleyl PAP [LL(1,2)PAP]. Dotted bars: $^{3}H$; lined bars: $^{14}C$. WAT, white adipose tissue; BAT, brown adipose tissue. Data are expressed as mean ± SEM.

between the rapid and late fate of the ingested products. Thus, after an initial time period (4 h) that allowed for rapid metabolism and excretion, the second time period, at 18 h, was intended to provide sufficient time for their complete distribution and/or metabolism.

Radioactivity was expressed as dpm measured per mg of tissue (or mL of fluid for urine). The major amount was initially concentrated in the urine. Other organs showing important amounts of radioactivity were the intestine at 4 h and BAT at 4 and 18 h. The mono- and diesters of PAP showed similar distribution profiles.

Figure 2 shows the percentage of radioactivity of $^{3}H$ and $^{14}C$ obtained in different samples compared to the proportion of radioactive forms in the two original oils administered: 35% $^{14}C$/65% $^{3}H$ for monoesters of PAP and 45% $^{14}C$/55% $^{3}H$ for diesters of PAP. All tissue samples at 4 h showed a clear predominance of $^{3}H$ in accordance with the radioactivity pattern of the original oil and in line with the overall lipophilicity of the organ (results are slightly affected by the different quenching present in different samples). On the other hand, the result found in urine at 4 h after administration indicated that the $^{14}C$, originally present in the aniline ring, was preferentially excreted. In urine samples obtained at 18 h after administration, the marked reversal in the proportions of $^{14}C$ and $^{3}H$ was no longer observed.

Figure 3 shows the radioactivity present in the aqueous phase of a Bligh and Dyer extraction of lipids in samples of tissue (liver, BAT, and white adipose tissue), plasma, and urine.

*Gastrointestinal biotransformation.* Figure 4 shows the fatty acid composition of the different PAP measured by liquid chromatography/mass spectrometry (LC/MS) in intestinal samples (jejunum) and liver after administration of L(1)PAP, and the differences observed as a function of the oil used as a vehicle. The

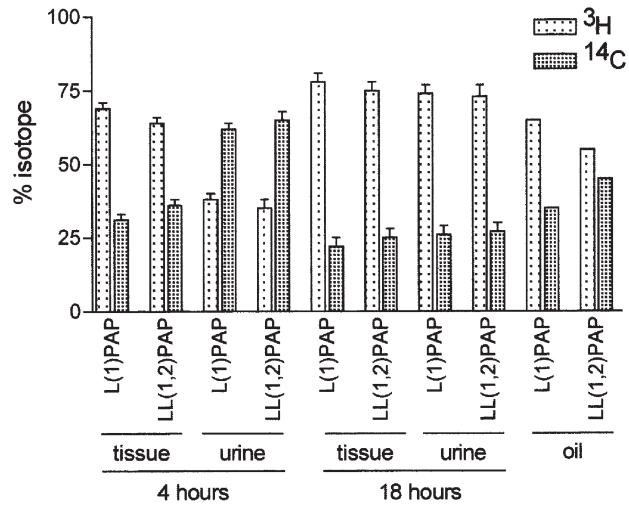



**FIG. 2.** Percentage of radioactivity corresponding to $^{3}H$ and $^{14}C$ obtained in tissue or urine 4 and 18 h after oral administration of radiolabeled L(1)PAP or LL(1,2)PAP. The original oils administered contained 65% $^{3}H$/35% $^{14}C$ for L(1)PAP and 55% $^{3}H$/45% $^{14}C$ for LL(1,2)PAP. For abbreviations see Figure 1. Data are expressed as mean ± SEM.

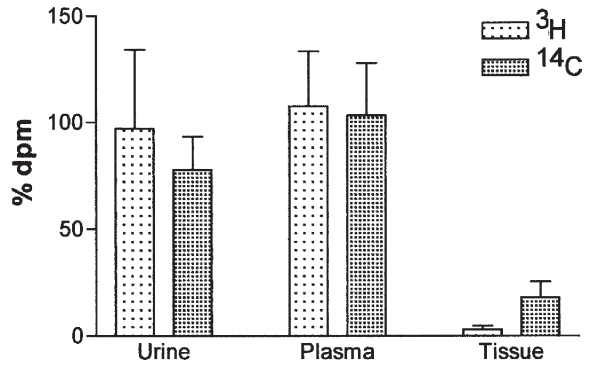



**FIG. 3.** Radioactivity present in the aqueous phase of a Bligh and Dyer (8) extraction of lipids. Results are presented as percentage of the dose administered. Data are expressed as mean ± SEM.

major amount of PAP is present as dPAP, indicating that during intestinal absorption, the monoesters of PAP are esterified in the *sn*-2 position. Lower amounts of mPAP are observed, with the original L(1)PAP predominating. The diesters detected maintain the linoleyl group but incorporate a new fatty acid that can be related to the main free fatty acids present in the oil used as vehicle: oleic acid for olive oil, palmitic acid for palm oil, and lauric and myristic acids for coconut oil. There is a good correlation between the percentage of free fatty acids present in the oils and the fatty acid taken up by the original molecule of L(1)PAP (Fig. 5). The presence of some mPAP species that do not include linoleic acid in their composition demonstrates the hydrolysis of the original linoleic acid moiety of the molecule.

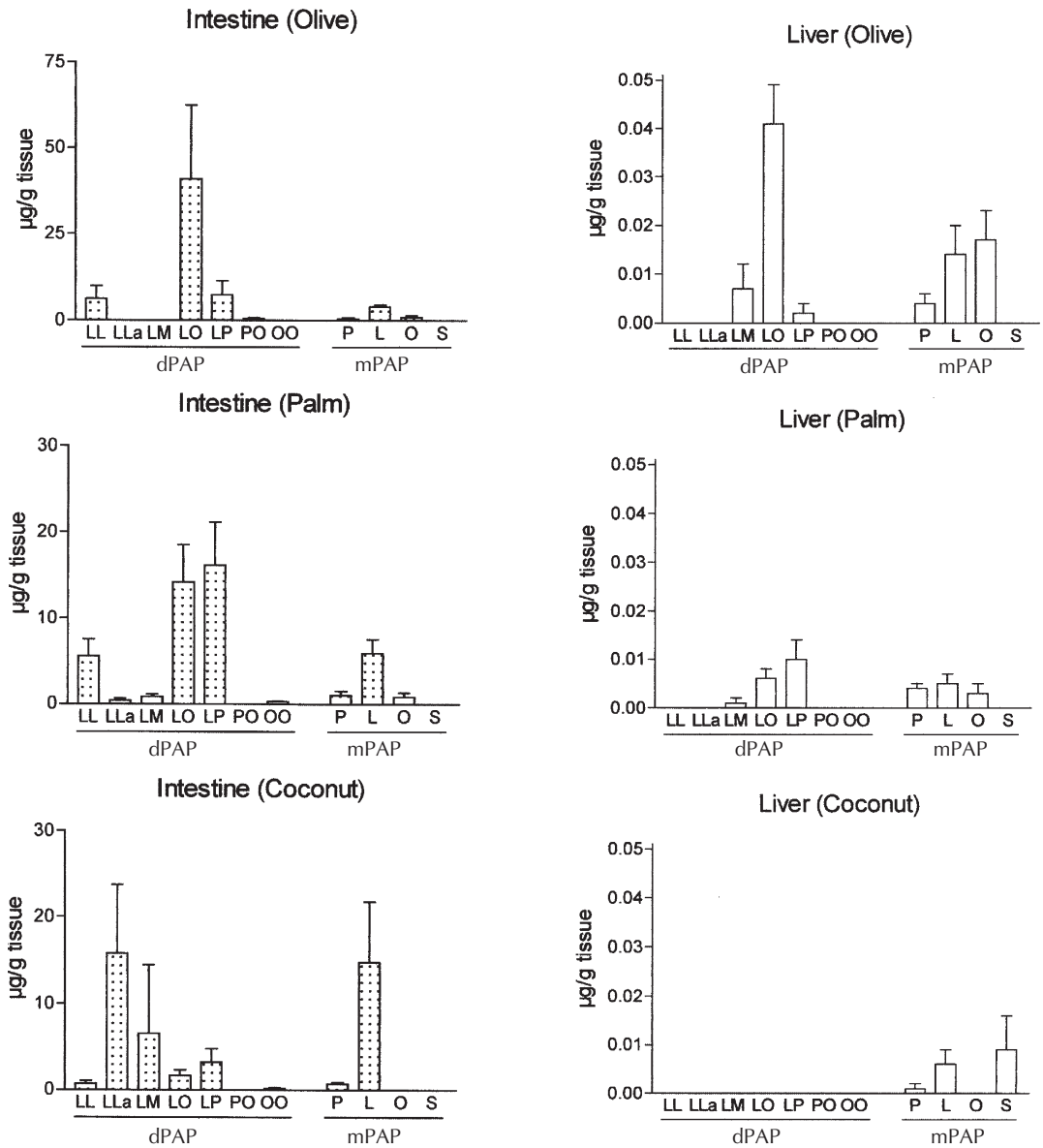



**FIG. 4.** Fatty acid composition of PAP observed in intestinal (left) and liver (right) samples after administration of linoleyl PAP, as a function of the oil administered as vehicle. The fatty acids present in the PAP esters are: P, palmitic; L, linoleic; O, oleic; S, stearic; La, lauric; M, myristic. Thus, LLa indicates that the DPAP incorporates linoleic and lauric acids. dPAP, diester of PAP; MPAP, monoester of PAP; for other abbreviations see Figure 1. Data are expressed as mean ± SEM.

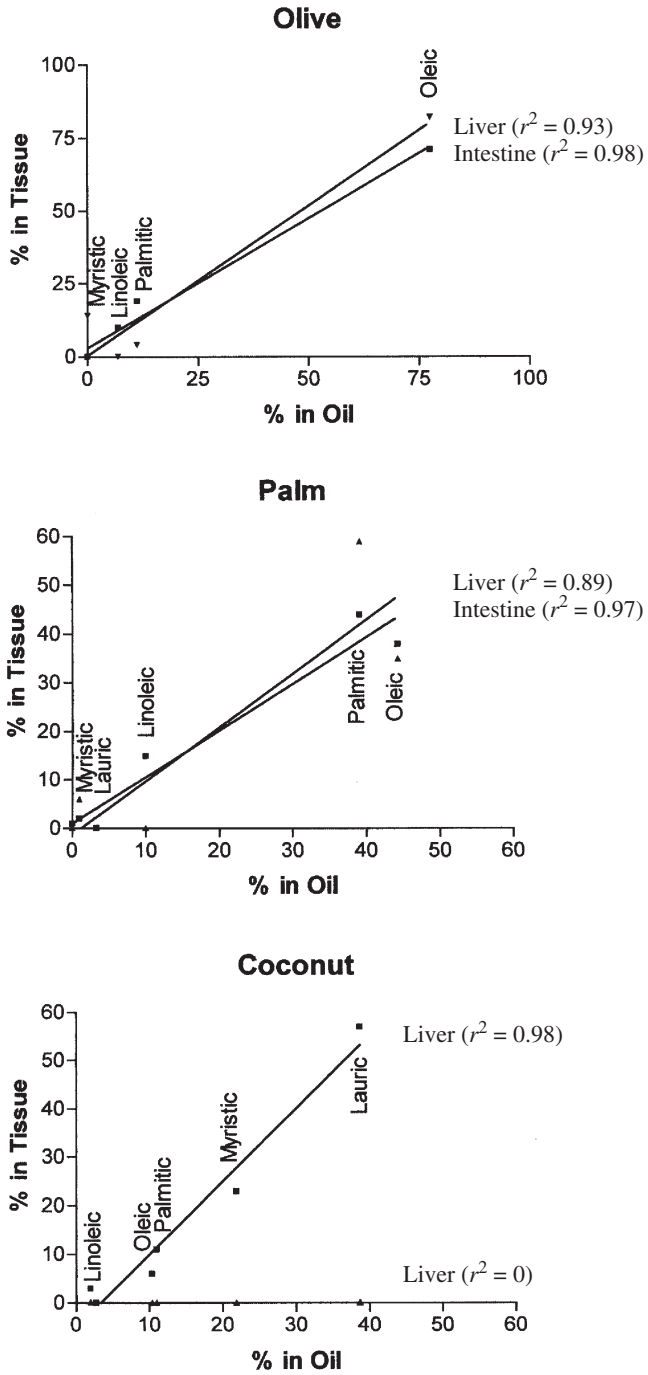



**FIG. 5.** Correlation between the percentage of free fatty acids present in the oils used as vehicle (x axis) and the fatty acid esterified in the original L(1)PAP found in tissue (y axis); intestine (■) or liver (▲). For abbreviations see Figure 1.

In the case of the liver, PAP were present in lower amounts than in jejunum. In this organ we found similar amounts of dPAP and mPAP [with the exception of LO(1,2)PAP for olive oil]. In addition, the predominance of L(1)PAP found in jejunum was not observed in the liver. The profiles found in dPAP in the liver were quite similar to those obtained in the jejunum with the exception of the coconut oil–treated group. In this case, only mPAP were found.

We did not find measurable amounts of any PAP metabolite in lung.

*Biological (PAF-like) activity: Changes in vascular permeability.* Figure 6 shows the changes in vascular permeability induced by superfusion of the different mediators analyzed. Lyso-PAF was used as an inactive control. As expected, superfusion of PAF greatly increased the vascular permeability of the mesentery. All other mediators evaluated showed no effect, except for the monoester of PAP with stearic acid [S(1)PAP]. This product induces a significant increase in vascular permeability. Nevertheless, the effect was very reduced when compared to PAF. To evaluate whether this effect could be related to the PAF receptors, we administered the PAF antagonist BN52021. This product completely inhibited the response to PAF but did not affect the increase in vascular permeability induced by S(1)PAP.

On the other hand, the possible effect of the diesters of PAP as antagonists of PAF was evaluated by cosuperfusion of these products with PAF (Fig. 7). The increase in vascular

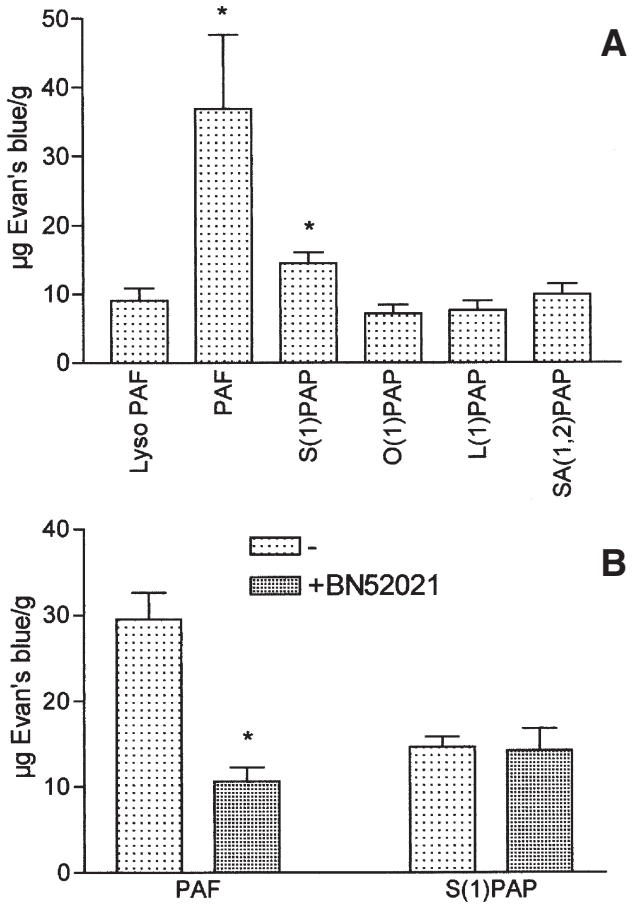



**FIG. 6.** Changes on vascular permeability (A) and effect of a platelet-activating factor (PAF) antagonist (B), measured as the accumulation of Evan's blue, after superfusion (100 nM, 30 min) of the different mediators analyzed. S(1)PAP, 1-stearyl-PAP; Oc(1)PAP, 1-octanyl-PAP; SA(1,2)PAP, 1-stearyl-2-acetyl PAP; for other abbreviations see Figure 1. Data are expressed as mean ± SEM. *$P < 0.05$ vs. Lyso PAF for Figure 6A; *$P < 0.05$ vs. untreated for Figure 6B.

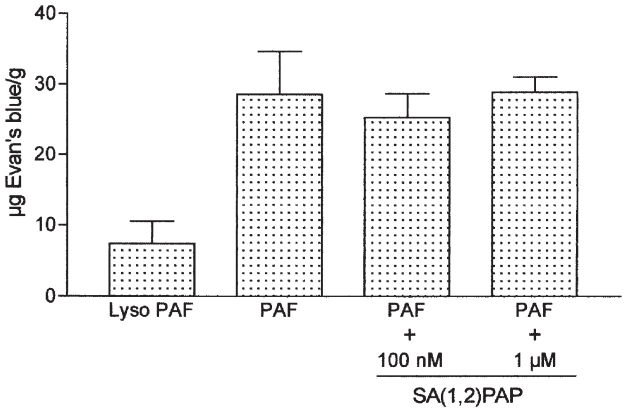



**FIG. 7.** Effect of dPAP on PAF-induced increases in vascular permeability. Effect of PAF (100 nM) was not modified by the presence of (100 nM) or (1 µM) SA(1,2)PAP in the superfusate. For abbreviations see Figures 1, 4, and 6.

permeability induced by 100 nM PAF was not modified by the presence of (100 nM) or (1 µM) SA(1,2)PAP in the superfusate.

*Effect of PAP on PAF synthesis.* The biosynthesis of PAF was induced in alveolar macrophages by LPS (Fig. 8). Mono- and diesters of PAP at 100 nM concentration inhibited the synthesis of PAF, with the exception of the monoester bearing octanoic acid in the *sn*-2 position.

## DISCUSSION

TOS, which occurred in Spain in 1981, was associated with the consumption of refined aniline-denatured rapeseed oil, illegally sold as olive oil (1). Further investigations reported that aniline and fatty acid anilides were present in TOS-related oils, and more recently the fatty esters of 3-(*N*-phenylamino)-1,2-propanediol have shown a strong association with TOS. There is also interest in these compounds because of their relation to the eosinophilia myalgia syndrome (EMS) that occurred in the United States in 1989 (15). That disease was associated with the consumption of L-tryptophan food supplements, and its clinical similarity to TOS suggested that a common biological pathway was affected in the TOS-associated case oils and the L-tryptophan case samples. *In vitro* conversion of (*N*-phenylamino)alanine, an EMS-associated contaminant (16), to 3-(*N*-phenylamino)1,2-propanediol has recently been reported (17). The latter compound forms the basic skeleton of the PAP esters.

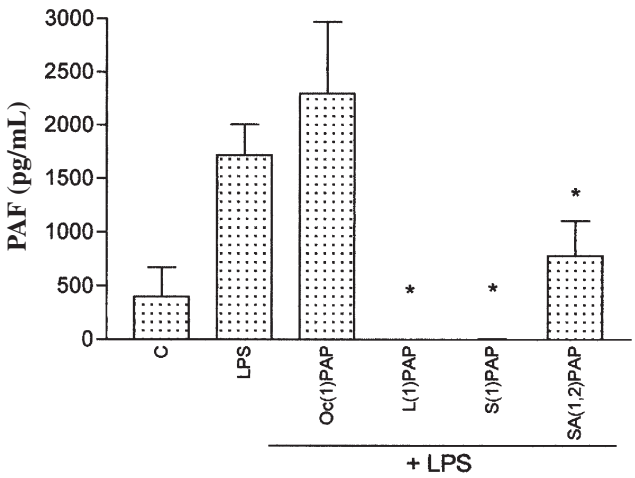



**FIG. 8.** Effect of mPAP and dPAP in the PAF synthesis induced by lipopolysaccharide (LPS) in alveolar macrophages. Oc(1)PAP, 1-octanyl PAP; C, control; for other abbreviations see Figures 1, 4, and 6. Data are expressed as mean ± SEM. *$P < 0.05$ vs. LPS.

The identification of PAP esters (Fig. 2) in TOS-related oil samples (2) suggested possible immunologic or inflammatory responses if these products were to be incorporated into cell membranes. This fact is also supported by the structural similarities of PAP esters and diacylglycerols (DAG). In addition, systemic bioactive phospholipids such as PAF are somewhat similar in structure. In this sense, it is important to understand not only the uptake and distribution of PAP esters but also the modification of these products upon gastrointestinal absorption.

The data obtained from rats after oral administration of radioactive doubly-labeled PAP indicate that these chemical species could be metabolized and distributed to different organs, being rapidly excreted in the urine (Fig. 3). Some organs appeared to be very active in the accumulation of these compounds, particularly the BAT, white adipose tissues, and the liver. The change in the isotopic composition found in urine 4 h after ingestion (Fig. 4) proved that these molecules were catabolized upon gastrointestinal absorption. Moreover, this observation suggests that the aniline-containing part of the molecule was preferentially excreted through urine, while constituent fatty acids were incorporated into the body pool of fatty acids. This observation would be in line with a recent report describing the biotransformation and clearance of the parent PAP, which was found to be highly metabolized in mice and excreted in urine in the form of 2-hydroxy-3-(phenylamino) propanoic acid (18). Results obtained measuring the radioactivity present in the aqueous phase of a Bligh and Dyer lipid extraction (Fig. 5) supported our conclusion. In tissue, radioactivity remained in the lipidic fraction so that little was recovered in the aqueous phase. By contrast, in plasma and urine radioactivity was totally extracted in the aqueous phase, indicating that the radioactivity present in plasma and urine corresponded to polar compounds or metabolic breakdown products. This result indicated that these compounds maintained their lipidic properties in the tissues, whereas in plasma and urine the radioactivity was mainly present in the form of hydrosoluble compounds. As shown in Figure 1, the preferential excretion of the $^{14}C$-labeled aniline ring is evident in the urine samples at 4 h, whereas in the urine collected after 18 h we mainly find $^{3}H$ excretion, probably in the form of catabolized end products of fatty acids, including $^{3}H$-labeled water.

The composition of PAP was found to change during intestinal absorption, and these changes were dependent on the vehicle used for their administration (Figs. 4 and 5). *sn*-1

Monoesters of PAP were esterified in the *sn*-2 position with a second fatty acid. The nature of this fatty acid was a function of the pattern of free fatty acids present in the oil administered as vehicle. The original monoester of the PAP administered was found only in low quantities, indicating that PAP were mostly incorporated in the form of dPAP. In addition, the presence of some esters of PAP that did not contain the original linoleic acid indicated that the intestine was able to hydrolyze the fatty acid originally present in the *sn*-1 position and re-esterify it with another fatty acid. These results suggest that the absorption and intestinal metabolism of PAP could be similar to that of DAG and open the possibility that PAP esters could interfere any of the cell pathways modulated or regulated by DAG.

When measuring the composition of PAP in liver (Figs. 4 and 5), we found that this organ showed a pattern of constituent fatty acids different from that of the PAP detected in the intestine, suggesting that the ability to modify the fatty acid composition of the PAP originally ingested is not restricted to the intestine. As in intestine, the liver showed a different profile of PAP as a function of the oil used as vehicle. For dPAP, changes observed in intestine were maintained in the liver, showing the original linoleic acid and a new fatty acid that was coincident with the fatty acid predominant in the oil. In contrast, mPAP showed clear differences when liver and intestine were compared. It could be suspected that PAP are absorbed by the gastrointestinal tract as dPAP, distributed, and stored in this form in the different organs, whereas mPAP are the result of dPAP metabolism.

On the other hand, the ability to modify the composition of the fatty acids present in the ingested PAP molecule implies that any physiological effects of PAP esters could be induced by PAP esters with a fatty acid composition different from that in the original PAP identified in the toxic oil samples.

When evaluating the potential biological PAF-like effects of PAP, we found a moderate increase in the vascular permeability of the mesentery when it was superfused with the monoester of stearic acid (Fig. 6). This effect was lower than the effect of PAF and was not affected by the presence of PAF receptor antagonists. Therefore, it seems that the effect is not mediated by the structural similarities between S(1)PAP and PAF. On the other hand, the diester of PAP with stearic acid in the *sn*-1 position and acetic acid in the *sn*-2 position (which is the most similar in its structure to PAF), is not able to modify the effect of PAF when assayed at equimolar or ×10 concentrations (Fig. 7).

The most remarkable result found in relation to PAF metabolism is the effect of different PAP on the synthesis of PAF (Fig. 8). When incubating alveolar macrophages with LPS to induce PAF synthesis in the presence of different PAP, we found an inhibitory effect by the esters that contained linoleic or stearic acid in the *sn*-1 position. In contrast, PAP esters containing shorter fatty acids (octanoic) had no effect. The presence of an acetyl group in the *sn*-2 position did not affect the inhibitory effect of the compound, and only the presence of a long ($C_{16}$–$C_{18}$) chain in the *sn*-1 position appeared to be important for this effect.

Some enzymes involved in PAF synthesis show low substrate specificity. For example, acetyltransferases can utilize both acyl-lysoglycerophosphate and acyl-lysoglycerophosphatidylcholine. For this reason, significant quantities of PAF analogs (acyl, alkyl, ethanolamine, and other analogs) have been reported to be formed in various cells (6,19). Taking into account this low substrate specificity, one could hypothesize that some enzymes involved in the pathway of PAF synthesis could be blocked by structurally similar compounds such as PAP esters with $C_{16}$ or $C_{18}$ in the *sn*-1 position.

Despite the low probability that the inhibitory effect could explain the toxic effect of oil samples, these results indicate that these compounds can be effectively incorporated into the lipid metabolic pathways and interfere with some of the enzymes involved in these pathways. However, this fact need not be restricted only to phospholipid analogs, since the incorporation of PAP esters into endogenous lipid metabolic pathways implies the possible generation of other families of mediators, such as modified DAG analogs, that could be suspected to contribute to pathogenesis of TOS. Taken together, the latter consideration plus the data reported herein open the way for new approaches to the study of the metabolically derived physiological effect of PAP esters.

## ACKNOWLEDGMENTS

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# Arachidyl Amido Cholanoic Acid (Aramchol) Is a Cholesterol Solubilizer and Prevents the Formation of Cholesterol Gallstones in Inbred Mice[1]

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**ABSTRACT:** We have recently synthesized fatty acid bile acid conjugates (FABAC) that were able to reduce and retard cholesterol crystallization in model and human biles. When given orally, they prevented the formation of cholesterol crystals in the bile of hamsters. The aim of the present study was to determine whether the FABAC are cholesterol solubilizers, whether they can dissolve pre-existing crystals, whether they can prevent the formation of cholesterol gallstones, and to investigate the optimal type of bond between the fatty acid and bile acid. The presence of cholesterol crystals was determined by light microscopy, and the total crystal mass of precipitated crystals was measured by chemical means. Inbred (C57J/L) mice on a lithogenic diet were used to evaluate cholesterol crystal formation, dissolution, and gallstone formation *in vivo*. Arachidyl amido cholanoic acid (Aramchol) was the FABAC used in the present experiments. At equimolar amounts, the cholesterol-solubilizing capacity of Aramchol was higher than that of taurocholate and similar to that of phosphatidylcholine. The addition of Aramchol dissolved approximately 50% of pre-existing crystals in model bile solutions. The same phenomenon was demonstrated in human bile *ex vivo*, with a dose–response effect. All inbred mice developed cholesterol crystals in bile after 10–14 d on the lithogenic diet. Thereafter, supplementation of the diet with Aramchol progressively reduced the proportion of mice with crystals to 25% after 28 d. On the lithogenic diet, 100% of inbred mice developed cholesterol gallstones in the gallbladder by day 21. None of the mice whose diet was supplemented with 0.5 mg or 1.0 mg of Aramchol/d developed stones or crystals. FABAC are a new class of molecules that are cholesterol solubilizers and which are able to dissolve cholesterol crystals in bile. Upon oral administration, they dissolve pre-existing cholesterol crystals and prevent the formation of gallstones in gallstone-susceptible mice.

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Fatty acid bile acid conjugates (FABAC) are a new class of synthetic molecules produced with the aim of reducing cholesterol crystallization in bile (1). It has been demonstrated that phospholipids, and not bile acids, are the major cholesterol solubilizers in bile (2–5). The FABAC were designed to bring parts of the phospholipid molecule (long-chain saturated fatty acids) into bile, using the very efficient absorption and biliary secretion of bile acids. The whole FABAC molecule has, however, characteristics that are different from those of its components.

The FABAC, and in particular 3-β-arachidylamido-7-α, 12-α-dihydroxy-5-β-cholan-24-oic acid (arachidyl amido cholanoic acid, Aramchol, $C_{20}$-FABAC), were shown to prolong the cholesterol nucleation time markedly and reduce the total crystal mass in model bile solutions (1). In native human gallbladder bile, FABAC were even more effective, preventing crystal formation for weeks and reducing the eventual crystal mass to minute proportions. Aramchol was also shown to prevent *in vivo* biliary cholesterol crystallization in experimental animals (1). Thus, almost three decades after the introduction of chenodeoxycholic acid for gallstone dissolution (6), FABAC raise again the possibility of a medical therapy for the prevention and/or dissolution of cholesterol gallstones. Many questions in relation to the mechanism of action and metabolism of the FABAC remain open. The present study was designed to answer four main questions: (i) Do FABAC act as cholesterol solubilizers in bile? (ii) Can they dissolve formed, pre-existing cholesterol crystals *in vitro* and *in vivo*? (iii) Can they prevent the formation of cholesterol gallstones *in vivo*? (iv) Does the kind of conjugation (ester vs. amide) influence the absorption and biliary secretion of the FABAC?

The experiments were performed with Aramchol, which is a conjugate of arachidic and cholic acid (at position 3) using an amide bond. This compound in particular was found to effectively inhibit *in vitro* cholesterol crystallization in our earlier study (1).

## MATERIALS AND METHODS

*Materials.* Egg yolk phosphatidylcholine (PC), 99% pure, was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Free fatty acids (FFA), cholesterol, sodium taurocholate (TC), and cholic acid (CA), >98% pure, were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol, TC, and CA were recrystallized prior to use. All other chemicals and

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Abbreviations: Aramchol, arachidyl amido cholanoic acid; CA, cholic acid; CSI, cholesterol saturation index; FABAC, fatty acid bile acid conjugates; FFA, free fatty acid; PC, phosphatidylcholine; TC, taurocholate; UDCA, ursodeoxycholic acid.

solvents were American Chemical Society or reagent grade. The glassware was acid washed, thoroughly rinsed in distilled water, and dried.

*Preparation of Aramchol.* Aramchol was prepared as previously described (1) by conjugating cholic acid with arachidic acid ($C_{20}$) in the 3-position, using an amide bond. The conjugation was in the β configuration. For the absorption studies (see below) an ester conjugate was similarly prepared. Both compounds were purified by silica gel chromatography and characterized by $^1$H nuclear magnetic resonance and mass spectrometry.

*Model biles.* The model biles were prepared from a mixture of cholesterol (in chloroform), PC (in chloroform), and TC (in methanol) in various concentrations (specified in the Results section), as previously described (7). The model bile solution was incubated under argon at 37°C, and aliquots were taken for analysis at predetermined times throughout the crystallization process.

*Human bile.* Human gallbladder biles were obtained from gallstone patients at cholecystectomy. The bile was aspirated after needle puncture of the gallbladder prior to mobilization of the gallbladder. Informed consent was obtained according to a protocol approved by the local institutional human subjects committee. Biles were ultracentrifuged (200,000 × *g*, 45 min, 25°C) prior to testing to get rid of crystals and cell debris.

*Solubilization experiments. (i) Cholesterol-solubilizing capacity.* Model biles (30 mM PC, 150 mM Na-TC) were prepared with two different cholesterol concentrations (12.2 and 9.9 mM) to yield a supersaturated [cholesterol saturation index (CSI), 1.1] and an undersaturated (CSI, 0.9) bile solution, respectively. They were then incubated overnight with 10-mM $^3$H-labeled dried cholesterol. The $^3$H-cholesterol solubilization after 24 h of incubation at 37°C was measured. The amount of cholesterol remaining in the supernatant solution after ultracentrifugation for 5 min at 76.000 × $g_{max}$ was the parameter for solubilization. Solubilization of cholesterol in the model bile was compared to that observed in biles that were supplemented with 5 mM Na-TC, PC, or Aramchol.

*(ii) Dissolution of pre-existing cholesterol crystals in bile.* Model bile (as above) or native bile from a cholesterol gallstone patient (after ultracentrifugation for 1 h at 200,000 × $g_{max}$ at room temperature to get rid of debris or crystals) was incubated at 37°C. After crystallization, *in vitro* or *ex vivo*, had progressed and reached an apparent equilibrium (no further increase in crystal mass), incubation was continued without as well as after adding FABAC. Serial microscopic observations and determinations of total crystal mass were performed.

*Evaluation of cholesterol crystal formation and growth. (i) Light microscopy.* Aliquots (5 μL) of bile or model bile were examined by a light microscope (Zeiss, Jena, Germany) under polarized light and with the differential interference mode as previously described (7). Cholesterol crystals were identified by their characteristic morphology and birefringence, and counted per microscopic field at 100-fold magnification.

*(ii) Measurement of crystal mass.* Chemical analyses of cholesterol were performed on model bile samples after *in situ* crystallization, as previously described (8). In human biles the mass of the pelleted crystals was quantitated after ultracentrifugation.

*Animal experiments.* All animal experiments related to cholesterol crystallization were performed with male inbred mice (C57J/L, 4 wk old, approximately 20 g). Cholesterol crystal and gallstone formation was induced by feeding the mice a lithogenic diet containing regular chow (Koffolk, Petach Tikva, Israel), to which butterfat 15%, cholesterol 1%, cholic acid 0.5%, and corn oil 2% (w/w) were added (9).

*Dissolution of preformed crystals.* After 10 and 14 d on the lithogenic diet, a group of mice was sacrificed and gallbladder bile was examined to ascertain the presence of cholesterol crystals. Subsequently, in the remaining animals, Aramchol mixed with saline was administered by gavage (*via* a feeding needle) at a dose of 3 mg/animal/d. Half of the remaining animals were sacrificed after 14 d and the rest after 28 d of Aramchol feeding. The presence of cholesterol crystals and stones in the gallbladder was examined at each time interval.

*Prevention of gallstone formation.* Mice were sacrificed after 3 wk of consuming the lithogenic diet with or without Aramchol (0.5 or 1.0 mg/animal/d). The percentage of animals harboring gallstones (and cholesterol crystals) in their gallbladders was determined by direct inspection and microscopy of their gallbladders.

*Comparison of absorption and biliary secretion of ester- and amide-bonded conjugates.* Male C57Black mice received 3 mg/animal of FABAC by intragastric administration; and heart blood, portal blood, and gallbladder bile were sampled in groups of three animals sacrificed after 1, 2, and 3 h. One group ($n$ = 9) received the regular Aramchol, with an amide (NH) bond between the bile acid and the fatty acid. Another group ($n$ = 9) received the compound with an ester bond. In a separate experiment, animals (3 in each group) were sacrificed 24 h after the intragastric administration of the ester or amide conjugate

*Analytical methods.* Biliary lipids were extracted by chloroform/methanol (2:1, vol/vol) and quantitated as previously described (10–12). Aramchol concentration was determined by high-performance liquid chromatography (Kontron) employing a Phenomenex Luna reversed-phase C-18 column as previously described (1). Samples were applied dissolved in methanol. The running phase was methanol 100%, at a flow rate of 0.9 mL/min. Aramchol was detected at 206 nm.

*Statistical analysis.* Student's $t$ test was used to compare the data. A $P$-value of $<0.05$ was considered significant.

## RESULTS

The cholesterol-solubilizing capacity of Aramchol was studied in two model bile solutions—one undersaturated (CSI, 0.9) and the other supersaturated (CSI, 1.1) with cholesterol. The amounts of cholesterol solubilized by Aramchol in excess of the amount solubilized by the model solutions alone were 0.9 and 1.4 mM, respectively. The corresponding amounts solubilized by NaTC were 0.6 and –0.4 mM, while those by PC were

0.9 and 0.9 mM, respectively. Thus, the cholesterol-solubilizing capacity of Aramchol was higher than that of NaTC and similar to that of PC. The effect was more marked in the supersaturated model.

The ability of Aramchol to dissolve (preformed) cholesterol crystals was studied in two model biles (model A: 15 mM cholesterol, 30 mM PC, 150 mM Na-TC; model B: 18 mM cholesterol, 36 mM PC, 120 mM Na-TC). Aramchol was added at a concentration of 7 mM to model A and at 10 mM to model B, based on the maximal solubility of Aramchol in these models. As shown in Figure 1, the cholesterol crystal mass in both model biles decreased after addition of Aramchol, from 4.4 to 2.7 μmol in model A ($P = 0.28$) and from 7 to 2.9 μmol in bile B ($P = 0.0004$), respectively. Figure 2 shows the effect of Aramchol on preformed cholesterol crystals in human bile. In the native bile, the addition of Aramchol also decreased the crystal mass significantly. The effect was dose-dependent in the dose range studied (10–30 mM). Fourteen days after the addition of Aramchol (10 and 30 mM), the total crystal mass decreased from 2.4 ± 0.1 to 1.5 ± 0.1 μmol ($P < 0.02$) and to 0.1 ± 0.01 μmol ($P = 0.01$), respectively. The decreases corresponded to 38 and 94%, respectively. Three human biles were studied. In all of them, the addition of 30 mM Aramchol resulted in the disappearance of plate-like cholesterol crystals while decreasing the total crystal mass to 6–62% of control. In two-thirds of the cases, the addition of Aramchol converted the plate-like (mature) crystals to filamentous (less mature) crystal forms (13) and reduced the total crystal mass in all three.

The ability of Aramchol to dissolve preformed cholesterol crystals *in vivo* was studied in inbred mice. Two sets of experiments, with a total of 36 mice, were performed. Figure 3 shows the percentage of mice with crystals in their gallbladders as a function of time of feeding the lithogenic diet with and without Aramchol. On days 10 and 14 all sacrificed animals ($n = 7$ and 8, respectively) had crystals in their gallbladders. However, when Aramchol (at 3 mg/animal/d) was then added to the diet, the percentage of animals with crystals gradually dropped to 75% on day 28 ($n = 12$) and to 25% on

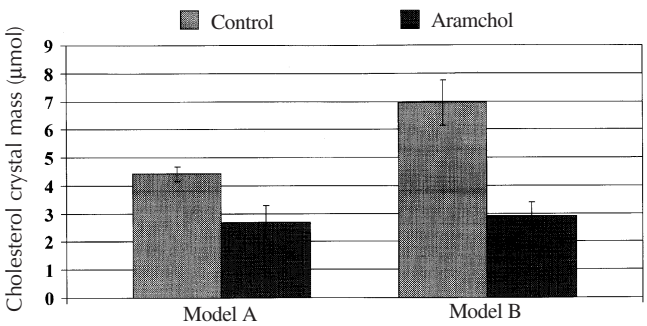


**FIG. 1.** Cholesterol crystal mass after 19 d of incubation of pre-existing crystals in model biles (model A: 15 mM cholesterol, 30m M egg lecithin, 150 mM Na-taurocholate; model B: 18 mM cholesterol, 36 mM egg lecithin, 120 mM Na-taurocholate) in the absence or presence of Aramchol (model A: 7 mM, model B: 10 mM; $n = 3$ for both). The decrease was 39% ($P = 0.28$) in model A and 58% ($P = 0.0004$) in model B.

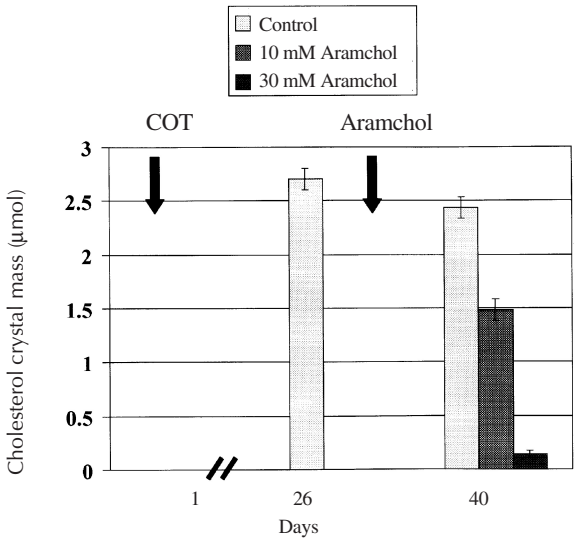


**FIG. 2.** Cholesterol crystal solubilization in human bile *ex vivo* by Aramchol, dose–response effect. Bile from a cholesterol gallstone patient was incubated at 37°C until apparent crystallization equilibrium (day 26). Incubation was then continued for another 14 d in the absence or presence of 10 or 30 mM Aramchol. Microscopic observations and determinations of the final crystal mass were performed. Aramchol decreased the crystal mass by 38 (grey bar: 10 mM) and 94% (black bar: 30 mM). COT, crystal observation time.

day 42 ($n = 9$). This observation contrasts with that following a continuation of the lithogenic diet without any other manipulations, which yields more crystals, and eventually stones (data not shown; Ref. 9).

The ability of Aramchol to prevent gallstone formation *in vivo* was also studied in inbred mice ($n = 17$). As seen in Figure 4, feeding of Aramchol together with the lithogenic diet completely prevented the formation of gallstones (as well as

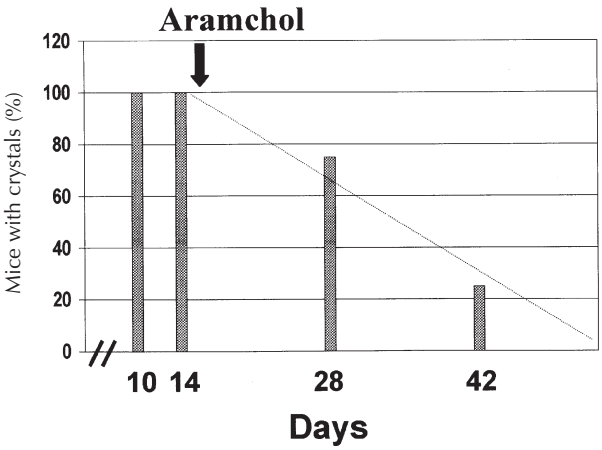


**FIG. 3.** Effect of fatty acid bile acid conjugate feeding on pre-formed cholesterol crystals *in vivo*. All mice ($n = 36$) were fed a lithogenic diet throughout the experiment. Seven animals were sacrificed after day 10 and another 8 after day 14: All had cholesterol crystals in their gallbladders. Thereafter, Aramchol (3 mg/d) was added to their diet. In these Aramchol-supplemented mice ($n = 21$), the proportion with crystals declined progressively to 75% after 14 d and to 25% after 28 d of Aramchol feeding.

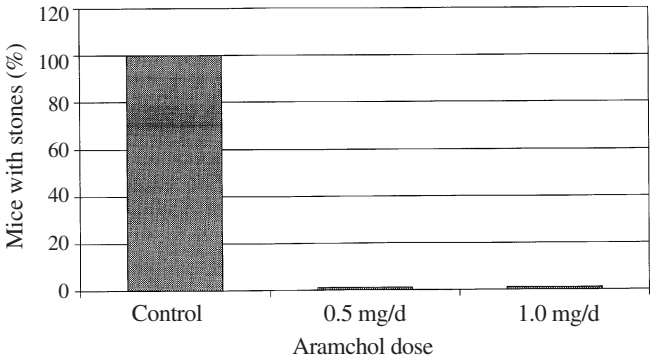


**FIG. 4.** Effect of Aramchol feeding on cholesterol gallstone formation *in vivo*, expressed as a percentage of inbred mice harboring cholesterol gallstones in their gallbladders after 3 wk of feeding a lithogenic diet, with or without Aramchol (0.5 or 1.0 mg/animal/d). At the end of the experiment, none of the Aramchol-fed mice ($n = 10$) had stones (or crystals) in their gallbladders; stones were found in all the controls ($n = 5$).

crystals) in the mice at both doses studied (0.5 and 1.0 mg/animal/d). All control animals developed both crystals and cholesterol gallstones by day 21, whereas none of the Aramchol- supplemented animals had either crystals or gallstones. The concentrations of Aramchol in the gallbladder bile of mice fed 0.5 mg/d varied between 0.03 and 0.17 mM (≥24 h after the last dose).

Bile and heart blood levels of amide- and ester-bonded Aramchol after a single intragastric dose of 3 mg/mouse are shown in Figure 5. In mice receiving the amide-bonded Aramchol, bile levels after 1, 2, and 3 h were 0.3 ± 0.02, 0.4 ± 0.03, and 0.7 ± 0.06 mM, respectively. In mice receiving the compound with an ester bond between the arachidic and cholic acids, bile levels remained below 0.07 mM at all times. Blood levels in the animals receiving the ester-bonded

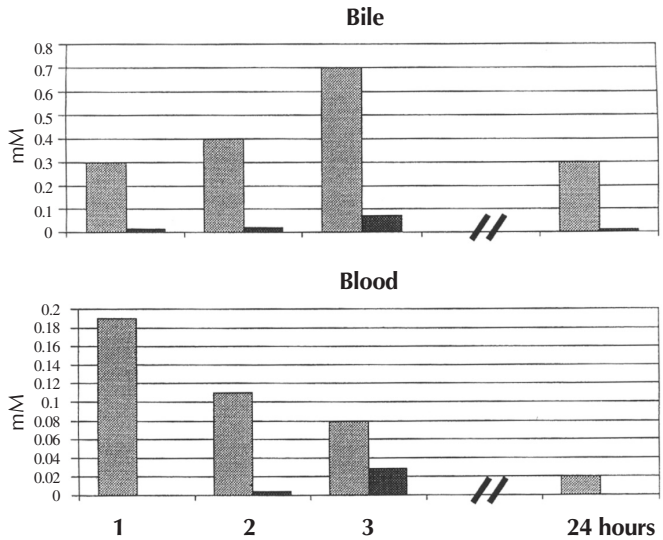


**FIG. 5.** Bile and heart blood levels of amide- (gray bars) and ester- (black bars) bonded Aramchol after a single intragastric administration of 3 mg/animal at 0 time. Three animals were tested at each time point with either conjugate. A separate experiment was performed to test levels at 24 h.

FABAC were below 0.03 mM at all times, whereas in the animals receiving the amide-bonded compound blood levels were mostly between 0.1 and 0.2 mM. Portal blood levels were also significantly higher with the amide-bonded Aramchol (0.1–0.3 mM) compared to the ester-bonded compound (0.001–0.1 mM). Twenty-four hours after an intragastric administration of 3 mg/animal, bile levels were 0.3 ± 0.03 mM with the amide and 0.01 ± 0.001 mM with the ester bond, while heart blood levels were 0.02 ± 0.002 and <0.0001 mM, respectively (Fig. 5).

## DISCUSSION

Aramchol represents a new group of synthetic molecules (FABAC), which were recently shown to inhibit cholesterol crystallization (1). In our previous study we showed that they prolong the crystal observation time and decrease the crystal mass in model and human biles. The FABAC were absorbed after intragastric administration and prevented crystal formation in experimental animals. Their mode of action (solubilization vs. inhibition of crystallization) was not studied. The present study clearly indicates that Aramchol is a cholesterol solubilizer. *In vitro* it dissolves solid amorphous cholesterol when added to either an undersaturated or supersaturated model bile solution. Aramchol dissolves formed cholesterol crystals in model bile and also in human gallbladder bile *ex vivo*. We have now demonstrated, for the first time, that upon oral administration it can dissolve preformed cholesterol crystals *in vivo* and, most significantly, prevent the development of cholesterol gallstones in inbred mice.

Many of the structure/function relationships of the FABAC have now been elucidated. Those with longer-chain fatty acids ($C_{18}$–$C_{22}$) were the most potent in terms of cholesterol solubilization (1). FABAC containing shorter-chain fatty acids ($C_6$–$C_{12}$) were not effective, while those with $C_{14}$, $C_{16}$, and $C_{24}$ fatty acids had a smaller effect (1). The Aramchol used in the present experiments is one of the more effective compounds.

Conjugation with cholic acid (at position 3) results in a dihydroxy compound. Conjugation with a dihydroxy bile acid (e.g., ursodeoxycholic acid) would result in a monohydroxy compound, which could be toxic. However, this is at the moment a speculative consideration, which is being further studied. Our results show that the bond between the fatty acid and the bile acid is very important. An ester bond is easily broken down by intestinal enzymes and bacteria, resulting in the separate absorption of a fatty acid and a bile acid. Thus, little of the intact ester conjugate reaches the bile (Fig. 5). Further breakdown may occur during enterohepatic cycling. Esters of fatty acids with bile acids have been found in the feces (14). These compounds have also been synthesized by Kritchevsky's group (15) in an attempt to delay the intestinal catabolism of chenodeoxycholic and ursodeoxycholic acids. The amide bond used in our experiments is a more stable bond, allowing the absorption and biliary secretion of the intact FABAC. The amide bond is, however, not exclusive. Any stable bond, permitting the absorption and biliary secretion of the intact

FABAC and providing effective cholesterol solubilization, is suitable. The bond between the fatty acid and bile acid can be *via* a bonding molecule, or it can be a direct bond (C=C).

It should be noted that the mechanism of action of the FABAC is quite different from that of chenodeoxycholic and ursodeoxycholic acids. These specific bile acids reduce the mole percentage of cholesterol in hepatic bile by an effect on biliary enzymes and/or cholesterol absorption in the gut (16). They are not cholesterol solubilizers. The direct, *ex vivo* addition of bile salts to human gallbladder bile does not prolong the nucleation time, but the addition of a cholesterol solubilizer, like phospholipids, markedly prolongs the nucleation time. This has been demonstrated by Jungst *et al.* (2). As shown in the present investigation, FABAC are cholesterol solubilizers. This was demonstrated *in vitro* where any potential effects on liver and intestines was excluded.

The cholesterol-solubilizing effect of FABAC was stronger in human bile as compared to model biles. In human bile FABAC have an effect at concentrations of 3–5 mM while in model biles concentrations of up to 30 mM are required (1). This higher effectiveness in human bile has now been confirmed many times, but the explanation is not clear at the moment.

Another major difference was observed in relation to the effect of FABAC on bile *in vivo* and *ex vivo*. To prolong the nucleation time and reduce the crystal mass in bile *ex vivo*, concentrations of 3–5 mM were required (1). To dissolve pre-existing crystals in bile *ex vivo* (using a single addition of FABAC to bile), concentrations of 10–30 mM were required. They dissolved approximately 50% or more of pre-existing crystals, usually converting the remaining crystals to earlier crystal forms (plates to filaments) (Fig. 2). After the administration of a single oral dose of 3 mg/mouse of Aramchol, biliary concentrations were 0.3–0.5 mM after 2 h and 0.1–0.6 mM after 3 h. On chronic oral administration of 3 mg/d of Aramchol, the concentrations in gallbladder bile (≥24 h after the last dose) were of the order of 0.2–0.6 mM. The dissolution of the pre-existing and continuously produced crystals was progressive (Fig. 3), with only 25% of animals having crystals after 28 d. An extrapolation of the curve predicts complete disappearance of crystals approximately 33 d after the start of Aramchol ingestion. The oral administration of 0.5 mg/d prevented the formation of crystals and gallstones in the gallbladder (Fig. 4). This was accomplished with biliary concentrations (≥24 h after the last dose) in the range of 0.03–0.17 mM.

The difference between the 3–5 mM effective in bile *ex vivo* and the 0.1–0.6 mM effective *in vivo* is marked. No definite explanation is presently available. An additional effect of the FABAC on liver, gallbladder, or intestine cannot be excluded. Studies using mass spectrometry demonstrated intact FABAC in blood and bile (Leinkin-Frenkel, A., unpublished data). We have at present no evidence for the presence of metabolites. Further research is ongoing.

The lithogenic tendency in inbred mice on a high-cholesterol diet is very strong. Under these conditions, close to 100% of the mice develop cholesterol crystals in bile within 7–10 d and gallstones within 3–8 wk (9). In human models, the process takes much longer and is less effective. During pregnancy, biliary sludge is formed in almost one-third of women but mostly during the second or third trimester, i.e., 4–9 mon (17). Cholesterol crystals are a major component of biliary sludge. In obese subjects ingesting a very low calorie diet or undergoing gastroplasty, gallstones develop in up to one-third of subjects, although usually only after several months (18). Also, patients receiving octreotide take months to develop gallstones and even then are in only some of the patients (19). The fact that FABAC almost completely prevent the formation of crystals and stones in lithogenic mice and accomplish this within 1–3 wk of ingestion suggests that their solubilizing effect is strong. Moreover, this is accomplished at doses very close to the doses (on a molar equivalence basis) used in bile salt therapy in humans (15 mg/kg/d) (16).

Unlike bile salts, FABAC are found in the systemic circulation at concentrations similar to or higher than those in the portal vein (1). This is probably due to transport *via* the lymph. Moreover, after a single oral dose they circulate in the vascular tree for over 48 h. In view of their cholesterol-solubilizing capacity, this raises the possibility of their applicability in atherosclerosis as well.

The use of chenodeoxycholic acid and ursodeoxycholic acid (UDCA) proved that cholesterol gallstones could be dissolved (16). The process was, however, too long and of low efficacy. It was further shown that for medium- and large-sized stones lithotripsy was needed. The addition of UDCA after lithotripsy did not accelerate fragment evacuation and/or dissolution (20). More importantly, UDCA therapy did not effectively prevent gallstone recurrence after successful non-surgical therapy (21,22). FABAC therapy has the potential for correcting some of these shortcomings.

In summary, the data to date suggest that FABAC may be a potential medical therapy to prevent and/or to dissolve cholesterol gallstones in humans.

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# Alterations in Fatty Acid Composition of Tissue Phospholipids in the Developing Retinal Dystrophic Rat

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**ABSTRACT:** Alterations in lipid composition occur in the retinal pigment epithelium and photoreceptor cells of the Royal College of Surgeons (RCS) dystrophic rat, a model for inherited retinal degeneration. With respect to lipid composition of nonretinal tissues, the developmental timing of lipid alterations and the incidence of dystrophy are unknown. We determined the fatty acid composition in choline phosphoglycerides (ChoGpl) and ethanolamine phosphoglycerides (EtnGpl) in the brain, liver, and retina from dystrophic RCS rats and from their nondystrophic congenics (controls) at the ages of 3 and 6 wk. At 3 wk, the fatty acid compositions were specific to individual phospholipid classes without any difference between dystrophic and nondystrophic tissues. In plasma phospholipids, there was an age-related increase in the relative contents of monounsaturated and n-3 polyunsaturated fatty acids, with only minor differences between dystrophic and nondystrophic rats. At 6 wk, the fatty acid compositions in ChoGpl and EtnGpl from dystrophic brain and retina were significantly different from those of nondystrophics. The effect of strain on developmental changes in brain fatty acid composition was significant for 18:0 and 22:6n-3 in EtnGpl and for 16:0, 18:0, 18:1n-9, and 20:4n-6 in ChoGpl. The brain ChoGpl fatty acid composition in nondystrophic rats was similar at 6 wk to that of normal rats, and there were almost no postweaning changes in the dystrophics. In retinal phospholipids, the effect of dystrophy was to increase the 20:4n-6 content in EtnGpl and to decrease 22:6n-3 in ChoGpl. The 18:2n-6 and 22:6n-3 contents in dystrophic liver ChoGpl were also significantly affected, while no difference was observed in the EtnGpl fraction. The dystrophy affected the phospholipid fatty acid developmental changes in a tissue- and class-specific manner. Fatty acid metabolism could be selectively altered in neural and nonneural tissues of developing dystrophic RCS rats.

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The retinal dystrophy observed in Royal College of Surgeons (RCS) rats is a classically used experimental model for studying a group of recessively inherited retinal degeneration diseases commonly called retinitis pigmentosa (1). The mutation results in the degeneration of the retinal photoreceptor cells and ultimately in blindness. The primary cause of retinal dystrophy in RCS rats is located in the retinal pigmented epithelium (RPE), which is unable to phagocytize the disk membranes of the photoreceptor cell rod outer segments (ROS). In normal conditions, the ROS membranes are shed with a circadian rhythm throughout the lifespan of the animal (2). The nondystrophic congenic RCS rat, which has the same genetic background except for the retinal dystrophy, retains phagocytic functions and serves as a control for the dystrophic animals. A deletion of RCS DNA that disrupts the gene encoding the receptor tyrosine kinase *Mertk* involved in the signaling of phagocytosis was recently discovered in dystrophic RCS rats (3). The phagocytic defect leads to an abnormal accumulation of ROS debris between the outer segment layer of the photoreceptor cells and the RPE that begins within 2 wk after birth (1,4). The defect is accompanied by a breakdown in the RPE cell tight junctions, neovascular formations, cell migrations, and abnormal accumulation of microglial cells (5). In addition, it has been shown that the neutral lipid and phospholipid head group compositions are altered in the ROS membranes of postweaning dystrophic rats (6). At the age of 3–5 wk, at which time the photoreceptor cells become severely damaged, the dystrophic ROS plasma membranes have a twofold lower cholesterol-to-phospholipid ratio than normal membranes, and the phospholipid headgroup composition of the disks differs from those of nondystrophics (6). The ROS fatty acid compositions were not analyzed in these studies. Anomalous phospholipid class distribution and fatty acid composition were reported in the RPE cells from young dystrophic rats (7,8). The dystrophic RPE cell plasma membranes have higher docosahexaenoic acid (22:6n-3) and lower arachidonic acid (20:4n-6) contents than those of nondystrophic rats (8). Moreover, the specific activities of $Na^+K^+$-ATPase and 5′-nucleotidase were found to be higher in the dystrophic RPE plasma membranes (8). These changes may contribute to the degenerative process by modifying the concentration of ions, fluids, and metabolites in the interphotoreceptor matrix (8). The relationship between the dystrophic mutation and the cause of alterations in structural lipids is unknown. Besides, the developmental timing of membrane lipid alterations has not been determined, and it is not known whether other neural or nonneural tissues in RCS rats present some modifications in fatty acid compositions. In this study, we analyzed the fatty acid composition of plasma, retina, brain, and liver phospholipids from dystrophic and nondystrophic rats during the critical period of retinal development. Analyses were performed at 3 wk, when the ROS morphology is still almost normal in dystrophic rats (1), and at a later

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Abbreviations: ChoGpl, choline phosphoglycerides; EtnGpl, ethanolamine phosphoglycerides; prcd, progressive rod-cone degeneration; RCS, Royal College of Surgeons; ROS, rod outer segments; RPE, retinal pigmented epithelium.

stage of dystrophy (6 wk), after the inner segments have entirely disappeared and the degeneration has begun to spread through all parts of the visual cells (1). The strain × age interaction was determined for the main fatty acids in choline phosphoglycerides (ChoGpl) and ethanolamine phosphoglycerides (EtnGpl), the two major phospholipid classes in neural and nonneural tissues.

*Animals and collection of tissues.* RCS rats came from breeding colonies supplied by INSERM Unit 450 (Paris, France). Dystrophic rats (rdy/rdy) were black-eyed (p/+) and black-hooded. They were compared to the nondystrophic congenic strain (rdy/+). Rats were housed in an air-conditioned animal room illuminated from 7:00 AM to 7:00 PM and maintained at 21°C. They were given free access to a pelleted diet (Extralabo, Provins, France) containing 4.8% by weight of fats. As shown in Table 1, the dietary lipids contained 20:4n-6 (0.3% of total fatty acids) and 4.3% of n-3 long-chain polyunsaturated fatty acids in the form of 20:5n-3, 22:5n-3, and 22:6n-3 (from fish powders). Rats were killed by decapitation at weaning (3 wk) or at the age of 6 wk. They fasted overnight, with water *ad libitum*. Blood was collected with heparin as an anticoagulant and the plasma was lyophilized after centrifugation. Liver and brain (cerebellum was discarded) were excised, rinsed with 9 g/L NaCl, and drained. The brain and the whole liver were homogenized and lyophilized before extraction of total lipids. The eyes were removed and the anterior segment, lens, and vitreous humor were discarded. The retina was detached from the retinal pigment epithelium, and the eyecups were incubated under gentle stirring at room temperature in a calcium-free Ringer buffer containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.17 mmol/L $KH_2PO_4$, 1.17 mmol/L $MgSO_4$, 5.6 mmol/L D-glucose, 35 mmol/L $NaHCO_3$, and 1.0 mmol/L EDTA, pH 7.4 (9). The retinas harvested from one litter (5 to 6 rats) were pooled and stored at –80°C until lipid extraction.

**TABLE 1**
**Fatty Acid Composition in Pelleted Diet Lipids (wt% of total fatty acids)**

| | |
|---|---|
| 14:0 | 2.4 |
| 15:0 | 0.3 |
| 16:0 | 15.7 |
| 18:0 | 6.9 |
| Σ ≥20 C | 0.5 |
| 16:1n-9 | 3.0 |
| 16:1n-7 | 0.1 |
| 18:1n-9 | 26.4 |
| 18:1n-7 | 2.0 |
| Σ >18 C | 2.2 |
| 18:2n-6 | 30.7 |
| 22:2n-6 | 0.1 |
| 20:4n-6 | 0.3 |
| 18:3n-3 | 3.8 |
| 20:5n-3 | 2.1 |
| 22:5n-3 | 0.4 |
| 22:6n-3 | 1.9 |

*Determination of fatty acid composition.* Total lipids were extracted from 150 mg of lyophilized tissue with 4 mL of chloroform/methanol (2:1, vol/vol) (10) in the presence of butylhydroxytoluene 0.005 g/100 mL. The plasma total phospholipids were separated from neutral lipids by filtration through silica cartridges (Waters, Milford, MA) (11). The plasma phospholipids, composed of 90% ChoGpl, were not further purified. The two major phospholipid classes in the different tissue samples, ChoGpl and EtnGpl, were separated by solid-phase extraction on a 500 mg prepacked aminopropyl cartridge (J.T. Baker, Deventer, The Netherlands) washed beforehand with 3 mL of hexane and equilibrated with 3 mL of eluent I (isopropanol/chloroform, 1:2, vol/vol) (adapted from Ref. 12). Each sample of total lipids was dried under nitrogen and resolubilized in 250 µL of eluent I. The sample was then applied to the cartridge by elution through the solid phase. In the cases of retina, liver, and plasma samples, the cartridge was eluted successively with 3 mL of eluent I (neutral lipid fraction), 3 mL of diethyl ether/acetic acid (98:2, vol/vol) (free fatty acid fraction), 1 mL of acetonitrile, 8 mL of acetonitrile/*n*-propanol (3:1, vol/vol) (ChoGpl fraction), 2 mL of acetonitrile/*n*-propanol (1:1, vol/vol) (sphingomyelin fraction), and 3 mL of methanol to recover the EtnGpl fraction. Owing to the high cerebral sphingomyelin content, the brain samples were eluted with 3 mL of eluent I, 3 mL of diethyl ether/acetic acid (3:1, vol/vol), 4 mL of acetone (ChoGpl fraction), 4 mL of acetonitrile/*n*-propanol (3:1, vol/vol), 4 mL of acetonitrile/*n*-propanol (1:1, vol/vol) (sphingomyelin fraction), and 3 mL of methanol (EtnGpl fraction).

Fatty acid methyl esters were produced by reacting ChoGpl for 20 min with $BF_3$, 10 g/100 mL, in methanol at 90°C (13) and EtnGpl 4 h with HCl (1:9, vol/vol), and dimethoxypropane, 4 g/100 mL, in methanol at 70°C (14). After adding 2 mL of distilled water, the methyl esters were extracted twice with 1 mL hexane, washed with distilled water, dried under nitrogen, and finally taken up in 30–50 µL isooctane for gas chromatography. An aliquot of 1 µL was injected through the on-column injector of a 9001 gas chromatograph (Chrompack, Middleburg, The Netherlands) equipped with a retention gap and a CP WAX 52 CB bonded fused-silica capillary column (0.3 mm i.d. and 50 m length). The oven temperature was programmed for 79–140–205°C at heating rates of 9°C/min and 3°C/min for the first and second stages, respectively. Peaks attributable to fatty acid methyl esters were automatically integrated and identified by comparing their equivalent chain length with standard compounds. All compositions were expressed as mol% of total fatty acids after subtraction of unidentified peaks and dimethylacetals.

*Statistical treatment.* The significance of fatty acid composition differences between 3- and 6-wk-old rats was individually evaluated within each strain using Statview SE™ (Abacus Concepts Inc., Berkeley, CA) for one-way analysis of variation followed by Fisher's test. The significance of the aging effect was tested at the levels of $P < 0.001$, $P < 0.01$, and $P < 0.05$ in both phospholipid fractions from each tissue.

The interaction of the strain (dystrophic or nondystrophic) with the fatty acid compositional changes with age (strain × age interaction) was then tested for two-way analysis of variance and Fisher's test.

## RESULTS

*Fatty acid composition in plasma phospholipids.* The age-related changes in the fatty acid composition of plasma phospholipids in RCS rats are reported in Table 2. As a whole, the dystrophic and nondystrophic rats presented the same pattern, although differences between 3 and 6 wk of age were globally less significant in the dystrophic group. In both groups, the sum of monounsaturated fatty acids increased between 3 and 6 wk of age (Table 2). The main observation was that plasma 20:4n-6 increased with age by 15% in nondystrophic rats ($P < 0.05$), whereas it did not change in the dystrophic group; the age × strain interaction was not significant. By contrast, the 22:6n-3 plasma content significantly increased with age in dystrophic rats ($P < 0.001$) but not in the nondystrophic ones; the age × strain interaction was significant ($P = 0.04$). The ratio of 22:6n-3 to 20:4n-6 tended to decrease in nondystrophic rats and to increase in dystrophic rats, with a significant interaction ($P = 0.02$). There was no other significant interaction between age and strain effects in plasma phospholipids.

*Fatty acid composition in brain phospholipids.* The developmental fatty acid changes in brain EtnGpl and ChoGpl are reported in Tables 3 and 4. At 3 wk, rats from both strains presented similar fatty acid compositions in EtnGpl (Table 3) and ChoGpl (Table 4). As observed in many other species, both strains presented higher 22:6n-3 contents in the brain EtnGpl fraction than in ChoGpl. The 22:6n-3 content accounted for around 25% of total EtnGpl fatty acids in both strains. However, the 22:6n-3 content decreased by 7% in the brain EtnGpl from the 6-wk-old dystrophic rats ($P < 0.001$), whereas it remained unchanged in the age-matched nondystrophics. The interaction of the age and strain effects was significant ($P = 0.005$, Table 3). Moreover, the 22:6n-3 to 22:5n-3 ratio slightly increased with age in the nondystrophic rats ($P < 0.01$) but not in the dystrophics ($P = 0.004$ for the age × strain interaction). The second major fatty acid in brain EtnGpl was 18:0. This fatty acid slightly decreased with age ($P < 0.01$) in the dystrophic group (Table 3), and this age-related change was strain-specific ($P = 0.007$, Table 3). At 3 wk, the 20:4n-6 content in the brain EtnGpl fractions from both strains was 1.3- to 1.4-fold lower than the content in 22:6n-3 (Table 3). The 20:4n-6 content decreased with age by around 20% in both strains ($P < 0.001$), and the 22:6n-3/20:4n-6 ratio concurrently increased. The 18:1n-9 contents in brain EtnGpl from dystrophic and nondystrophic rats, which both increased

**TABLE 2**
**Fatty Acid Composition in Plasma Total Phospholipids from Nondystrophic and Dystrophic Rats at the Ages of 3 and 6 wk (mol%, mean ± standard deviation)**

| | Nondystrophics | | Dystrophics | | |
|---|---|---|---|---|---|
| | 3 wk $n = 10$ | 6 wk $n = 6$ | 3 wk $n = 10$ | 6 wk $n = 6$ | Interaction[a] ($P$ value) |
| 15:0 | 0.2 ± 0.1 | 0.1 ± 0.04 | 0.2 ± 0.1 | 0.2 ± 0.1 | |
| 16:0 | 28.0 ± 1.0 | 26.0 ± 1.7[b] | 28.8 ± 1.3 | 27.4 ± 1.0[b] | ns |
| 17:0 | 0.7 ± 0.1 | 1.1 ± 0.1[c] | 0.7 ± 0.3 | 0.9 ± 0.05 | ns |
| 18:0 | 17.9 ± 1.2 | 17.5 ± 0.8 | 18.5 ± 1.5 | 17.6 ± 1.4 | |
| ∑ SFA[d] | 47.2 ± 1.6 | 45.2 ± 2.3 | 48.6 ± 2.6 | 46.5 ± 2.3 | |
| 16:1n-9 | 0.6 ± 0.1 | 1.0 ± 0.2[c] | 0.6 ± 0.1 | 0.6 ± 0.1 | 0.05 |
| 18:1n-9 | 4.8 ± 1.2 | 7.3 ± 1.0[c] | 6.0 ± 0.9 | 7.3 ± 0.7[b] | ns |
| 18:1n-7 | 1.3 ± 0.1 | 1.6 ± 0.2[c] | 1.6 ± 0.1 | 1.7 ± 0.2 | ns |
| ∑ MUFA[e] | 7.4 ± 1.6 | 10.8 ± 1.0[c] | 8.9 ± 1.4 | 10.6 ± 1.1[b] | ns |
| ∑ NE PUFA[f] | 0.9 ± 0.1 | 1.3 ± 0.1[c] | 1.0 ± 0.3 | 1.2 ± 0.2 | ns |
| 18:2n-6 | 26.1 ± 2.2 | 21.8 ± 1.6[c] | 23.8 ± 1.9 | 21.8 ± 1.4[b] | ns |
| 20:2n-6 | 0.3 ± 0.05 | 0.4 ± 0.05 | 0.2 ± 0.05 | 0.3 ± 0.1 | |
| 20:4n-6 | 11.4 ± 0.8 | 13.1 ± 1.3[b] | 11.3 ± 1.7 | 11.4 ± 1.3 | ns |
| 22:4n-6 | 0.1 ± 0.05 | 0.3 ± 0.1 | 0.2 ± 0.02 | 0.2 ± 0.02 | |
| ∑ n-6 PUFA | 37.9 ± 2.8 | 35.5 ± 2.5 | 35.5 ± 3.0 | 33.6 ± 2.3 | |
| 18:3n-3 | 0.1 ± 0.06 | 0.2 ± 0.06 | 0.3 ± 0.1 | 0.3 ± 0.1 | |
| 20:5n-3 | 0.7 ± 0.1 | 0.8 ± 0.05 | 0.5 ± 0.1 | 0.6 ± 0.1 | |
| 22:5n-3 | 0.7 ± 0.1 | 1.0 ± 0.1[c] | 0.6 ± 0.2 | 0.9 ± 0.2[b] | ns |
| 22:6n-3 | 5.2 ±0.5 | 5.3 ± 0.5 | 4.7 ± 0.5 | 6.3 ± 1.4[b] | 0.04 |
| ∑ n-3 PUFA | 6.6 ± 0.5 | 7.2 ± 0.3 | 6.1 ± 0.8 | 8.0 ± 1.6[c] | ns |
| 22:6n-3/20:4n-6 | 0.5 ± 0.05 | 0.4 ± 0.01[b] | 0.4 ± 0.05 | 0.6 ± 0.2[b] | 0.02 |
| 22:6n-3/22:5n-3 | 7.4 ± 1.5 | 5.3 ± 0.5[b] | 7.8 ± 4.0 | 7.0 ± 1.0 | ns |

[a]Age effect x dystrophy effect (ns: not significant).
[b]$P < 0.05$.
[c]Age effect (6 vs. 3 wk) inside nondystrophic or dystrophics group significant at $P < 0.01$.
[d]Saturated fatty acids.
[e]Monounsaturated fatty acids (including fatty acids with 20 to 24 carbons).
[f]Nonessential polyunsaturated fatty acids: 20:3n-9, 20:3n-7, and 22:3n-9.

**TABLE 3**
**Fatty Acid Composition in Brain Ethanolamine Glycerophospholipids from Nondystrophic and Dystrophic Rats at the Ages of 3 and 6 wk (mol%, mean ± standard deviation)**

| | Nondystrophics | | Dystrophics | | |
|---|---|---|---|---|---|
| | 3 wk $n = 20$ | 6 wk $n = 6$ | 3 wk $n = 22$ | 6 wk $n = 6$ | Interaction[a] ($P$ value) |
| 16:0 | 7.6 ± 0.2 | 7.0 ± 0.1[b] | 7.6 ± 0.5 | 7.1 ± 0.5 | ns |
| 17:0 | 0.2 ± 0.05 | 0.1 ± 0.06 | 0.2 ± 0.05 | 0.2 ± 0.02 | |
| 18:0 | 22.2 ± 0.4 | 22.6 ± 0.6 | 21.7 ± 1.0 | 20.7 ± 0.5[c] | 0.007 |
| 20:0 | 0.3 ± 0.1 | 0.2 ± 0.02 | 0.2 ± 0.04 | 0.3 ± 0.02 | |
| ∑ Total SFA[d] | 30.5 ± 0.9 | 30.2 ± 1.0 | 30.1 ± 1.5 | 30.1 ± 0.8 | |
| 16:1n-7 | 0.2 ± 0.1 | 0.2 ± 0.04 | 0.2 ± 0.05 | 0.2 ± 0.05 | |
| 18:1n-9 | 8.7 ± 0.4 | 13.0 ± 0.6[b] | 9.1 ± 0.5 | 13.5 ± 0.5[b] | ns |
| 18:1n-7 | 1.4 ± 0.1 | 1.9 ± 0.1[b] | 1.4 ± 0.1 | 2.0 ± 0.1[b] | ns |
| 20:1n-9 | 0.5 ± 0.1 | 1.3 ± 0.1[b] | 0.5 ± 0.1 | 1.3 ± 0.1[b] | ns |
| 20:1n-7 | 0.2 ± 0.1 | 0.4 ± 0.03[b] | 0.2 ± 0.04 | 0.4 ± 0.05[b] | ns |
| ∑ Total MUFA[e] | 13.6 ± 0.9 | 19.2 ± 0.4[b] | 14.6± 1.5 | 21.4 ± 0.7[b] | ns |
| ∑ Total NE PUFA[f] | 0.4 ± 0.2 | 0.1 ± 0.02[b] | 0.5 ± 0.09 | 0.2 ± 0.05[b] | ns |
| 18:2n-6 | 0.5 ± 0.1 | 0.4 ± 0.1 | 0.5 ± 0.1 | 0.1 ± 0.2[b] | <0.0001 |
| 20:2n-6 | 0.2 ± 0.1 | 0.3 ± 0.02 | 0.1 ± 0.04 | 0.1 ± 0.0[b] | |
| 20:3n-6 | 0.8 ± 0.1 | 0.6 ± 0.02 | 0.7 ± 0.04 | 0.6 ± 0.02 | |
| 20:4n-6 | 19.9 ± 0.4 | 15.9 ± 0.4[b] | 19.4 ± 0.9 | 15.2 ± 0.4[b] | ns |
| 22:4n-6 | 6.0 ± 0.2 | 5.7 ± 0.3 | 5.9 ± 0.2 | 5.5 ± 0.1[b] | ns |
| 22:5n-6 | 1,1 ± 0.04 | 0.8 ± 0.1[b] | 1.0 ± 0.1 | 0.8 ± 0.02[b] | ns |
| ∑ Total n-6 PUFA | 28.6 ± 0.8 | 23.8 ± 0.7[b] | 27.7 ± 0.4 | 23.2 ± 0.4[b] | ns |
| 22:5n-3 | 0.6 ± 0.1 | 0.4 ± 0.01[c] | 0.5 ± 0.08 | 0.5 ± 0.02 | |
| 22:6n-3 | 26.5 ± 0.8 | 26.3 ± 0.7 | 26.5 ± 1.3 | 24.7 ± 0.9[b] | 0.005 |
| ∑ Total n-3 PUFA | 26.9 ± 0.8 | 26.7 ± 0.5 | 27.1 ± 0.8 | 25.1 ± 0.6[b] | 0.004 |
| 22:6n-3/20:4n-6 | 1.3 ± 0.04 | 1.7 ± 0.02[b] | 1.4 ± 0.05 | 1.6 ± 0.02[b] | 0.02 |
| 22:6n-3/22:5n-3 | 44.2 ± 8.0 | 61.5 ± 5.3[c] | 56.5 ± 9.2 | 55.2 ± 4.0 | 0.004 |

[a]Age effect × dystrophy effect (ns, not significant).
[b]Age effect (6 vs. 3 wk) inside nondystrophic or dystrophic group significant at $P < 0.001$.
[c]$P < 0.01$.
[d]Saturated fatty acids.
[e]Monounsaturated fatty acids (including odd and very long chain fatty acids).
[f]Nonessential polyunsaturated fatty acids (20:3n-9, 20:3n-7, and 22:3n-9).

with age by around 48%, had exactly the opposite age-related changes as 20:4n-6 contents (Table 3). Brain EtnGpl also contained substantial amounts of 22:4n-6, whose molar contents slightly decreased with age (significant in dystrophic rats only). In summary, the data mainly showed that the strain's origin determined the age-related changes of 22:6n-3 and 18:0, the two major fatty acids in brain EtnGpl, whereas the age-related changes in 20:4n-6, the second major polyunsaturated fatty acid, were not altered by the dystrophy.

The brain ChoGpl was much richer in saturated fatty acids than the EtnGpl fraction (Table 4). In the nondystrophic rats, the developmental effect in ChoGpl was a sharp increase of 16:0 ($P < 0.001$) accompanied by a decrease in 18:0 ($P < 0.01$) (Table 4). In this group, the variations of the 20:4n-6 and 18:1n-9 contents were the same as those observed in the EtnGpl fraction, with a 45% decrease and a 43% increase in the 20:4n-6 and 18:1n-9 contents, respectively. In addition, the 22:6n-3 content decreased with age by 37% ($P < 0.001$) in the nondystrophic brain ChoGpl (whereas it remained unchanged in the EtnGpl fraction). The developmental changes in the brain ChoGpl fatty acid composition from the dystrophic rats were very different from those in the nondystrophics. There were in fact only minor changes in the dystrophic brain ChoGpl fatty acid composition between the ages of 3 and 6 wk. The result of this was that the age × strain interaction was highly significant for the changes in 14:0, 16:0, 18:0, 18:1n-9, 18:1n-7, 18:2n-6, and 20:4n-6 (Table 4). Moreover, the age-related changes in the 22:6n-3/20:4n-6 ratio were opposite in the nondystrophic and dystrophic rats ($P = 0.002$, Table 4).

*Fatty acid composition in retina phospholipids.* At weaning, the retinal EtnGpl from dystrophic and nondystrophic rats contained between 40 and 42% of total fatty acids as 22:6n-3 (Table 5). At the age of 6 wk, the 22:6n-3 content tended to increase in nondystrophics, and to decrease in dystrophic rats, but these variations did not reach a significant level. The age × strain interaction was not significant either. At the same time, the retinal EtnGpl 20:4n-6 level decreased by 17% in nondystrophic rats ($P < 0.01$), while it remained almost unchanged in dystrophics; the age × strain interaction was significant with $P = 0.004$. In addition, the 22:5n-3 content underwent a slight but significant ($P < 0.05$) age-related decrease in the nondystrophic EtnGpl fraction; the interaction with the strain effect was significant ($P = 0.01$). The opposite variations of retinal n-3 fatty acids observed in the EtnGpl fraction from nondystrophic and dystrophic rats resulted in opposite age-related changes in the ratio of 22:6n-3 to

**TABLE 4**
**Fatty Acid Composition in Brain Choline Glycerophospholipids from Nondystrophic and Dystrophic Rats at the Ages of 3 and 6 wk (mol%, mean ± standard deviation)**

| | Nondystrophics | | Dystrophics | | |
|---|---|---|---|---|---|
| | 3 wk $n = 17$ | 6 wk $n = 6$ | 3 wk $n = 23$ | 6 wk $n = 6$ | Interaction[a] ($P$ value) |
| 14:0 | 1.5 ± 0.5 | 0.6 ± 0.1[b] | 1.3 ± 0.2 | 2.6 ± 0.5[c] | <0.0001 |
| 15:0 | 0.3 ± 0.1 | 0.2 ± 0.02 | 0.2 ± 0.1 | 0.5 ± 0.1 | |
| 16:0 | 31.5 ± 5.4 | 43.8 ± 1.2[c] | 32.5 ± 3.1 | 29.7 ± 1.8 | <0.0001 |
| 17:0 | 0.5 ± 0.08 | 0.3 ± 0.02 | 0.4 ± 0.04 | 0.5 ± 0.07 | |
| 18:0 | 21.2 ± 4.1 | 13.0 ± 0.3[b] | 20.0 ± 2.3 | 20.1 ± 2.2 | 0.001 |
| ∑ Total SFA[d] | 55.4 ± 2.5 | 58.1 ± 1.2 | 54.8 ± 2.5 | 53.9 ± 3.1 | |
| 16:1n-9 | 0.8 ± 0.09 | 0.5 ± 0.08 | 0.8 ± 0.1 | 0.7 ± 0.1 | |
| 16:1n-7 | 0.8 ± 0.2 | 0.8 ± 0.1 | 0.7 ± 0.3 | 1.2 ± 0.1 | |
| 18:1n-9 | 13.1 ± 2.0 | 18.7 ± 0.5[c] | 13.6 ± 1.4 | 13.2 ± 1.7 | <0.0001 |
| 18:1n-7 | 3.3 ± 0.4 | 5.5 ± 0.1[c] | 3.1 ± 0.2 | 4.0 ± 0.2[c] | <0.0001 |
| ∑ Total MUFA[e] | 18.3 ± 2.0 | 26.3 ± 0.3[c] | 18.7 ± 1.4 | 20.0 ± 2.1 | <0.0001 |
| 18:2n-6 | 1.7 ± 0.4 | 1.4 ± 0.1 | 1.3 ± 0.1 | 1.9 ± 0.7[c] | 0.0008 |
| 20:2n-6 | 0.3 ± 0.1 | 0.2 ± 0.01 | 0.2 ± 0.04 | 0.1 ± 0.06[c] | |
| 20:3n-6 | 0.5 ± 0.07 | 0.3 ± 0.03 | 0.5 ± 0.04 | 0.4 ± 0.02 | |
| 20:4n-6 | 16.1 ± 1.9 | 8.9 ± 0.3[c] | 15.7 ± 1.3 | 16.7 ± 2.9 | <0.0001 |
| 22:4n-6 | 0.9 ± 0.3 | 0.6 ± 0.1[f] | 1.2 ± 0.2 | 0.8 ± 0.08[c] | ns |
| 22:5n-6 | 0.3 ± 0.1 | 0.3 ± 0.08 | 0.3 ± 0.04 | 0.2 ± 0.1 | |
| ∑ Total n-6 PUFA[g] | 19.7 ± 2.3 | 11.6 ± 0.3[c] | 19.1 ± 1.3 | 20.1 ± 2.7 | <0.0001 |
| 20:5n-3 | 0.2 ± 0.1 | 0.1 ± 0.01 | 0.1 ± 0.04 | 0.2 ± 0.09 | |
| 22:5n-3 | 0.2 ± 0.1 | 0.1 ± 0.03 | 0.2 ± 0.04 | 0.2 ± 0.04 | |
| 22:6n-3 | 6.3 ± 0.7 | 3.8 ± 0.6[c] | 7.2 ± 0.8 | 5.7 ± 1.4[b] | ns |
| ∑ Total n-3 PUFA[g] | 6.7 ± 0.7 | 4.0 ± 0.6[c] | 7.4 ± 0.8 | 6.1 ± 1.2[f] | ns |
| 22:6n-3/20:4n-6 | 0.39 ± 0.06 | 0.43 ± 0.07 | 0.46 ± 0.06 | 0.34 ± 0.09[b] | 0.002 |
| 22:6n-3/22:5n-3 | 31.5 ± 6.4 | 38.0 ± 5.5 | 36.0 ± 7.3 | 28.5 ± 7.0 | |

[a]Age effect × dystrophy effect (ns, not significant).
[b]$P < 0.01$.
[c]Age effect (6 vs. 3 wk) inside nondystrophic or dystrophic group significant at $P < 0.001$.
[d]Saturated fatty acids.
[e]Monounsaturated fatty acids (including odd and very long chain fatty acids).
[f]$P < 0.05$.
[g]Polyunsaturated fatty acids.

22:5n-3. The changes in this product-to-substrate ratio could be indicative of changes in the final steps of metabolic conversion (and EtnGpl acylation) within the n-3 family; from 3 to 6 wk of age, the 22:6n-3/22:5n-3 ratio increased by 50% ($P < 0.05$) in the nondystrophic rats, whereas it decreased by 20% ($P < 0.05$) in the dystrophics, and the age × strain interaction was significant with $P = 0.001$ (Table 5). The 22:6n-3 to 20:4n-6 ratio (Table 5) tended to increase with age in nondystrophics and to decrease in dystrophics, but none of these variations reached a significant level.

In the ChoGpl fraction, the 22:6n-3 content was two- to threefold lower than in the EtnGpl counterpart (Table 6). The ChoGpl 22:6n-3 content significantly increased with age in the nondystrophic rats (from 11.1 to 18.9%, $P < 0.05$), while it tended to decrease in the dystrophic group. As a result of these opposite changes, the age × strain interaction was significant for 22:6n-3 in the ChoGpl fraction ($P = 0.008$). Moreover, the 22:6n-3/22:5n-3 ratio increased with age (by a factor of 2.4, $P < 0.05$) in the nondystrophic group only; the age × strain interaction was slightly significant with $P = 0.025$ (Table 6). As observed in the EtnGpl fraction, the ChoGpl 20:4n-6 content decreased with age only in the nondystrophic rats, but the corresponding age × strain interaction was not significant (Table 6). Thus, the 22:6n-3 and 20:4n-6 ChoGpl contents evolved differently with age in the nondystrophic group, whereas the dystrophic rats had the same ChoGpl fatty acid composition at 3 and 6 wk, resulting in a significant age × strain interaction for the ratios of 22:6n-3 to 20:4n-6 ($P = 0.001$).

In summary, a significant age × strain interaction in the retina was essentially found for 20:4n-6 in the EtnGpl fraction and for 22:6n-3 in the ChoGpl fraction. In both phospholipid classes, the age-related increase of the 22:6n-3/22:5n-3 ratio was significantly altered by the dystrophy, while the 22:6n-3/20:4n-6 ratio increase was altered in the ChoGpl fraction only.

*Fatty acid composition in liver phospholipids.* The changes in liver EtnGpl fatty acid composition between 3 and 6 wk were almost identical in both strains (Table 7). Globally, the fatty acids with 18 carbons significantly increased (18:1n-9, 18:1n-7, and 18:2n-6) or tended to increase (18:0) in the EtnGpl fraction from both strains, while the 16:0 contents decreased. The two major long-chain polyunsaturated fatty acids in liver EtnGpl were 22:6n-3, which significantly decreased with age (by 20–26%) in both strains ($P < 0.001$),

**TABLE 5**
**Fatty Acid Composition in Retina Ethanolamine Glycerophospholipids from Nondystrophic and Dystrophic Rats at the Ages of 3 and 6 wk (mol%, mean ± standard deviation)**

| | Nondystrophics | | Dystrophics | | |
|---|---|---|---|---|---|
| | 3 wk 4 pups | 6 wk 3 pups | 3 wk 4 pups | 6 wk 3 pups | Interaction[a] (*P* value) |
| 16:0 | 8.8 ± 1.6 | 9.8 ± 1.4 | 9.3 ± 2.9 | 10.1 ± 2.0 | |
| 17:0 | 0.2 ± 0.06 | 0.3 ± 0.09 | 0.2 ± 0.02 | 0.3 ± 0.05 | |
| 18:0 | 22.2 ± 2.4 | 22.7 ± 1.8 | 21.7 ± 1.7 | 24.3 ± 1.8 | |
| 20:0 | 0.2 ± 0.08 | 0.2 ± 0.01 | 0.2 ± 0.06 | 0.2 ± 0.05 | |
| ∑ Total SFA[b] | 31.4 ± 3.3 | 33.0 ± 1.5 | 31.4 ± 1.7 | 34.9 ± 1.1 | |
| 16:1n-7 | 0.2 ± 0.07 | 0.1 ± 0.1 | 0.4 ± 0.1 | 0.2 ± 0.02 | |
| 18:1n-9 | 4.3 ± 0.8 | 4.0 ± 0.7 | 5.6 ± 3.1 | 4.4 ± 0.7 | |
| 18:1n-7 | 1.2 ± 0.06 | 1.3 ± 0.07 | 1.6 ± 0.4 | 1.3 ± 0.07 | |
| ∑ Total MUFA[c] | 5.8 ± 0.9 | 5.4 ± 0.6 | 7.5 ± 3.4 | 6.0 ± 0.7 | |
| 18:2n-6 | 1.2 ± 0.2 | 1.4 ± 0.1 | 0.9 ± 0.6 | 1.1 ± 0.1 | |
| 20:3n-6 | 0.4 ± 0.08 | 0.4 ± 0.06 | 0.4 ± 0.1 | 0.3 ± 0.06 | |
| 20:4n-6 | 15.5 ± 0.6 | 12.6 ± 1.0[d] | 13.8 ± 1.0 | 14.5 ± 1.0 | 0.004 |
| 22:4n-6 | 2.8 ± 0.7 | 2.0 ± 0.09 | 2.5 ± 0.1 | 2.6 ± 0.1 | |
| 22:5n-6 | 0.7 ± 0.09 | 2.5 ± 2.2 | 0.8 ± 0.1 | 0.5 ± 0.04 | |
| ∑ Total n-6 PUFA[e] | 19.4 ± 1.3 | 17.6 ± 3.3 | 17.4 ± 1.6 | 17.9 ± 1.4 | |
| 22:5n-3 | 1.2 ± 0.1 | 0.8 ± 0.1[f] | 1.0 ± 0.1 | 1.1 ± 0.1 | 0.01 |
| 22:6n-3 | 40.9 ± 3.8 | 41.7 ± 3.2 | 41.8 ± 5.7 | 39.0 ± 1.9 | |
| ∑ Total n-3 PUFA[e] | 42.1 ± 3.3 | 42.5 ± 3.2 | 42.8 ± 5.6 | 40.1 ± 1.9 | |
| 22:6n-3/20:4n-6 | 2.6 ± 0.3 | 3.3 ± 0.6 | 3.0 ± 0.5 | 2.7 ± 0.3 | ns |
| 22:6n-3/22:5n-3 | 33.1 ± 1.4 | 49.5 ± 9.9[f] | 43.8 ± 3.8 | 34.9 ± 2.9[f] | 0.001 |

[a]Age effect × dystrophy effect (ns, not significant).
[b]Saturated fatty acids.
[c]Monounsaturated fatty acids, including very long chain fatty acids.
[d]Age effect (6 vs. 3 wk) inside nondystrophic group significant at $P < 0.01$.
[e]Polyunsaturated fatty acids, including very long chain fatty acids.
[f]$P < 0.01$.

and 20:4n-6, which slightly increased in the nondystrophic group ($P < 0.05$) and tended to increase in the dystrophic one. These variations were apparently not related to the dystrophy, inasmuch as none of the age × strain interactions reached a significant level (Table 7).

In the liver ChoGpl fraction, the fatty acid changes with age were almost the same as those observed in EtnGpl (Table 8). The main observations were that the ChoGpl 18:2n-6 content increased significantly in nondystrophic rats only ($P = 0.003$), and that 22:6n-3 decreased by a greater factor in the nondystrophic group (1.9 vs. 1.4, $P = 0.025$). Besides, minor monounsaturated fatty acids (16:1n-7 and 18:1n-7) increased with age in both strains, with a lower amplitude in the dystrophic group (Table 8). In summary, the origin of the strain had no influence on the variations with age of fatty acid composition in liver EtnGpl, while a slight but significant age × strain interaction was found for 22:6n-3 and for 18:2n-6 in the ChoGpl fraction. Changes in liver phospholipids were mainly determined by development and apparently not related to those observed in neural tissues.

## DISCUSSION

*Brain fatty acid composition.* Two major postweanling fatty acid changes occurred in the nondystrophic brain phospholipids: (i) the relative contents in 20:4n-6 decreased with age both in the ChoGpl and EtnGpl fractions, while those of 18:1n-9 increased in compensation, and (ii) the 16:0 and 18:0 contents also evolved differently in ChoGpl. Opposite changes in 20:4n-6 and 18:1n-9 contents occurred as well in the dystrophic brain EtnGpl, but not in the ChoGpl counterpart. Changes in 16:0 and 18:0 contents were also nearly abolished in the dystrophic brain ChoGpl. Thus, postweanling changes in the brain ChoGpl fatty acid composition were not preserved in dystrophic rats, especially as regards 16:0, 18:0, 18:1n-9, and 20:4n-6.

In developing organs, changes in relative contents with age are likely to arise from differential accretion rates of fatty acids, rather than from losses in their absolute amounts. The finding that the brain ChoGpl fraction from dystrophic rats had the same fatty acid composition at 3 and 6 wk, while the nondystrophics changed their composition, implies that the accretion rates of ChoGpl fatty acids occurred concurrently during the development of the dystrophic brain, whereas they occurred differentially in the nondystrophic. It was recently shown that 22:6n-3 is deposited in the rat fetal brain at a high rate within embryonic days 14 to 17, specifically in the phosphatidylserine and EtnGpl fractions (15), prior to the formation of synapses from growth cones (16). After birth, the accretion of saturated and monounsaturated fatty acids (mainly, 16:0, 18:0, and 18:1n-9) occurs at a low rate, while that of 20:4n-6 and 22:6n-3 remained unchanged up to postnatal day

**TABLE 6**
**Fatty Acid Composition in Retina Choline Glycerophospholipids from Nondystrophic and Dystrophic Rats at the Ages of 3 and 6 wk (mol%, mean ± standard deviation)**

| | Nondystrophics | | Dystrophics | | |
|---|---|---|---|---|---|
| | 3 wk 5 pups | 6 wk 3 pups | 3 wk 4 pups | 6 wk 3 pups | Interaction[a] (*P* value) |
| 14:0 | 2.0 ± 0.2 | 0.9 ± 0.1[b] | 2.1 ± 0.4 | 1.6 ± 0.8 | ns |
| 15:0 | 0.5 ± 0.1 | 0.5 ± 0.08 | 0.6 ± 0.04 | 0.5 ± 0.02 | |
| 16:0 | 37.6 ± 4.4 | 36.8 ± 0.7 | 37.3 ± 3.7 | 40.6 ± 1.8 | |
| 17:0 | 0.4 ± 0.06 | 0.3 ± 0.1 | 0.2 ± 0.2 | 0.3 ± 0.05 | |
| 18:0 | 12.6 ± 1.9 | 15.3 ± 0.6 | 12.7 ± 1.4 | 14.4 ± 2.2 | |
| Σ Total SFA[c] | 53.1 ± 4.7 | 53.8 ± 1.6 | 52.9 ± 4.9 | 57.3 ± 3.6 | |
| 16:1n-9 | 1.1 ± 0.2 | 0.9 ± 0.1 | 1.7 ± 0.2 | 1.3 ± 0.3 | |
| 16:1n-7 | 0.9 ± 0.4 | 0.8 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.3 | |
| 18:1n-9 | 13.3 ± 1.1 | 13.2 ± 1.2 | 14.1 ± 1.0 | 14.6 ± 1.7 | |
| 18:1n-7 | 3.2 ± 0.2 | 2.6 ± 0.3 | 3.0 ± 0.4 | 3.0 ± 0.3 | |
| 20:1n-9 | 0.2 ± 0.06 | 0.2 ± 0.07 | 0.3 ± 0.03 | 0.2 ± 0.1 | |
| Σ Total MUFA[d] | 18.6 ± 0.8 | 17.7 ± 0.6 | 20.2 ± 1.2 | 20.2 ± 1.5 | |
| 18:2n-6 | 2.2 ± 0.4 | 2.2 ± 0.3 | 2.1 ± 0.4 | 2.2 ± 0.8 | |
| 20:3n-6 | 0.6 ± 0.2 | 0.2 ± 0.01 | 0.3 ± 0.02 | 0.3 ± 0.04 | |
| 20:4n-6 | 9.9 ± 2.4 | 6.1 ± 0.4[b] | 8.1 ± 1.1 | 6.7 ± 1.1 | ns |
| 22:4n-6 | 0.7 ± 0.3 | 0.3 ± 0.04 | 0.6 ± 0.1 | 0.4 ± 0.05 | |
| 22:5n-6 | 0.3 ± 0.1 | 0.5 ± 0.1 | 0.3 ± 0.06 | 0.3 ± 0.1 | |
| Σ Total n-6 PUFA[e] | 16.6 ± 5.2 | 9.4 ± 0.9 | 11.3 ± 1.3 | 9.9 ± 1.8 | |
| 22:5n-3 | 0.5 ± 0.1 | 0.3 ± 0.01 | 0.4 ± 0.1 | 0.3 ± 0.02 | |
| 22:6n-3 | 11.1 ± 4.2 | 18.9 ± 0.7[b] | 15.2 ± 2.4 | 12.2 ± 2.0 | 0.008 |
| Σ Total n-3 PUFA[e] | 11.7 ± 4.2 | 19.1 ± 0.7[b] | 15.6 ± 2.7 | 12.6 ± 2.0 | 0.01 |
| 22:6n-3/20:4n-6 | 1.2 ± 0.6 | 3.1 ± 0.3[f] | 1.9 ± 0.1 | 1.9 ± 0.4 | 0.001 |
| 22:6n-3/22:5n-3 | 24.0 ± 14.9 | 57.9 ± 5.1[b] | 39.8 ± 6.8 | 44.9 ± 8.2 | 0.025 |

[a]Age effect × dystrophy effect (ns, not significant).
[b]Age effect (6 vs. 3 wk) inside nondystrophic group significant at $P < 0.05$.
[c]Saturated fatty acids.
[d]Monounsaturated fatty acids, including very long chain fatty acids.
[e]Polyunsaturated fatty acids, including very long chain fatty acids.
[f]$P < 0.01$.

16 (15). The opposite changes in the relative contents of 20:4n-6 and 18:1n-9 observed in both classes of brain phospholipids from nondystrophic rats could signify that accretion rate of 20:4n-6 in the brain decreased during the 3-wk postweaning period, whereas that of 18:1n-9 increased. Differential deposition rates of 20:4n-6 and 18:1n-9 in brain should be affected by the dystrophy in the ChoGpl fraction only. The low level of 16:0 in EtnGpl at 3 wk (around 8% of total fatty acids) and its high level in ChoGpl (around 32%) suggest that fetal and postnatal deposition of 16:0 in brain phospholipids occurred preferentially in ChoGpl, whereas 18:0 was almost equally distributed in EtnGpl and ChoGpl (22% of total fatty acids in each class being 18:0). Other reports indicate that incorporation of 18:0 in neonatal rat brain phospholipids preferentially occurs in the phosphatidylserine and phosphatidylinositol fractions (17). After weaning, 16:0 continued to accumulate preferentially in ChoGpl, mainly to the detriment of 18:0, whose relative content decreased while that of 16:0 increased. The absence of age-related changes in the 16:0 and 18:0 relative contents in the brain ChoGpl from dystrophic rats may imply that, in this group, both fatty acids were concurrently deposited between 3 and 6 wk of age.

Thus, postweaning changes in fatty acid composition were nearly abolished in the dystrophic brain ChoGpl, while the EtnGpl fraction was much less affected (with a very slight incidence of dystrophy on the 18:0 and 22:6n-3 contents only). Assessment of the impact of these biochemical alterations may be attempted by comparing the brain fatty acid compositions of RCS rats with those from other strains. The fatty acid composition in the brain ChoGpl from 6-wk-old nondystrophic rats was actually very close to that reported for Sprague-Dawley rats of the same age (18) and for Long-Evans rats aged 9 wk (19). In particular, the 16:0 and 18:0 relative contents in the brain ChoGpl fractions were approximately 40–45% and 13–14%, respectively by weight of total fatty acids in nondystrophic RCS, Sprague-Dawley (18), and Long-Evans (19) rats. As presented above, the dystrophic rats of the same age did not match this composition. Therefore, the fatty acid composition in the brain ChoGpl of dystrophic rats appeared to be specifically altered in comparison to normal rats from other strains. In the EtnGpl fraction, the differences in saturated fatty acid contents between dystrophic rats and rats from other strains were of much lower amplitude. Differences in the 20:4n-6 and 22:6n-3 EtnGpl contents exist between both strains of RCS rats (dystrophic and nondystrophic) and rats from other strains that could be attributed to the prevalent influence of the respective rearing diets in composing the brain EtnGpl. Taking into account the fact that

**TABLE 7**
**Fatty Acid Composition in Liver Ethanolamine Glycerophospholipids from Nondystrophic and Dystrophic Rats at the Ages of 3 and 6 wk (mol%, mean ± standard deviation)**

| | Nondystrophics | | Dystrophics | | |
|---|---|---|---|---|---|
| | 3 wk $n = 20$ | 6 wk $n = 6$ | 3 wk $n = 23$ | 6 wk $n = 6$ | Interaction[a] ($P$ value) |
| 16:0 | 24.2 ± 1.5 | 21.0 ± 1.6[b] | 24.1 ± 1.1 | 21.7 ± 1.9[b] | ns |
| 17:0 | 0.8 ± 0.1 | 1.3 ± 0.2[b] | 0.9 ± 0.08 | 1.2 ± 0.1[b] | ns |
| 18:0 | 20.0 ± 0.9 | 20.4 ± 0.5 | 20.5 ± 0.5 | 21.2 ± 0.5[c] | ns |
| 22:0 | 0.3 ± 0.07 | 0.2 ± 0.08 | 0.3 ± 0.1 | 0.4 ± 0.06 | |
| ∑ Total SFA[d] | 45.2 ± 1.4 | 42.9 ± 1.8 | 45.7 ± 1.5 | 44.5 ± 2.1 | |
| 16:1n-7 | 0.1 ± 0.06 | 0.7 ± 0.05[b] | 0.2 ± 0.05 | 0.5 ± 0.1[b] | ns |
| 18:1n-9 | 2.2 ± 0.4 | 4.5 ± 0.7[b] | 2.0 ± 0.3 | 4.6 ± 1.2[b] | ns |
| 18:1n-7 | 1.1 ± 0.3 | 1.8 ± 0.8[e] | 1.1 ± 0.2 | 1.7 ± 0.2[b] | ns |
| 20:1n-9 | 0.1 ± 0.08 | 0.2 ± 0.1 | 0.1 ± 0.04 | 0.2 ± 0.09 | |
| ∑ Total MUFA[f] | 3.5 ± 0.7 | 7.2 ± 1.6[b] | 3.4 ± 0.3 | 7.0 ± 1.1[b] | ns |
| ∑ Total NE PUFA[g] | 0.3 ± 0.4 | 0.2 ± 0.04 | 0.3 ± 0.2 | 0.2 ± 0.04 | |
| 18:2n-6 | 5.0 ± 0.9 | 6.8 ± 0.7[e] | 5.2 ± 0.5 | 6.5 ± 1.0[b] | ns |
| 20:3n-6 | 0.5 ± 0.08 | 0.7 ± 0.02 | 0.5 ± 0.04 | 0.6 ± 0.04 | |
| 20:4n-6 | 17.6 ± 2.1 | 19.9 ± 0.4[c] | 17.3 ± 1.4 | 17.8 ± 0.2 | ns |
| 22:4n-6 | 0.5 ± 0.4 | 0.9 ± 0.6 | 0.5 ± 0.1 | 0.7 ± 0.4 | |
| 22:5n-6 | 0.2 ± 0.08 | 0.5 ± 0.2 | 0.2 ± 0.04 | 0.5 ± 0.1 | |
| ∑ Total n-6 PUFA | 23.8 ± 3.1 | 28.7 ± 0.4[e] | 23.7 ± 1.4 | 26.0 ± 0.4[b] | ns |
| 20:5n-3 | 0.9 ± 0.2 | 1.3 ± 0.03[e] | 0.8 ± 0.3 | 1.2 ± 0.1[e] | ns |
| 22:5n-3 | 2.4 ± 0.8 | 2.1 ± 0.2 | 2.3 ± 0.4 | 2.0 ± 0.1 | |
| 22:6n-3 | 23.9 ± 2.4 | 17.7 ± 1.1[b] | 23.9 ± 1.3 | 19.1 ± 1.3[b] | ns |
| ∑ Total n-3 PUFA | 27.3 ± 2.8 | 21.0 ± 0.8[b] | 26.9 ± 1.7 | 22.3 ± 1.1[b] | ns |
| 22:6n-3/20:4n-6 | 1.4 ± 0.3 | 0.9 ± 0.1[e] | 1.4 ± 0.2 | 1.1 ± 0.1[e] | ns |
| 22:6n-3/22:5n-3 | 10.0 ± 1.4 | 8.5 ± 1.1 | 10.5 ± 1.7 | 9.6 ± 1.3 | |

[a]Age effect × dystrophy effect (ns, not significant).
[b]Age effect (6 vs. 3 wk) inside nondystrophic or dystrophic group significant at $P < 0.001$.
[c]$P < 0.05$.
[d]Saturated fatty acids.
[e]$P < 0.01$.
[f]Monounsaturated fatty acids.
[g]Nonessential polyunsaturated fatty acids (20:3n-9, 20:3n-7, and 22:3n-9).

ChoGpl is the major phospholipid class in the brain, it may be supposed that suppression of compositional changes in ChoGpl has an influence on the behavioral development of dystrophic rats. However, the dystrophy-associated alterations in the brain fatty acid composition were not similar to those resulting from dietary deficiencies or imbalances in polyunsaturated fatty acids that are well known to alter the cognitive performance and behavioral development in rodents (20–22). The content in brain ChoGpl 22:6n-3 was not specifically altered by the dystrophy, and it seems unlikely that the 7% decrease in EtnGpl 22:6n-3 that we observed at the age of 6 wk in the dystrophic brains is sufficient *per se* to generate behavioral alterations. However, it should be noted that Christensen *et al.* (23) found that feeding neonatal Wistar rats a structured marine oil–enriched diet induced the brain EtnGpl 22:6n-3 content to increase from 24 to 27% (by weight of total fatty acids) and concomitantly augmented the auditory brainstem response of the adults, the latter event possibly causally related to the former. In their study, learning ability was not modified by the dietary treatment.

*Retina fatty acid composition.* During postnatal development, the normal photoreceptor cells shift their fatty acid composition to more unsaturated species. The photoreceptors rapidly and selectively accumulate 22:6n-3 in nascent outer segments, resulting in much higher 22:6n-3 contents in adult cells than in immature ones (24). High levels of 22:6n-3 are supposed to favor membrane functional dynamics in the renewing photoreceptor disk membranes. The di-22:6n-3 molecular species of ChoGpl normally present in the mammalian retina (25) have been postulated to associate specifically with the seven α-helical segments of rhodopsin and to play an important role in modulating membrane dynamics involved in the rhodopsin-mediated phototransduction process (26,27). In addition, membrane 22:6n-3 can also serve as a precursor for the biologically active docosanoids required in maturing cells (28). It was shown that 22:6n-3 incorporation in retinal phospholipids is lowered in rodent and canine models of inherited retinal degeneration. Photoreceptor cells from the 10-d-old mutant *rd* mouse contained only about two-thirds of the level of 22:6n-3 observed in normal cells (24). In the progressive rod-cone degeneration (prcd) miniature poodle, the ROS 22:6n-3 level was reduced by 20% in prcd-affected dogs as compared to the nonaffected ones (29), although the affected retina retained its ability to synthesize 22:6n-3 from its upstream precursor (30). In dystrophic RCS rats aged 3–5 wk, Boesze-Battaglia *et al.* (6) showed that the distribution of

**TABLE 8**
**Fatty Acid Composition in Liver Choline Glycerophospholipids from Nondystrophic and Dystrophic Rats at the Ages of 3 and 6 wk (mol%, mean ± standard deviation)**

| | Nondystrophics | | Dystrophics | | |
|---|---|---|---|---|---|
| | 3 wk $n = 20$ | 6 wk $n = 6$ | 3 wk $n = 23$ | 6 wk $n = 6$ | Interaction[a] ($P$ value) |
| 14:0 | 1.4 ± 0.4 | 0.7 ± 0.04[b] | 1.7 ± 0.5 | 0.7 ± 0.06[b] | ns |
| 15:0 | 0.4 ± 0.1 | 0.7 ± 0.1 | 0.5 ± 0.05 | 0.6 ± 0.05 | |
| 16:0 | 29.5 ± 1.4 | 28.0 ± 2.4 | 28.1 ± 1.0 | 26.9 ± 0.8[c] | ns |
| 17:0 | 0.6 ± 0.09 | 0.8 ± 0.2 | 0.7 ± 0.05 | 0.8 ± 0.05 | |
| 18:0 | 16.0 ± 0.9 | 15.6 ± 0.9 | 15.3 ± 2.4 | 17.3 ± 0.2 | |
| Σ Total SFA[d] | 47.9 ± 2.3 | 45.9 ± 2.0 | 46.3 ± 2.5 | 46.3 ± 0.7 | |
| 16:1n-9 | 0.3 ± 0.1 | 0.4 ± 0.02 | 0.3 ± 0.05 | 0.4 ± 0.02 | |
| 16:1n-7 | 0.6 ± 0.1 | 1.6 ± 0.05[e] | 0.6 ± 0.1 | 1.0 ± 0.1[e] | <0.0001 |
| 18:1n-9 | 6.8 ± 1.8 | 8.5 ± 0.7[c] | 8.0 ± 2.4 | 8.5 ± 0.7 | ns |
| 18:1n-7 | 1.6 ± 0.3 | 2.8 ± 0.2[e] | 1.7 ± 0.4 | 2.1 ± 0.1[c] | 0.002 |
| Σ Total MUFA[f] | 9.3 ± 1.8 | 13.3 ± 1.0[e] | 10.6 ± 2.8 | 12.1 ± 0.7 | ns |
| 18:2n-6 | 12.8 ± 1.3 | 16.5 ± 0.2[e] | 14.3 ± 1.4 | 15.1 ± 1.0 | 0.003 |
| 20:2n-6 | 0.3 ± 0.04 | 0.4 ± 0.02 | 0.3 ± 0.08 | 0.2 ± 0.02 | |
| 20:3n-6 | 0.8 ± 0.1 | 1.1 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 | |
| 20:4n-6 | 13.6 ± 1.2 | 14.2 ± 1.1 | 13.1 ± 0.9 | 14.3 ± 0.2[b] | ns |
| 22:4n-6 | 0.2 ± 0.07 | 0.1 ± 0.02 | 0.3 ± 0.08 | 0.2 ± 0.02 | |
| 22:5n-6 | 0.1 ± 0.03 | 0.2 ± 0.1 | 0.2 ± 0.04 | 0.4 ± 0.04 | |
| Σ Total n-6 PUFA[g] | 27.8 ± 2.1 | 32.4 ± 1.1[e] | 29.0 ± 1.4 | 31.0 ± 1.1[b] | 0.02 |
| 20:5n-3 | 0.8 ± 0.1 | 0.8 ± 0.1 | 0.7 ± 0.1 | 0.9 ± 0.1 | |
| 22:5n-3 | 1.6 ± 0.4 | 0.9 ± 0.1[f] | 1.7 ± 0.2 | 1.0 ± 0.04[b] | ns |
| 22:6n-3 | 12.7 ± 1.9 | 6.8 ± 1.4[e] | 11.8 ± 1.6 | 8.7 ± 0.4[e] | 0.025 |
| Σ Total n-3 PUFA[g] | 15.0 ± 2.3 | 8.4 ± 1.6[e] | 14.1 ± 1.7 | 10.6 ± 0.4[e] | 0.03 |
| 22:6n-3/20:4n-6 | 0.93 ± 0.19 | 0.48 ± 0.07[e] | 0.90 ± 0.11 | 0.60 ± 0.02[e] | ns |
| 22:6n-3/22:5n-3 | 8.1 ± 2.0 | 7.8 ± 0.8 | 7.1 ± 1.3 | 8.4 ± 0.8[c] | 0.03 |

[a]Age effect × dystrophy effect (ns, not significant).
[b]$P < 0.01$.
[c]$P < 0.05$.
[d]Saturated fatty acids.
[e]Age effect (6 vs. 3 wk) inside nondystrophic or dystrophic group significant at $P < 0.001$.
[f]Monounsaturated fatty acids.
[g]Polyunsaturated fatty acids.

cholesterol between disk and ROS plasma membranes was detrimental to the ROS membranes. The phospholipid class distribution in the dystrophic disk membranes also differed from that of nondystrophics (6). In addition, anomalous phospholipid class distribution before weaning (7) and fatty acid composition after weaning (8) were reported in dystrophic RPE cells. In particular, the RPE cell plasma membranes of dystrophic rats aged 4–5 wk have 2.2-fold higher and 1.4-fold lower contents of 22:6n-3 and 20:4n-6, respectively, than those from nondystrophic rats aged 5–6 wk (8). In a previous study, Organisciak and Noell (31) compared the fatty acid composition in the EtnGpl and ChoGpl fractions of the ROS debris from adult dystrophic RCS rats with that of normal ROS from Sprague -Dawley rats (with an average age of 3 mon). Their data are summarized in Table 9, where it can be seen that the ChoGpl fatty acid composition in ROS debris was globally more altered at 3 mon than its EtnGpl counterpart. As compared to the normal ROS ChoGpl composition at 3 mon, the contents of 16:0, 18:1n-9, and 20:4n-6 increased in the dystrophic ROS ChoGpl, while those of 18:0 and 22:6n-3 decreased. In the present study we confirm that dystrophic retina fatty acid composition was altered more in the ChoGpl fraction than in EtnGpl, and we show that divergence from normal composition occurred precociously. In fact, the dystrophic retina fatty acid composition that we observed at the age of 3 wk was almost identical to that previously reported (31) for the ROS debris in dystrophic adult rats (Table 9). At 3 wk and 3 mon of age, the main fatty acid contents in dystrophic retina ChoGpl were equal to 37.3 and 38.1% 16:0, 12.7 and 15.9% 18:0, 14.1 and 14.7% 18:1n-9, 2.1 and 1.3% 18:2n-6, 8.1 and 7.3% 20:4n-6, and 15.2 and 17.1% 22:6n-3, respectively (data expressed in mol% of total fatty acids). The very similar ChoGpl fatty acid composition in dystrophic retina at 3 wk and in ROS debris at 3 mon suggests that the ChoGpl fatty acid composition in ROS debris did not differentiate from that of the whole dystrophic retina, whatever the age considered. From the data reported in Table 9, it can be estimated that the ChoGpl 22:6n-3 content increased by only 13% between 3 wk and 3 mon of age in dystrophic rats, whereas it increased by 224% in nondystrophic rats, possibly reflecting the normal accretion of 22:6n-3 in ROS ChoGpl during development. It cannot be excluded that different dietary conditions used in two distinct studies may explain the difference in 22:6n-3 contents observed in nondy-

strophic retinas at 3 wk and 3 mon. However, it is a fact that lower ChoGpl 22:6n-3 contents were related to the dystrophy, and the analysis was precociously performed either on the whole retina (Table 6) or on ROS debris at 3 mon (Table 9). In the EtnGpl fraction, the 22:6n-3 content was also identical in the ROS debris at 3 mon and in the dystrophic retina at 3 wk, i.e., 41.1 vs. 41.8% (Table 9), while, as noted for the ChoGpl fraction, the EtnGpl 22:6n-3 content was higher in 3-mon normal ROS than in 3-wk nondystrophic RCS retina (48 vs. 40.9%). However, the other fatty acids in EtnGpl changed with age in dystrophic as well as in normal rats, suggesting that 22:6n-3 was specifically affected in dystrophic EtnGpl, while most of the fatty acids (including 22:6n-3) were affected in the ChoGpl fraction. In simple terms, the fatty acid composition became globally and precociously fixed in dystrophic ChoGpl, while developmental changes continued to occur in the dystrophic EtnGpl, with the noticeable exception of 22:6n-3. These observations have to be compared to the previous finding of Boesze-Battaglia *et al.* (6) that the ROS disk membranes from 3 to 5-wk-old RCS dystrophic rats contained around 1.3-fold more ChoGpl than those from nondystrophics, while the EtnGpl contents were not statistically different (6). Together, the data imply that phospholipid metabolism in the retina of young dystrophic rats is affected in a class-specific manner. In retina as well as in brain and liver, the ChoGpl fraction appeared more deeply affected than the EtnGpl counterpart.

It is noteworthy that the 20:4n-6 contents significantly decreased with age in both the EtnGpl and ChoGpl fractions from nondystrophic rats, in apparent compensation for the concomitant increase in 22:6n-3. Opposite changes in the 20:4n-6 and 22:6n-3 contents were recently shown to occur in ROS phospholipids during postnatal development of albino Sprague-Dawley rats (32). The data of Suh *et al.* (32) indicate that the 22:6n-3/20:4n-6 ratio in the ChoGpl and EtnGpl fractions from ROS phospholipids increases by around approximately twofold between 3 and 6 wk of age (32). Moreover, the 22:6n-3/20:4n-6 ratio increased with age even though the rearing diet contained 1% of fat as 20:4n-6 (32). The authors suggested that a critical balance between n-3 and n-6 long-chain polyunsaturated fatty acids could be necessary throughout the weanling period for photoreceptor cells to achieve proper fatty acid composition and concomitantly to develop optimal rhodopsin content (32). It should be noted that, in neonatal RCS dystrophic rats reared under ordinary conditions of cyclic illumination, the eye rhodopsin content increases up to 4 wk of age and thereafter sharply decreases (1). Our data indicate that the 22:6n-3/20:4n-6 ratio significantly increased between 3 and 6 wk of age in the nondystrophic retinal ChoGpl and suggest that the dystrophy abolished this change. The comparative data reported in Table 9 also show evidence that the age-related increase of the 22:6n-3/20:4n-6 ratio (3 mon vs. 3 wk) was dramatically reduced in the dystrophic retina glycerophospholipids. Moreover, we found a significant age × strain interaction for the 22:6n-3/20:4n-6 ratio in both classes of brain phospholipids. It is possible that alterations in the retinal balance of n-3 and n-6 fatty acids could be related to the dystrophy-induced alterations in the rhodopsin content. Furthermore, the 22:6n-3/22:5n-3 ratio significantly increased with age in both phospholipid classes of nondystrophic retina, and the dystrophy clearly suppressed this change. Considering that alterations in this product-to-substrate ratio may be indicative of alterations in the terminal steps of n-3 synthesis (33), it could be suggested that dystrophic retina failed to synthesize proper amounts of 22:6n-3. However, lowered phospholipid 22:6n-3 contents may also arise from specific alteration in its uptake, trafficking, acylation, recycling, or turnover, and further experiments are needed to evaluate the impact of dystrophy on these pathways. In addition, alterations of lipid compositions observed in dystrophic RPE and ROS could be associated with the defect in phagocytic and recycling functions (7,8), possibly through damaging lipid peroxidation or anomalous membrane receptor interactions between the RPE and photoreceptor cells (35,36). The recent finding that a receptor tyrosine kinase (*Mertk*) plays an essential role in the signaling of phagocytosis in RCS rats suggests a molecular model in which a Mer-family receptor-binding protein (*Gas6*) mediates the interactions between RPE plasma membranes and phosphatidylserine molecules exposed at the outer leaflet of the shedding disks (3). It is not explained how the dystrophic mutation is involved in alterations of lipid metabolism. Anderson *et al.* (36) assumed that the primary defect ultimately leads to structural and biochemical adaptive changes in photoreceptor cells, possibly in response to the increasing risk of oxidative stress (36). The occurrence of some form of communication between the retina and other tissues is postulated.

**TABLE 9**
**Fatty Acid Composition (mol%) in Retina Glycerophospholipids from Nondystrophic and Dystrophic Rats at the Age of 3 wk (present study) and 3 mo (data from 31)**

| | Nondystrophics[a,b] | | | Dystrophics[a] | | |
|---|---|---|---|---|---|---|
| | | | | | 3 mon[a] | |
| | 3 wk[a] retina | 3 mon[b] ROS[d] | Variation[c] % | 3 wk[a] retina | ROS[d] debris | Variation[c] % |
| ChoGpl | | | | | | |
| 16:0 | 37.6 | 23.5 | –38 | 37.3 | 38.1 | 2 |
| 18:0 | 12.6 | 27.3 | 117 | 12.7 | 15.9 | 25 |
| 18:1n-9 | 13.3 | 5.2 | –61 | 14.1 | 14.7 | 4 |
| 18:2n-6 | 2.2 | 0.6 | –73 | 2.1 | 1.3 | –38 |
| 20:4n-6 | 9.9 | 2.2 | –78 | 8.1 | 7.3 | –10 |
| 22:6n-3 | 11.1 | 36.0 | 224 | 15.2 | 17.1 | 13 |
| 22:6/20:4 | 1.2 | 16.4 | 1267 | 1.9 | 2.3 | 21 |
| EtnGpl | | | | | | |
| 16:0 | 8.8 | 9.7 | 10 | 9.3 | 11.2 | 20 |
| 18:0 | 22.2 | 28.3 | 27 | 21.7 | 29.8 | 37 |
| 18:1n-9 | 4.3 | 6.3 | 47 | 5.6 | 9.9 | 77 |
| 18:2n-6 | 1.2 | 0.5 | –58 | 0.9 | 0.3 | –67 |
| 20:4n-6 | 15.5 | 1.4 | –91 | 13.8 | 3.0 | –78 |
| 22:6n-3 | 40.9 | 48.3 | 18 | 41.8 | 41.1 | –2 |
| 22:6/20:4 | 2.6 | 34.5 | 1227 | 3.0 | 13.7 | 357 |

[a]Royal College of Surgeons strain.
[b]Sprague-Dawley strain.
[c]Age-related variation (3 mon vs. 3 wk) in % of the value at 3 wk.
[d]Rod outer segments.

Our data show that changes in the phospholipid fatty acid composition of brain and retina of RCS rats were precociously altered in the developmental process. Age-related changes in plasma and liver phospholipids were apparently not linked to those occurring in neural tissues. Our finding that the EtnGpl and ChoGpl compositions were differently affected suggests that the fatty acid uptake by neural tissues and/or fatty acid-acylation activities could be impaired in a phospholipid class-specific manner. Tissue-specific alterations in phospholipid synthesis and fatty acid metabolism could be one of the defects precociously associated to the retinal dystrophy.

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# Biosynthesis and Tissue Deposition of Docosahexaenoic Acid (22:6n-3) in Rainbow Trout *(Oncorhynchus mykiss)*

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**ABSTRACT:** Rainbow trout *(Oncorhynchus mykiss)* weighing *ca.* 5 g and previously acclimated for 8 wk on a diet comprising vegetable oil (11%), fish meal (5%), and casein (48%) as the major constituents were fed a pulse of diet containing deuterated ($D_5$) (17,17,18,18,18)-18:3n-3 ethyl ester. The synthesis and tissue distribution of $D_5$-22:6n-3 was determined 3, 7, 14, 24, and 35 d after the pulse. The whole-body accumulation of $D_5$-22:6n-3 was linear over the first 7 d, corresponding to a rate of 0.54 ± 0.12 μg $D_5$-22:6n-3/g fish/mg $D_5$-18:3n-3 eaten/d. Maximal accretion of $D_5$-22:6n-3 was 4.3 ± 1.2 μg/g fish/mg of $D_5$-18:3n-3 eaten after 14 d. The amount of $D_5$-22:6n-3 peaked in liver at day 7, in brain and eyes at day 24, and plateaued after day 14 in visceral and eye socket adipose tissue and in the whole fish. The majority of $D_5$-22:6n-3 was found in the carcass (remaining tissues minus the above tissues analyzed separately) at all times. On a per milligram lipid basis, liver and eyes had the highest concentration of $D_5$-22:6n-3. The experimental diet also contained 21:4n-6 ethyl ester as a marker to estimate the amount of food eaten by individual fish. From such estimates it was calculated that the great majority of the $D_5$-tracer was catabolized, with the combined recovery of $D_5$-18:3n-3 plus $D_5$-22:6n-3 being 2.6%. The recovery of 21:4n-6 was 57.6%. The concentration of 22:6n-3 in the fish decreased during the 13-wk period, and the amount of 22:6n-3 synthesized from 18:3n-3 was only about 5% of that obtained directly from the fish meal in the diet.

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Docosahexaenoic acid (22:6n-3) is the functional end product of the n-3 series of polyunsaturated fatty acids (PUFA) and has a crucial role in membrane structure and function, being concentrated in excitable membranes, e.g., synapses, retina, sarcoplasmic reticulum (1–3). It is also abundant in sperm (4) where 22:6n-3-rich phospholipids may be crucial to the fusion process during fertilization (5). Docosahexaenoic acid is therefore a critical fatty acid in vertebrates and, in those species that are unable to synthesize 22:6n-3 from its precursor linolenic acid (18:3n-3), it must be supplied in the diet (6), i.e., it is a dietary essential fatty acid.

The extent to which different species can synthesize 22:6n-3 from 18:3n-3 or are reliant on a direct dietary input has been the subject of much debate and study (3,7). It is now recognized that there is a large demand for 22:6n-3 during early development when neural tissue (brain and retina) is being formed and that, in some species that convert 18:3n-3 to 22:6n-3, the rate of conversion may be insufficient to meet demand at this crucial time (7). This has led to the recommendation that infant formula feeds should be supplemented with 22:6n-3 (8).

Marine fish that are obligate carnivores (e.g., gadoids, clupeoids) have an absolute requirement for 22:6n-3 in the diet (9) and normally receive an excess from their prey. Freshwater fish, including salmonids, are able to form 22:6n-3 from 18:3n-3 and are therefore not thought to have such a requirement (10). The pathway whereby 18:3n-3 is converted to 22:6n-3 in fish is inducible and is repressed in the presence of dietary fish oil containing 20:5n-3 and 22:6n-3 (11). However, in spite of the importance of 22:6n-3, there is little quantitative information available on the rate of its formation from 18:3n-3 in vertebrates. *In vitro* assays using hepatocytes and $^{14}$C-labeled fatty acids cannot reliably be extrapolated to living animals because such assays commonly generate major amounts of intermediates in the pathway and minor amounts of end product.

This study measured the rate of formation of 22:6n-3 from 18:3n-3 in rainbow trout *in vivo* using a deuterated tracer and quantitation by gas chromatography–mass spectrometry (GC–MS) as described by Pawlosky *et al.* (12). The tissue deposition of newly formed 22:6n-3 was also determined over a 5-wk period.

## MATERIALS AND METHODS

*Chemicals.* Chloroform, methanol, ethanol, isohexane, and diethyl ether were high-performance liquid chromatography grade from Fisher (Loughborough, Leicestershire, United Kingdom). Diisopropylamine, anhydrous acetonitrile, and pentafluorobenzyl bromide were obtained from Aldrich (Gillingham, Dorset, United Kingdom). $D_5$ (17,17,18,18,18)-linolenic acid was purchased from Cambridge Isotope Laboratories (Andover, MA) as the fatty acid ethyl ester (FAEE). Linseed oil was from ICN (Basingstoke, Hampshire, United Kingdom) and refined olive oil was from Tesco supermarkets. High oleic acid sunflower oil was a gift from Croda Chemicals (Goole, United Kingdom). Fish meal was from Biomar (Grangemouth, United Kingdom). All other chemicals were from Sigma (Poole, Dorset, United Kingdom).

*Synthesis of 21:4n-6.* Heneicosatetraenoic acid (Δ6,9,12,15-

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Abbreviations: ANOVA, analysis of variance; $D_n$, deuterated; FAEE, fatty acid ethyl ester; FAME, fatty acid methyl ester; GC–MS, gas chromatography–mass spectrometry; PUFA, polyunsaturated fatty acid; tri23:0, tritricosanoyl glycerol.

21:4) ethyl ester was prepared by a one-carbon addition to 20:4n-6 fatty acid (13). The product was obtained in 51.2% yield and was 98.9% pure by GC and GC–MS of the fatty acid methyl ester (FAME) and pentafluorobenzyl ester (see following).

*Fish and diets.* Rainbow trout, approximately 2 g size, were obtained from a commercial hatchery and kept in a running freshwater aquarium at 6.5 to 12.5°C on a 14 h/10 h light/dark cycle. Fish were fed a diet based on casein and a blend of vegetable oil containing predominantly oleic acid with 18:2n-6 and 18:3n-3 at approximately 1% each to maximize 22:6n-3 synthesis and satisfy the essential fatty acid requirements. The final diet provided 50% crude protein and 11% oil blended to give 0.99% 18:2n-6, 1.02% 18:3n-3, and 0.12% highly unsaturated fatty acids (20:5n-3 and 22:6n-3) from the fish meal that was added to make the diet palatable and readily accepted by the fish. The remaining fatty acids were predominantly 16:0 (1.02%) and 18:1n-9 (7.18%). The full composition of the diet was (g/kg): vitamin-free casein 480, starch 150, fish meal 50, mineral mix 47, vitamin mix 10, arginine 4, methionine 3, cystine 2, leucine 4, orange G 1, chromic oxide 10, α-cellulose 129.6, blended vegetable oil 110, antioxidant mix 0.4. The composition of the mineral mix, vitamin mix, and antioxidant mix were described previously (14). The fish were fed this diet for 8 wk before starting the experiments.

*Preparation of labeled diet.* A small portion of diet containing $D_5$-18:3n-3 FAEE and 21:4n-6 FAEE was prepared as follows. An oil sample containing 10 mg $D_5$-18:3n-3 FAEE, 2.5 mg 21:4n-6 FAEE, 153 mg of high oleic acid sunflower oil, and 61 µg antioxidant was dissolved in 0.82 mL isohexane, and 1.335 g of dry diet mix was added. The isohexane was then removed at 37°C under nitrogen and the diet desiccated *in vacuo* for 18 h. The diet was mixed thoroughly, 0.95 mL water was added, and was mixed to a stiff paste. This was extruded through a 1-mL disposable syringe, dried at room temperature for 2–3 h and cut into 3–4 mm lengths. The diet was stored under argon at –20°C and was used within 3 d.

*Experimental protocol.* Twenty-six fish were acclimated in a 100-L circular tank with running water for 4 d then starved for 24 h. They were then fed the labeled diet, all of which appeared to be eaten. The fish were then fed a known amount of diet twice a day and sampled at days 3, 7, 14, 24, and 35. The temperature rose from 10.5 to 12.5°C over the 5 wk of the experiment. Two fish died on day 27 and were frozen at –20°C.

Fish were anesthetized with ethyl 3-aminobenzoate methane sulfonate and bled from the tail vein into a weighed vial containing heparinized saline (100 U/mL of 0.9% NaCl). The fish were weighed and the following tissues removed: liver, brain, eyes, visceral adipose tissue, and eye socket adipose tissue. The remaining carcass was chopped up. Samples were homogenized in chloroform/methanol 2:1 (vol/vol) using a Potter or Ultra-Turrax homogenizer and an extract according to Folch *et al.* (15) was prepared. Tritricosanoyl glycerol (tri23:0) standard was added to each tissue sample before homogenization. The amount of tri23:0 standard added varied with the weight of the fish and the lipid content of the respective tissues, e.g., for day 7: liver 2.0 µg tri23:0, brain 1.0 µg, eyes 1.0 µg, blood 0.5 µg, carcass 100 µg, eye socket fat 5 µg, visceral fat 20 µg. Samples were kept on ice under nitrogen during workup and were stored at –20°C under argon.

*Quantitation of fatty acids.* One milligram of total lipid was saponified with 2 mL of 0.1 M KOH in 95% (vol/vol) ethanol under nitrogen for 1 h at 78°C. Nonsaponifiable material was removed by extracting with isohexane/diethyl ether (2:1, vol/vol), the aqueous phase was acidified, then free fatty acids were extracted with diethyl ether. Pentafluorobenzyl esters were then prepared from 100 µg free fatty acid using acetonitrile/diisopropylamine/pentafluorobenzyl bromide (1000:10:1, by vol) at 60°C for 30 min under nitrogen as described by Pawlosky *et al.* (12). Excess reagent and solvent were then removed under nitrogen, and samples were dissolved in isohexane and stored at –20°C under argon until analysis.

Calibration standards of individual fatty acids (18:3n-3, 21:4n-6, and 22:6n-3) with 23:0 were prepared by varying the amount of unknown fatty acid while keeping the 23:0 constant and plotting the peak area ratio against the mass ratio of the different fatty acids. Sample volumes for analysis were adjusted such that the amount of 23:0 injected onto the GC–MS was constant. Pentafluorobenzyl esters were chromatographed and quantitated on a Fisons MD 800 GC–MS fitted with an on-column injector and a Chrompack CP wax 52CB column (30 m × 0.32 mm i.d., 0.25 µm film thickness; Burke Analytical, Alva, Clackmannanshire, United Kingdom) using helium as carrier gas (column head pressure 7 psi) and running in negative chemical ionization mode with methane as reagent gas (pressure 7 psi). The temperature program was 80–190°C at 40°C/min, 190–240°C at 1.5°C/min, then 240°C for 10 min. Fatty acids were identified by selective ion scanning for the required masses using a dwell time of 80 ms and cycle time of 20 ms and were quantitated by reference to the appropriate fatty acid calibration curve.

*Fatty acid analysis.* FAME were prepared by acid-catalyzed transesterification using 1% (vol/vol) $H_2SO_4$ in methanol at 50°C for 16 h under nitrogen in the presence of 17:0 free fatty acid standard. FAME were chromatographed on a Fisons GC 8000 gas chromatograph fitted with an on-column injector and a fused-silica capillary column (CP wax 52CB, 30 m × 0.32 mm i.d., 0.25 µm film thickness, Chrompack) using hydrogen as carrier gas (16).

*Analysis of results.* Mean values at different times were compared using one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test (Prism statistical package, GraphPad Software Inc., San Diego, CA).

## RESULTS

Twenty-six fish were fed the labeled diet; of those, two died during the experiment while three others were found to have eaten less than 1% of the tracer and were not analyzed further. The size of the fish varied between 3.2 and 15.6 g by the end of the experiment with group means of 5.6 ± 1.0 g at day 3, 6.6 ± 2.1 g at day 7, 7.3 ± 1.9 g at day 14, 7.9 ± 2.4 g at day 24, and 10.4 ± 4.4 g at day 35. A representative range of sizes was

selected at each sample time. Over the whole time course the recovery of deuterated fatty acids as $D_5$-18:3n-3 was 1.2% and as $D_5$-22:6n-3 was 1.4%. The recovery of 21:4n-6 was 57.6%.

The accretion of newly synthesized $D_5$-22:6n-3 with time in the different tissues is shown in Figure 1, where the units are μg $D_5$-22:6n-3/g fish/mg $D_5$-18:3n-3 eaten. Following the pulse of $D_5$-18:3n-3, the rate of accumulation of $D_5$-22:6n-3 was 0.54 ± 0.12 μg/g fish/mg $D_5$-18:3n-3 eaten/d over the first 7 d. Accretion peaked at day 14 in the whole fish (4.3 ± 1.2 μg $D_5$-22:6n-3/g fish/mg $D_5$-18:3n-3 eaten) then plateaued and possibly declined by day 35 (Fig. 1A). Carcass, defined here as the remainder of the fish following removal of the tissues analyzed separately, showed an identical pattern (not shown). The liver accumulated $D_5$-22:6n-3 to a level of 0.5 μg $D_5$-22:6n-3/g fish/mg $D_5$-18:3n-3 eaten by day 7, followed by a sharp decline over the next 4 wk (Fig. 1B). The accumulation of $D_5$-22:6n-3 in both brain and eyes was slow, peaking around day 24 at 0.1 and 0.4 μg $D_5$-22:6n-3/g fish/mg $D_5$-18:3n-3 eaten, respectively (Figs. 1C,1D). The values in adipose tissue plateaued by day 14 at 0.25–0.30 μg $D_5$-22:6n-3/g fish/mg $D_5$-18:3n-3 eaten for visceral adipose tissue (Fig. 1E) and around 0.12 μg $D_5$-22:6n-3/g fish/mg $D_5$-18:3n-3 eaten for eye socket adipose tissue (Fig. 1F).

The deposition of the substrate $D_5$-18:3n-3 showed a very different pattern over time (Fig. 2). Amounts in the whole fish (Fig. 2A), brain (Fig. 2C), eyes (Fig. 2D), visceral adipose tissue (Fig. 2E), and eye socket adipose tissue (Fig. 2F) remained essentially constant throughout the period. Liver showed a sharp decrease from day 3 (0.018 μg $D_5$-18:3n-3/g fish/mg $D_5$-18:3n-3 eaten) onward (Fig. 2B). The amount of $D_5$-18:3n-3 was very low in brain at all times, and in liver from day 14 onward.

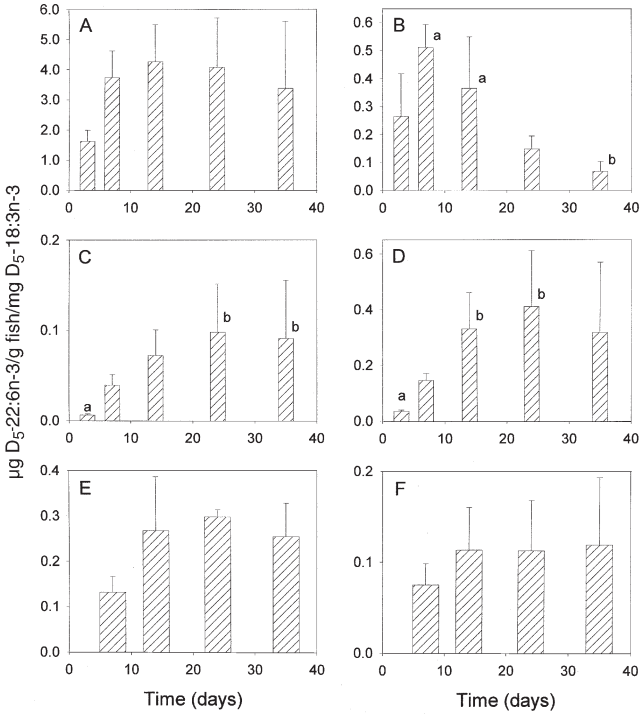


**FIG. 1.** The amount of deuterated $D_5$-22:6n-3 in different tissues with time. Data are expressed as mean ± SD. A = whole fish, B = liver, C = brain, D = eyes, E = visceral adipose tissue, F = eye socket adipose tissue. Note the different scales for different tissues. $n$ = 5 for day 3, 4 for day 7, 5 for day 14, 3 for day 24 and 4 for day 35. One-way analysis of variance (ANOVA) gave differences in mean values of $P$ = 0.0012 for liver, $P$ = 0.0114 for brain, and $P$ = 0.0109 for eyes. Values with different superscripts are significantly different at $P < 0.05$.

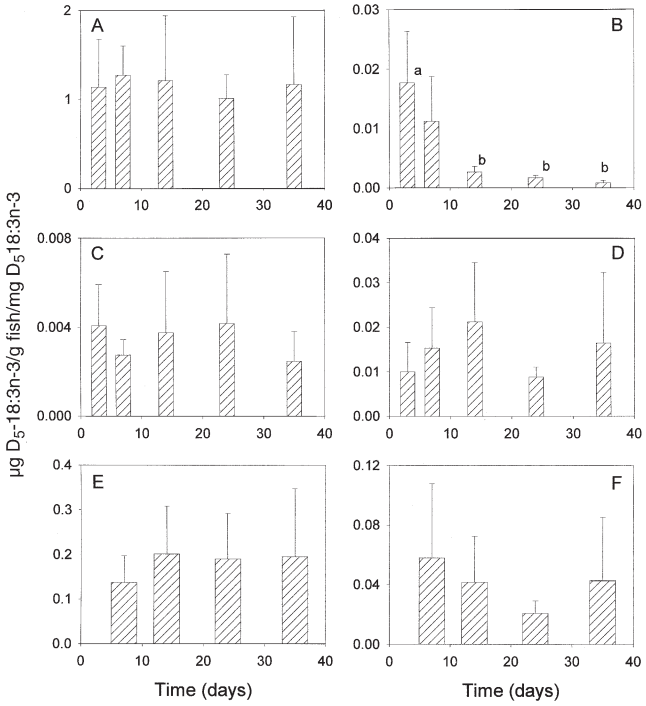


**FIG. 2.** The amount of $D_5$-18:3n-3 in different tissues with time. Data are expressed as mean ± SD. Other details are as Figure 1. One-way ANOVA gave a difference in mean values for liver of $P$ = 0.0009. Values with different superscripts are significantly different at $P < 0.01$. For abbreviations see Figure 1.

When the data are expressed on a per lipid weight basis in the respective tissues, a different pattern emerges (Table 1). The highest concentration of $D_5$-22:6n-3 was found in liver, up to 1.1 μg $D_5$-22:6n-3/mg lipid/mg $D_5$-18:3n-3 eaten at day 7, followed by the eyes, 0.94 μg $D_5$-22:6n-3/mg lipid/mg $D_5$-18:3n-3 eaten at day 24, and the blood, 0.52 μg $D_5$-22:6n-3/mg lipid/mg $D_5$-18:3n-3 eaten at day 14. Brain reached 0.21 μg $D_5$-22:6n-3/mg lipid/mg $D_5$-18:3n-3 eaten after 24 d, whereas both adipose tissues, carcass, and the whole fish contained low concentrations of $D_5$-22:6n-3 on a per milligram lipid basis. The fish grew over the experimental period so that lipid deposition at the later time points diluted the tracer. For $D_5$-18:3n-3 the highest concentrations were in the liver and blood at day 3, 0.038 and 0.051 μg $D_5$-18:3n-3/mg lipid/mg $D_5$-18:3n-3 eaten, respectively, with sharp falls thereafter (Table 2). The levels in the other tissues were similar (about 0.02 to 0.03 μg $D_5$-18:3n-3/mg lipid/mg $D_5$-18:3n-3 eaten) (Table 2), apart from brain where the concentration remained between 0.006 and 0.009 μg $D_5$-18:3n-3/mg lipid/mg $D_5$-18:3n-3 (Table 2).

The majority of the $D_5$-22:6n-3 was found in the carcass at all time points (71–79%) (Fig. 3). Liver was the next most enriched site containing 16.9% at day 3, falling to 2.1% by day

**TABLE 1**
**Amounts[a] of $D_5$-22:6n-3 in Different Tissues with Time**

| | μg $D_5$-22:6n-3/mg lipid/mg $D_5$-18:3n-3 eaten | | | | | | |
|---|---|---|---|---|---|---|---|
| Time (d) | Liver | Blood | Brain | Eyes | Visceral adipose tissue | Eye socket adipose tissue | Carcass |
| 3 | 0.54 ± 0.20[c] | 0.19 ± 0.10[a] | 0.01 ± 0.01[a] | 0.07 ± 0.02[a] | ND | ND | 0.027 ± 0.007[a] |
| 7 | 1.10 ± 0.23[a] | 0.38 ± 0.24 | 0.09 ± 0.02 | 0.28 ± 0.05 | 0.015 ± 0.006 | 0.068 ± 0.019 | 0.091 ± 0.032[b] |
| 14 | 0.62 ± 0.22[b,c] | 0.52 ± 0.18[b] | 0.17 ± 0.06[b] | 0.61 ± 0.27 | 0.040 ± 0.031 | 0.097 ± 0.039 | 0.078 ± 0.029 |
| 24 | 0.28 ± 0.12[b,c] | 0.23 ± 0.15 | 0.21 ± 0.09[b] | 0.94 ± 0.40[b] | 0.035 ± 0.013 | 0.114 ± 0.070 | 0.077 ± 0.043 |
| 35 | 0.15 ± 0.09[b] | 0.17 ± 0.12[a] | 0.21 ± 0.10[b] | 0.57 ± 0.50 | 0.036 ± 0.028 | 0.112 ± 0.099 | 0.055 ± 0.031 |

[a]Values are mean ± SD. ND = not determined. $n$ = 5 (day 3), 4 (day 7), 5 (day 14), 3 (day 24), and 4 (day 35). One-way analysis of variance (ANOVA) gave differences in mean values of $P < 0.0001$ for liver, $P = 0.023$ for blood, $P = 0.0013$ for brain, $P = 0.008$ for eyes, and $P = 0.0299$ for carcass. Within columns, values with different superscript roman letters are different at $P < 0.05$.

**TABLE 2**
**Amounts[a] of $D_5$-18:3n-3 Remaining in Tissues with Time**

| | μg $D_5$-18:3n-3/mg lipid/mg $D_5$-18:3n-3 eaten | | | | | | |
|---|---|---|---|---|---|---|---|
| Time (d) | Liver | Blood | Brain | Eyes | Visceral adipose tissue | Eye socket adipose tissue | Carcass |
| 3 | 0.038 ± 0.017[a] | 0.051 ± 0.023[a] | 0.008 ± 0.004 | 0.020 ± 0.015 | ND | ND | 0.023 ± 0.012 |
| 7 | 0.024 ± 0.016 | 0.010 ± 0.006[b] | 0.006 ± 0.001 | 0.028 ± 0.011 | 0.014 ± 0.004 | 0.049 ± 0.038 | 0.034 ± 0.013 |
| 14 | 0.005 ± 0.002[b] | 0.006 ± 0.004[b] | 0.008 ± 0.005 | 0.037 ± 0.023 | 0.024 ± 0.009 | 0.032 ± 0.017 | 0.023 ± 0.013 |
| 24 | 0.003 ± 0.001[b] | 0.003 ± 0.002[b] | 0.009 ± 0.006 | 0.021 ± 0.004 | 0.019 ± 0.004 | 0.020 ± 0.011 | 0.020 ± 0.009 |
| 35 | 0.002 ± 0.001[b] | 0.003 ± 0.003[b] | 0.006 ± 0.002 | 0.028 ± 0.026 | 0.027 ± 0.029 | 0.034 ± 0.027 | 0.020 ± 0.012 |

[a]Values are means ± SD. Abbreviations and number of samples as in Table 1. One-way ANOVA gave differences in mean values of $P = 0.0008$ for liver and $P < 0.0001$ for blood. Within columns, values with different superscript roman letters are different at $P < 0.01$.

35. The eyes contained up to 9.7% of the $D_5$-22:6n-3 (day 24). Visceral adipose tissue showed an increase from 3.7% at day 7 to 9.6% at day 35, while eye socket adipose tissue contained 2.2 to 3.6% of the $D_5$-22:6n-3. Accretion into brain was very slow, with 2.6% of total body $D_5$-22:6n-3 by day 35 (Fig. 3). The great majority of the residual $D_5$-18:3n-3 was in the carcass, decreasing from 82.0% at day 7 to 75.2% by day 35 and, in the visceral adipose tissue, increasing from 11.2% at day 7 to 19.7% at day 35 (Fig. 4). Eye socket adipose tissue contained 2.1–4.6% of $D_5$-18:3n-3 with lesser amounts in the eyes, brain, and liver. The tissue distribution of 21:4n-6 was very similar to that of $D_5$-18:3n-3, largely being in the two tissues containing the bulk of body lipid, i.e., the carcass (*ca.* 85%) and visceral adipose tissue (6.4–12.7%). Liver contained up to 4.9% 21:4n-6 at day 7, falling to 0.5% by day 35. Brain, eyes, and eye socket adipose tissue contained under 2% each.

As expected, the ratios of end product fatty acid to substrate fatty acid varied widely in the different tissues. In the liver the ratio of $D_5$-22:6n-3 to $D_5$-18:3n-3 was 8.6 at day 3, increasing to 86 at day 14, then falling back to 55 by day 35.

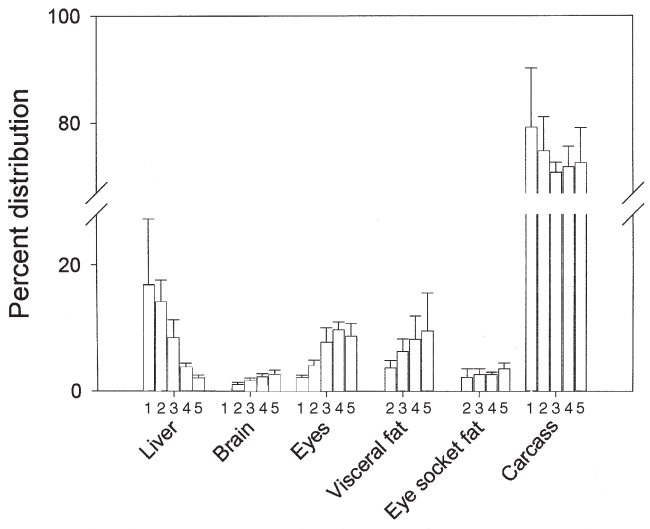


**FIG. 3.** The changes in tissue distribution of $D_5$-22:6n-3 with time. Means ± SD of the number of fish given are in Figure 1. Number key: 1, day 3; 2, day 7; 3, day 14; 4, day 24; 5, day 35. For abbreviation see Figure 1.

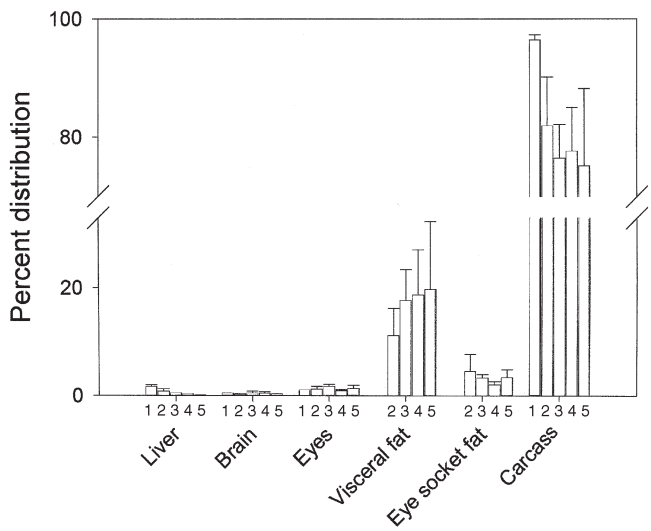


**FIG. 4.** The changes in tissue distribution of $D_5$-18:3n-3 with time. Means ± SD of the number of fish are given in Figure 1. Other details are in Figure 3. For abbreviation see Figure 1.

**TABLE 3**
**Change in the Content[a] of 22:6n-3 with Time in Fish on the Experimental Diet**

| Time on diet (d) | Time post tracer (d) | Number of fish | Weight of fish (g) | 22:6n-3 content (mg) | mg 22:6n-3/g fish |
|---|---|---|---|---|---|
| 0 | — | 5 | 3.04 ± 0.67 | 22.6 ± 7.7 | 7.3 ± 1.1 |
| 62 | 3 | 5 | 5.57 ± 1.03 | 28.3 ± 5.6 | 5.1 ± 0.6 |
| 66 | 7 | 5 | 6.65 ± 2.10 | 24.9 ± 9.1 | 3.8 ± 0.6 |
| 73 | 14 | 5 | 7.33 ± 1.90 | 23.2 ± 5.0 | 3.2 ± 0.4 |
| 83 | 24 | 5 | 7.92 ± 2.43 | 27.7 ± 7.9 | 3.6 ± 1.0 |
| 94 | 35 | 4 | 10.41 ± 4.40 | 30.5 ± 8.9 | 3.1 ± 0.6 |

[a]Values are mean ± 1 SD.

In blood the ratio was 1.9 at day 3, rising to 43 by day 14, then plateauing. Brain and eye showed a preferential accumulation of $D_5$-22:6n-3 with the ratio of $D_5$-22:6n-3 to $D_5$-18:3n-3 increasing to 18.6 by day 35 in brain, and to 44.8 by day 24 in the eyes. The ratio in the visceral adipose tissue and carcass showed little change, remaining between 1 and 2 from day 7 onward. Eye socket adipose tissue showed a slight preferential accumulation of $D_5$-22:6n-3 with a ratio between 3.9 and 5.4 from day 14 onward.

The content of 22:6n-3 in the fish was determined before the fish were fed the experimental diet and for each time point of the tracer experiment (Table 3). The amount of 22:6n-3 rose from 22.6 to 30.5 mg per fish, but the concentration of 22:6n-3 decreased with time (Table 3), giving a regression line with a slope of –0.04747 and a correlation coefficient of 0.870, i.e., the amount of 22:6n-3 decreased at a rate of 0.0475 mg/g fish/d.

## DISCUSSION

Most freshwater fish can desaturate and elongate 18:2n-6 and 18:3n-3 to their $C_{20}$ and $C_{22}$ homologs (17), but these processes have not been quantified. Past studies have used isolated hepatocytes or subcellular fractions, usually with [1-$^{14}$C]-labeled substrate fatty acids. Such assays are complicated because of the difficulties in presenting substrate, and they commonly result in relatively large amounts of pathway intermediates and small amounts of end product 22:6n-3. Therefore, their validity for the whole animal is suspect. Formation of 22:6n-3 from 18:3n-3 by trout hepatocytes was enhanced when fish were reared on diets lacking 22:6n-3 (11). In a study with salmon smolts, borage oil containing γ-linolenic acid (18:3n-6) enhanced the desaturation of both [1-$^{14}$C]18:2n-6 and [1-$^{14}$C]18:3n-3 in hepatocytes (18).

The method used here was developed by Pawlosky (12) to study essential fatty acid metabolism in cats (19). The use of stable isotope tracers offers several advantages over radiolabeled substrates. First, since stable isotopes are a mass tracer, they can be used more easily for quantitative measurements. Second, they are nonhazardous and are more amenable for *in vivo* studies, including those with humans. The metabolism of deuterated linoleic and linolenic acids was studied in human infants (20) and of deuterated linoleic acid in human adults (21). [U$^{13}$C]$C_{18}$ PUFA have been employed to examine PUFA recycling in rats (22) as well as desaturase activity in human infants (23), rats (24), and rhesus monkeys (25).

In this study we used $D_5$ (17,17,18,18,18)-18:3n-3 to determine the rate of formation of 22:6n-3 in rainbow trout *in vivo*. The fish had been reared on a low-PUFA diet (1% each of 18:2n-6 and 18:3n-3 with 0.038% 20:5n-3 and 0.079% 22:6n-3) sufficient to satisfy the essential fatty acid requirements for n-6 and n-3 PUFA (26) and to stimulate the conversion of 18:3n-3 to 22:6n-3 (11). The $D_5$-18:3n-3 tracer was delivered in the diet rather than by injection so that we could minimize stress from handling, which can stop fish eating and alter lipid metabolism, and so that we could feed a group of fish together, again eliminating stress. A marker was required to indicate how much diet individual fish in the experimental group had eaten and for this we used 21:4n-6. The rationale was that a fatty acid mimicking a $C_{20}$ essential fatty acid (arachidonic acid 20:4n-6) would be less likely to be catabolized and, therefore, give a better recovery than a saturated or monounsaturated fatty acid. This proved to be the case in that the recovery of 21:4n-6 in the experimental group as a whole was 57.6% compared with 2.6% for $D_5$-18:3n-3 plus $D_5$-22:6n-3. Other $D_5$-PUFA known to be intermediates in the biosynthetic pathway were not measured. The amount of dietary 21:4n-6 recovered in individual fish varied from 15.9 to 212.9 μg, and the proportion of the total 21:4n-6 recovered in the group as a whole was then used to calculate the amount of diet, and therefore the amount of $D_5$-18:3n-3, eaten by individual fish. The amount of deuterated substrate eaten varied from 0.11 to 1.46 mg per fish, excluding the three fish that did not eat the labeled portion. These values were then used to calculate the amount of $D_5$-22:6n-3 formed per milligram of substrate eaten. We previously used the same methodology to measure the rate of formation of 20:5n-3 in the sea urchin, where the recovery of the marker $D_8$-20:4n-6 was also much higher than that of $D_5$-18:3n-3 and its anabolites (27).

A notable feature of this study was the slow rate at which $D_5$-22:6n-3 was synthesized and deposited in tissues. The amount of newly synthesized $D_5$-22:6n-3 peaked in liver at day 7, whole body accretion peaked at day 14, and brain and eyes continued to accumulate $D_5$-22:6n-3 up to day 24. This suggests that it takes 2 to 4 wk for a pulse of 18:3n-3 to be metabolized to 22:6n-3 in trout.

The highest concentration of $D_5$-22:6n-3 on a per milligram lipid basis was found in liver at day 7 (1.1 μg/mg lipid/mg $D_5$-18:3n-3 eaten), as expected for a tissue actively synthesizing this fatty acid. The concentration in blood followed the trend for liver except that the values for days 7 and 14 were reversed. The eyes accumulated $D_5$-22:6n-3 to day 24, giving

a value of 0.94 μg $D_5$-22:6n-3/mg lipid/mg $D_5$-18:3n-3 eaten, the second-highest value for any tissue or time point, after liver at day 7. The concentration in eye socket adipose tissue at days 24 and 35 was *ca.* 3 times higher than that in visceral adipose tissue, suggesting a preferential deposition of 22:6n-3 for use by the retina. In fish of this size the eyes are still growing, so there is a continuing large demand for 22:6n-3 (28,29). In contrast, brain growth is much slower by this stage, and thus 22:6n-3 accretion by brain was much slower. There was much less tissue variation in the concentration of $D_5$-18:3n-3 on a per milligram lipid basis than for $D_5$-22:6n-3. The concentration of $D_5$-18:3n-3 in brain was notably low at all times, suggesting that either 18:3n-3 does not cross the blood brain barrier or it is metabolized very quickly. The latter seems unlikely since the amount of $D_5$-22:6n-3 was also low in brain and it is, therefore, more likely that 22:6n-3 synthesis in brain is either inherently low or is substrate limited. However, several studies have shown brain to be active in 22:6n-3 biosynthesis. Trout astrocytes in primary culture converted 0.7% of [1-$^{14}$C]18:3n-3 to 22:6n-3 (30) while [U-$^{13}$C]$C_{16}$ and $C_{18}$ PUFA were elongated and desaturated to 22:6n-3 in the brain of rats (31). In the feline, following a dose of $D_5$-18:3n-3, metabolites up to 22:5n-3 were present in liver and plasma, but metabolites from 24:5n-3 onward were only present in brain, suggesting that liver and brain act in tandem to synthesize 22:6n-3 (19). In the present study, the tissue ratios of $D_5$-22:6n-3 to $D_5$-18:3n-3 were highest in those tissues synthesizing 22:6n-3 (liver), transporting newly synthesized fatty acid (blood), and accreting 22:6n-3 (eyes and brain).

The results here clearly show that most of the $D_5$-18:3n-3 was catabolized, the recovery of $D_5$-18:3n-3 plus $D_5$-22:6n-3 being only 2.6% compared with 57.6% of 21:4n-6. This catabolism had largely occurred by the first time point at day 3, since the amount of $D_5$-18:3n-3 recovered in the whole fish was essentially unchanged over the 5-wk time course. The fish were growing, and it was expected that fatty acids would be oxidized extensively to provide energy for growth. However, it was rather unexpected that so much of an essential fatty acid would be oxidized, given that the diet was designed to satisfy the essential fatty acid requirement of the fish and no more (26). However, Cunnane *et al.* (22) showed that there was substantial carbon recycling in rat liver from linoleate into products of *de novo* lipogenesis even under conditions of extreme linoleate deficiency. This suggests that any control over the selection of fatty acids for oxidation is mainly dependent on chain length rather than their potential as key precursors for functionally essential fatty acids. Most of the remaining $D_5$-18:3n-3 was found in tissues other than the liver and was therefore unavailable as a substrate for 22:6n-3 biosynthesis without remobilization from other tissues. We could not determine from this experiment whether the PUFA biosynthetic pathway was substrate limited, but it is possible that if more substrate had been available the rate of formation of 22:6n-3 might have been elevated. The extent to which oxidation and deposition in other tissues controls the amount of substrate fatty acid available to the liver for 22:6n-3 biosynthesis requires investigation.

The synthesis of 22:6n-3 from 18:3n-3 was slow, giving a rate of 0.54 μg 22:6n-3/g fish/mg 18:3n-3 eaten/day over 7 d and peaking at 4.3 μg/g fish/mg 18:3n-3 eaten after 14 d. This can be put into context by considering the amount of 22:6n-3 in the fish and the amount obtained from the diet. The fish increased in size from a mean of 6.65 to 10.41 g over 28 d (from day 7 to day 35). From the regression line, the content of 22:6n-3 was 4.19 mg/g fish and 2.86 mg/g fish, respectively, at days 7 and 35, so for an average fish there was an increase in 22:6n-3 of 1.91 mg $[(10.41 \times 2.86) - (6.65 \times 4.19)]$ over this period. The fish ate 1.66% body weight/day for 28 d, which for an 8.5-g fish is 3.95 g diet containing 37.8 mg of 18:3n-3 and 3.13 mg 22:6n-3. The production of 22:6n-3 was 4.3 μg/g fish/mg 18:3n-3 eaten, giving 0.163 mg 22:6n-3 from 37.8 mg 18:3n-3 ingested. Therefore, for an average fish the total input was 3.29 mg—3.13 mg from the small amount of fish meal in the diet and 0.163 mg from biosynthesis. Thus, the contribution from biosynthesis was only about one-twentieth that from the diet. However, the measured increase in 22:6n-3 from the fatty acid composition was 1.91 mg. Therefore, over the 28 d there was a loss of 1.38 mg (3.29 – 1.91) of 22:6n-3 equivalent to 0.049 mg/d. The fish contained *ca.* 28.5 mg of 22:6n-3, so the turnover of 22:6n-3 was 0.17%/d. A number of assumptions were made in this calculation, and the final values are approximate. However, they are a good starting point for devising experiments to check the extent to which dietary supply and turnover affect the final concentration of 22:6n-3 in the fish.

Our results suggest that the extent to which trout can elongate and desaturate 18:3n-3 to 22:6n-3 may have been exaggerated and that the majority of 22:6n-3 may be obtained from the diet. Freshwater invertebrates that are natural food organisms for salmonids contain 10–15% 20:5n-3 but generally only trace amounts (<0.3%) of 22:6n-3 (32). However, freshwater oligochaetes and gammarids contain 1.0–1.4% 22:6n-3 (32), so it is possible that these species may be important as a source of 22:6n-3 for freshwater fish. Both 18:2n-6 and 18:3n-3 were abundant in all 10 species of invertebrates examined in that study (32). It can be calculated from our data that, in order to maintain the 22:6n-3 level constant in trout flesh, the amount of fish meal in the diet should be increased six- or sevenfold. Thus, a diet comprising vegetable oil and meal consisting of at least two-thirds fish meal should maintain tissue concentrations of 22:6n-3 in rainbow trout.

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# Modulation of Arachidonic Acid Distribution by Conjugated Linoleic Acid Isomers and Linoleic Acid in MCF-7 and SW480 Cancer Cells

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**ABSTRACT:** The relationship between growth and alterations in arachidonic acid (AA) metabolism in human breast (MCF-7) and colon (SW480) cancer cells was studied. Four different fatty acid preparations were evaluated: a mixture of conjugated linoleic acid (CLA) isomers (*c*9,*t*11, *t*10,*c*12, *c*11,*t*13, and minor amounts of other isomers), the pure *c*9,*t*11-CLA isomer, the pure *t*10,*c*12-CLA isomer, and linoleic acid (LA) (all at a lipid concentration of 16 μg/mL). $^{14}$C-AA uptake into the monoglyceride fraction of MCF-7 cells was significantly increased following 24 h incubation with the CLA mixture ($P < 0.05$) and *c*9,*t*11-CLA ($P < 0.02$). In contrast to the MCF-7 cells, $^{14}$C-AA uptake into the triglyceride fraction of the SW480 cells was increased while uptake into the phospholipids was reduced following treatment with the CLA mixture ($P < 0.02$) and *c*9,*t*11-CLA ($P < 0.05$). Distribution of $^{14}$C-AA among phospholipid classes was altered by CLA treatments in both cell lines. The *c*9,*t*11-CLA isomer decreased ($P < 0.05$) uptake of $^{14}$C-AA into phosphatidylcholine while increasing ($P < 0.05$) uptake into phosphatidylethanolamine in both cell lines. Both the CLA mixture and the *t*10,*c*12-CLA isomer increased ($P < 0.01$) uptake of $^{14}$C-AA into phosphatidylserine in the SW480 cells but had no effect on this phospholipid in the MCF-7 cells. Release of $^{14}$C-AA derivatives was not altered by CLA treatments but was increased ($P < 0.05$) by LA in the SW480 cell line. The CLA mixture of isomers and *c*9,*t*11-CLA isomer inhibited $^{14}$C-AA conversion to $^{14}$C-prostaglandin $E_2$ ($PGE_2$) by 20–30% ($P < 0.05$) while increasing $^{14}$C-$PGF_{2\alpha}$ by 17–44% relative to controls in both cell lines. LA significantly ($P < 0.05$) increased $^{14}$C-$PGD_2$ by 13–19% in both cell lines and increased $^{14}$C-$PGE_2$ by 20% in the SW480 cell line only. LA significantly ($P < 0.05$) increased 5-hydroperoxyeicosatetraenoate by 27% in the MCF-7 cell line. Lipid peroxidation, as determined by increased levels of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$), was observed following treatment with *c*9,*t*11-CLA isomer in both cell lines ($P < 0.02$) and with *t*10,*c*12-CLA isomer in the MCF-7 cell line only ($P < 0.05$). These data indicate that the growth-promoting effects of LA in the SW480 cell line may be associated with enhanced conversion of AA to $PGE_2$ but that the growth-suppressing effects of CLA isomers in both cell lines may be due to changes in AA distribution among cellular lipids and an altered prostaglandin profile.

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Compelling evidence indicates that conjugated linoleic acid (CLA), a derivative of linoleic acid found in milk and ruminant fats, is among the more potent naturally occurring anticarcinogens. *In vivo* model studies of experimental carcinogenesis have revealed that a synthetic mixture of CLA isomers containing 21% *c*11,*t*13, 29% *t*10,*c*12, 29.5% *c*9,*t*11, and 12.3% *c*8,*t*10 (1) possesses powerful inhibitory effects on mammary, colon, forestomach, and skin carcinogenesis in rodents (2–8). Other physiological benefits include a reduction in severity of atherosclerotic plaques, improvement of glucose tolerance in diabetic animals, body fat reduction, enhanced immune responses, and positive effects on bone formation, all of which have been well documented in numerous reviews (9–12). The specific CLA isomers that possess biological activity have not yet been clearly identified. Most of the mechanistic work to explain the potent anticancer effects of CLA has involved a commercial free fatty acid preparation containing up to 16 different CLA isomers (1). The availability of the pure isomers of *c*9,*t*11- and *t*10,*c*12-CLA has paved the way for determining the magnitude of biological responses to these isomers; these two are predominantly present in the synthetic CLA mixture and are produced by ruminants (13), and consequently they are found in the human diet. A study by Ip *et al.* (14) revealed that CLA-enriched butterfat, containing predominantly the *c*9,*t*11-CLA isomer, had a powerful protective effect against the risk of mammary cancer development in rodents.

The mechanisms by which CLA exerts its anticarcinogenic effects have not yet been fully elucidated. Induction of apoptosis by CLA *via* downregulation of a membrane protein bcl-2 has been reported to be the mode of cell death in cultured mammary tumor cells and in differentiated colonies of mammary epithelial organoids (15). The incorporation of CLA isomers into membrane phospholipids of breast, skin, and liver tissue has been reported but with variable levels of displacement from membranes of linoleic acid (LA) and arachidonic acid (AA) (7, 16–19). This suggests that CLA may influence the fatty acid

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Abbreviations: AA, arachidonic acid; CLA, conjugated linoleic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoate; $IP_3$, inositol triphosphate; LA, linoleic acid; $LTB_4$, leukotriene $B_4$; MG, monoglyceride; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; $PGD_2$, prostaglandin $D_2$; $PGE_2$, prostaglandin $E_2$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PI, phosphatidylinositol; PKC, protein kinase C; PL, phospholipid; $PLA_2$, phospholipase $A_2$; PLC, phospholipase C; PS, phosphatidylserine; TG, triglyceride; TLC, thin-layer chromatography.

composition of cell membranes, resulting in alterations in eicosanoid production and other signal transduction pathways downstream of the cell membrane. Eicosanoids, comprising prostaglandins and leukotrienes, are a family of membrane-derived lipid mediators that have been an attractive target for cancer chemoprevention (20). Research has shown that CLA can affect the synthesis of eicosanoids, in particular prostaglandin $E_2$ ($PGE_2$) (16), a prostanoid that has been shown to promote growth and metastasis in many experimental tumors (21).

Previously we used mammary (MCF-7) and colon (SW480) tumor cell lines as *in vitro* models to investigate the mechanisms by which CLA may affect breast and colon cancer. The MCF-7 epithelial cell line retains several characteristics of differentiated mammary epithelium including the ability to process estradiol *via* cytoplasmic estrogen receptors (22). The SW480 cell line, which was established from a primary adenocarcinoma of the colon, is a dedifferentiated cell line that expresses elevated levels of the p53 protein and small amounts of carcinoembryonic antigen (23). We previously demonstrated that the mixture of CLA isomers induced a dose- and time-dependent cytotoxicity against both cell lines and provided evidence that this effect may be due in part to increased lipid peroxidation (24). The anticancer effect of CLA may be due in part to a redistribution of AA among cellular lipids, which might influence oxidative susceptibility of particular membrane phospholipids and/or alter eicosanoid synthesis during tumor growth. This study was undertaken to examine the modulatory effects of CLA isomers on cell viability in addition to AA uptake, distribution, release, and conversion to eicosanoid classes in breast (MCF-7) and colon (SW480) human cancer cell lines.

## MATERIALS AND METHODS

*Materials.* $^{14}$C-AA (specific activity, 55 mCi/mmol), Biotrak enzyme immunoassay kit for leukotriene $B_4$ ($LTB_4$), and radioreceptor kit for inositol triphosphate ($IP_3$) were purchased from Nycomed Amersham (Little Chalfort, Buckinghamshire, United Kingdom). The CLA mixture (21% *c*11,*t*13, 29% *t*10, *c*12, 29.5% *c*9,*t*11, and 12.3% *c*8,*t*10) (1) was obtained from Nu-Chek-Prep (Elysian, MN). Individual CLA isomers, *c*9, *t*11 and *t*10,*c*12, were purchased from Matreya (Pleasant Gap, PA). LA, authentic $PGE_2$, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin $D_2$ ($PGD_2$), 5-hydroperoxyeicosatetraenoate (5-HPETE), phospholipid (PL) standards, and Supelclean LC-18 SPE columns were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Silica Sep-Pak columns were obtained from Waters Corporation (Milford, MA). The Bioxytech immunoassay kit for 8-epi-$PGF_{2\alpha}$ was obtained from Bio-Stat (Stockport, United Kingdom). DC-Alufliien Kiesegel 60 thin-layer chromatography (TLC) plates were obtained from Lennox (Dublin, Ireland). The CellTitre®$AQ_{ueous}$ Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Southampton, United Kingdom). All other chemicals and solvents used were high-performance liquid chromatography grade.

*Cell culture.* Human breast (MCF-7) and colon (SW480) cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Culture media and supplements were purchased from GIBCOBRL (Paisley, Scotland). Both cell lines were maintained in Dulbecco's minimal essential medium supplemented with fetal bovine serum (5% by vol), 0.2 mM L-glutamine, 1 mM HEPES, and 1 unit/mL penicillin and streptomycin. The MCF-7 cells required an additional supplement of 10 mM sodium pyruvate. Cells were grown in Falcon T-25 $cm^2$ flasks and maintained as previously described (24).

*Quantification of cell numbers.* The comparative effects of four different fatty acid preparations on cell viability were evaluated: (i) the CLA mixture of isomers, (ii) the pure *c*9,*t*11-CLA isomer, (iii) the pure *t*10,*c*12-CLA isomer, and (iv) LA. MCF-7 and SW480 cells were seeded in 96-well plates at densities of $1 \times 10^3$/well and $5 \times 10^2$/well, respectively. Cells were cultured for 24 h to allow the cells to attach to the substratum. The medium was then replaced with medium supplemented with either the CLA mixture of isomers, the pure *c*9,*t*11-CLA, the pure *t*10,*c*12-CLA, or LA at two different lipid concentrations: 5 and 16 μg/mL corresponding to 17.8 and 57 μM, respectively. The CLA concentrations used have been reported to be within the physiological range of concentrations of the *c*9,*t*11-isomer in human PL (25), plasma, bile, and duodenal juice (26) and have been previously used in cell culture work (27). The fatty acids were dissolved in ethanol and, therefore, control wells were supplemented with equivalent volumes of ethanol (0.25 or 0.8% by vol). After 24 h and 4 d of incubation, viable cell numbers were quantified using the MTS assay kit (CellTitre®$AQ_{ueous}$ Non-Radioactive Cell Proliferation Assay).

*Lipid extraction and fractionation.* Cells were seeded in T-25 $cm^2$ flasks at a density of $2 \times 10^5$/flask and grown to 90% confluency. The medium was then replaced with medium containing $^{14}$C-AA at 0.2 μCi along with either the CLA mixture of isomers, the pure *c*9,*t*11-CLA, the pure *t*10,*c*12-CLA, or LA, all at a lipid concentration of 16 μg/mL (57 μM). The CLA mixture of isomers at a lipid concentration of 16 μg/mL yielded a *c*9,*t*11-CLA and *t*10,*c*12-CLA concentration of approximately 4.7 μg/mL (17 μM) each. Control flasks were supplemented with an equivalent volume of ethanol (0.8% by vol). After 24 h incubation, cells were harvested using phosphate-buffered saline (PBS) containing trypsin (0.25% by vol). Total lipids were extracted from the cell pellet as described (28), dried under nitrogen, redissolved in chloroform, and applied to a silica Sep-Pak column to separate the triglyceride (TG), monoglyceride (MG), and PL fractions as described (29). An aliquot of each fraction was counted in a Beckman LS6500 scintillation counter before being dried under nitrogen. The PL fraction was separated using normal-phase TLC. Samples were co-migrated with authentic standards of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE). Chloroform/methanol/acetic acid (65:45:4, by vol) was used to separate the PL (30). Iodine vapors were used to identify the position of the PL compared with standards, and these bands were removed from TLC plates and placed in vials for counting by liquid scintillation.

*Phospholipase C (PLC) activity.* $IP_3$ was used as an index of PLC activity. Cells were treated with the four different fatty acid treatments (all at 16 μg/mL) or ethanol as described above. After 24 h incubation the cells were harvested as described above, and $IP_3$ was extracted from cells using a perchloric acid extraction method previously described by Palmer *et al.* (31). A radioreceptor assay kit (Biotrak D-*myo*-inositol 1,4,5-triphosphate assay system) was used to quantify $IP_3$ levels according to the manufacturer's instructions. This assay is based on competition between a [$^3$H]-$IP_3$ tracer and unlabeled $IP_3$ in the samples for binding to a bovine adrenal cortex protein.

*Release of $^{14}$C-AA derivatives.* Cells were seeded in T-25 $cm^2$ flasks at a density of $2 \times 10^5$/flask and grown to 80% confluency. Medium was replaced with medium containing $^{14}$C-AA (0.2 μCi) and incubated for 24 h. After removal of media, cells were washed three times with PBS before addition of medium containing the four different fatty acid treatments (all at 16 μg/mL) or ethanol as described earlier. After 24 h, medium containing the released $^{14}$C-AA derivatives was removed and an aliquot was counted by liquid scintillation.

*Primary PG and 8-epi-$PGF_{2\alpha}$.* Cells were seeded and treated with $^{14}$C-AA at 0.2 μCi along with the four different fatty acid treatments (all at 16 μg/mL) or ethanol as described previously. After 24 h incubation the medium was removed from the flasks, and eicosanoids were extracted twice with ethyl acetate from medium acidified to pH 3.0 with 0.1 N HCl as described (17). Eicosanoid extracts were dried under nitrogen, redissolved in ethyl acetate, and applied onto normal-phase TLC plates. Ethyl acetate/iso-octane/glacial acetic acid/water (55:25:10:50, by vol) was used to separate PG (16). Samples were comigrated with authentic standards of $PGE_2$, $PGF_{2\alpha}$, and $PGD_2$. Iodine vapors were used to identify the position of each PG compared with the standards. Bands of $PGE_2$, $PGF_{2\alpha}$, and $PGD_2$ were removed from TLC plates and placed in vials for counting by liquid scintillation. For the 8-epi-$PGF_{2\alpha}$ assay, culture medium was collected after 24 h incubation with the fatty acid treatments described earlier and 8-epi-$PGF_{2\alpha}$ was extracted as described (32). Briefly, ethanol was added to the medium to a final concentration of 15% (vol/vol) and acidified to pH 3.0 with formic acid (98% by vol). The sample was applied to Supelclean LC-18 SPE columns and washed with water (adjusted to pH 3.0 with formic acid), 15% (vol/vol) ethanol in water (pH 3.0), and hexane. Ethyl acetate containing 1% (vol/vol) methanol was used to elute 8-epi-$PGF_{2\alpha}$. The eluate was dried under nitrogen and resuspended in assay buffer (Bioxytech), and a competitive horseradish peroxidase enzyme-linked immunoassay kit (Bioxytech 8-isoprostane assay system) was used to quantify 8-epi-$PGF_{2\alpha}$ levels according to the manufacturer's instructions.

*$LTB_4$ and 5-HPETE.* Cells were seeded and treated with the four different fatty acid treatments (all at 16 μg/mL) or ethanol as described earlier. For the 5-HPETE assay, cells were lysed using Triton-X 100 (0.1% vol/vol). The assay was initiated by the addition of 50 μL of AA (70 mM prepared in 50 mM Tris-HCl buffer, pH 4) to 50 μL of cell lysate in an ice-cold 96-well plate and incubated at 37°C for 10 min. The reaction was terminated by the addition of 100 μL of the FOX reagent: sulfuric acid (25 mM), xylenol orange (100 μM), iron(II) sulfate (100 μM), methanol/water (9:1 vol/vol) (33). Absorbance was measured at 620 nm using an Anthos 2010 plate reader. For the $LTB_4$ assay, eicosanoids were extracted from the medium as described earlier and dried under nitrogen. An enzyme immunoassay kit (Biotrak $LTB_4$ enzyme immunoassay system) was used to quantify $LTB_4$ levels according to the manufacturer's instructions. This assay is based on the competition between unlabeled $LTB_4$ and a fixed quantity of peroxidase-labeled $LTB_4$ for binding sites on a $LTB_4$-specific antibody.

*Statistical analysis.* Three independent experiments were performed in triplicate. Student's *t* test was used to determine significant differences between treatments.

## RESULTS

*Effect of CLA isomers on cell viability.* MCF-7 and SW480 cells were incubated for 24 h and 4 d with the CLA mixture of isomers, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and LA at two different lipid concentrations (5 and 16 μg/mL corresponding to 17.8 and 57 μM, respectively). None of the fatty acids at either 5 or 16 μg/mL significantly altered cell viability after 24 h. The CLA mixture of isomers (16 μg/mL) caused a reduction in cell viability after 4 d in both cell lines, with a greater reduction noted in MCF-7 cells (58%) (Fig. 1A) compared with SW480 cells (52%) (Fig. 1B). The *c*9,*t*11-CLA isomer caused a similar reduction (~50%) in cell viability to the CLA mixture of isomers following 4 d of incubation at both 5 and 16 μg/mL. In both cell lines, the *t*10,*c*12-CLA isomer at 5 and 16 μg/mL reduced viability by 38–39 and 50–60%, respectively, following 4 d of incubation. Incubation of SW480 cells with LA (16 μg/mL) for 4 d increased cell viability by 23%, but the lower concentration of 5 μg/mL had no effect at either time point (Fig. 1B). LA (5 and 16 μg/mL) had no effect on the viability of MCF-7 cells following 4 d of incubation in this study.

*Effect of CLA isomers on incorporation of $^{14}$C-AA into cellular lipid fractions.* One of the mechanisms involved in growth suppression is an alteration in the AA cascade of events leading to eicosanoid production (17). In order to examine if cellular AA distribution was altered by CLA, we investigated the effect of CLA isomers on incorporation of $^{14}$C-AA into cellular lipid fractions. $^{14}$C-AA was preferentially incorporated into the PL fraction in untreated and CLA-treated MCF-7 cells and SW480 cells (Table 1). Levels of uptake into PL, TG, and MG were 60, 33, and 7%, respectively, in control MCF-7 cells (Table 1). $^{14}$C-AA uptake into the MG fractions was increased in MCF-7 cells treated with the CLA mixture ($P < 0.05$) (7.2%) and the pure *c*9,*t*11-CLA isomer ($P < 0.02$) (16.6%). None of the fatty acid treatments had any effect on uptake of $^{14}$C-AA into the TG and PL fractions of the MCF-7 cell line.

Levels of uptake into PL, TG, and MG were 76, 21, and 3%,

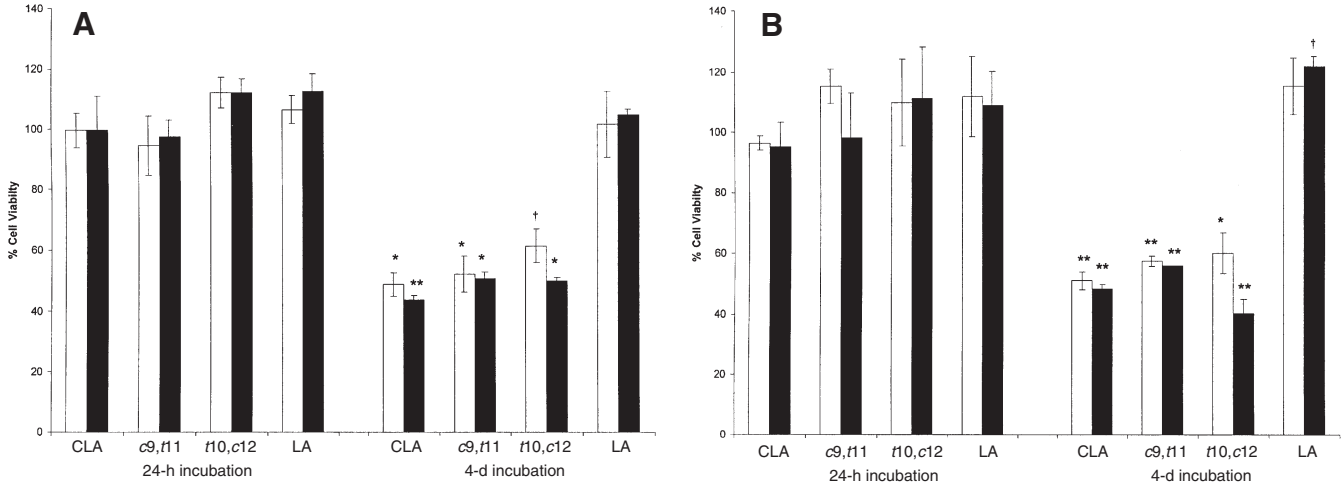


**FIG. 1.** Cell viability for (A) MCF-7 (human breast cancer) and (B) SW480 (human colon cancer) cells incubated with 5 (open bar) or 16 μg/mL (solid bar) conjugated linoleic acid (CLA) mixture, *c*9,*t*11-CLA, *t*10,*c*12-CLA, linoleic acid (LA), or ethanol control for 24 h and 4 d. Data represent cell viability expressed as a percentage of the control, which was taken to be 100% (**$P < 0.001$, *$P < 0.02$, and $^{\dagger}P < 0.05$). Error bars represent standard deviation.

respectively, in control SW480 cells (Table 1). In contrast with MCF-7 cells, uptake of $^{14}$C-AA into PL was significantly lowered ($P < 0.02$) (~25%) in the SW480 cells treated with the CLA mixture and *c*9,*t*11-CLA, while both the CLA mixture and *c*9,*t*11-CLA increased AA uptake into TG (25–30%) ($P < 0.05$). These data suggest that $^{14}$C-AA uptake into TG occurred at the expense of PL in the SW480 cell line. None of the fatty acid treatments had any effect on uptake of $^{14}$C-AA into the MG lipid fraction of the SW480 cells. The *t*10,*c*12-CLA isomer and LA (both at 16 μg/mL) had no effect on $^{14}$C-AA incorporation into any of the lipid fractions in either cell line.

*Effect of CLA isomers on $^{14}$C-AA distribution among PL fractions.* Having shown that $^{14}$C-AA was preferentially incorporated into the PL fraction of CLA-treated cells, we examined the effect of CLA isomers on $^{14}$C-AA distribution among individual PL. PC and PE were the predominant PL classes in which $^{14}$C-AA was taken up by control cells. Levels of uptake into PC, PI, PS, and PE were 45, 8, 6, and 41%, respectively, in the MCF-7 control cells and 34, 3, 3, and 60%, respectively, in the SW480 control cells (Tables 2 and 3). Of all the treatments, only incubation with the pure *c*9,*t*11-CLA isomer altered the distribution of $^{14}$C-AA among PL classes in the MCF-7 cells (Table 2). The *c*9,*t*11-CLA treatment at 16 μg/mL significantly ($P < 0.05$) reduced uptake of $^{14}$C-AA into PC (32%) and increased uptake into PE (41%). The CLA mixture at 16 μg/mL (which yielded a *c*9,*t*11-CLA isomer concentration of 4.7 μg/mL) had no effect. The *t*10,*c*12-CLA isomer at 16 μg/mL had no effect on the incorporation of $^{14}$C-AA into any of the PL fractions in the MCF-7 cells.

Incubation of the SW480 cell line with the *c*9,*t*11 CLA isomer (16 μg/mL) decreased uptake of $^{14}$C-AA into PC by 24% ($P < 0.01$) and increased uptake into PE by approximately 20% ($P < 0.01$) (Table 3). In contrast with MCF-7 cells, both the CLA mixture at 16 μg/mL (which yielded a *t*10,*c*12-CLA isomer concentration of 4.7 μg/mL) and the *t*10,*c*12 isomer (16 μg/mL) increased uptake into PS by 12–15% ($P < 0.05$) in the SW480 cell line. LA treatment had no effect on $^{14}$C-AA distribution among PL fractions in either cell line. None of the CLA isomers or LA had any effect on the uptake of $^{14}$C-AA in PI.

*Effect of CLA isomers on AA release.* AA can be released by two major pathways, the first through the action of phospholipase $A_2$ ($PLA_2$), which catalyzes the hydrolysis of *sn*-2 fatty acyl bond of membrane PL to liberate free AA (34), and the second by sequential cleavage of PI by PLC and diacylglyceride lipase (35). $IP_3$ levels were used as an index of PLC

**TABLE 1**
**Effect of Fatty Acid Treatments on Incorporation of $^{14}$C-AA into Lipid Fractions[a]**

| | MCF-7 | | | SW480 | | |
|---|---|---|---|---|---|---|
| Fatty acid | MG | TG | PL | MG | TG | PL |
| Control | 7.0 ± 1.3 | 33.5 ± 5.2 | 59.5 ± 6.2 | 2.9 ± 0.9 | 21.0 ± 0.6 | 76.1 ± 1.5 |
| CLA mixture | 14.8 ± 1.6[b] | 28.4 ± 1.2 | 56.8 ± 2.8 | 3.8 ± 1.0 | 47.2 ± 3.2[a] | 48.9 ± 2.2[a] |
| *c*9,*t*11-CLA | 23.6 ± 1.0[a] | 26.4 ± 4.1 | 49.9 ± 5.1 | 4.7 ± 1.3 | 45.7 ± 6.1[b] | 49.6 ± 7.2[a] |
| *t*10,*c*12-CLA | 6.2 ± 0.8 | 34.4 ± 4.5 | 59.5 ± 5.1 | 4.0 ± 1.9 | 22.1 ± 4.2 | 73.4 ± 5.7 |
| LA | 10.3 ± 2.0 | 31.2 ± 3.3 | 58.4 ± 5.2 | 3.8 ± 2.0 | 26.0 ± 9.1 | 70.1 ± 7.6 |

[a]Data represent the mean (± SD) percentage of total cellular lipids. Superscript roman letters indicate values that are significantly different compared to controls ([a] denotes $P < 0.02$ and [b] denotes $P < 0.05$). MCF-7, human breast cancer cells; SW480, human colon cancer cells; CLA, conjugated linoleic acid; AA, arachidonic acid; MG, monoglyceride; TG, triglyceride; PL, phospholipid; LA, linoleic acid.

**TABLE 2**
**Effect of Fatty Acid Treatments on Incorporation of $^{14}$C-AA into MCF-7 Phospholipid Fractions[a]**

| Fatty acids | PC | PI | PS | PE |
|---|---|---|---|---|
| Control | 44.4 ± 9.7 | 8.0 ± 2.8 | 6.2 ± 4.04 | 1.4 ± 8.2 |
| CLA mixture | 33.5 ± 3.9 | 6.7 ± 3.7 | 11.4 ± 5.6 | 48.4 ± 3.3 |
| *c*9,*t*11-CLA | 11.6 ± 2.7[a] | 4.3 ± 0.6 | 1.8 ± 0.5 | 82.2 ± 3.5[b] |
| *t*10,*c*12-CLA | 27.5 ± 6.9 | 5.5 ± 1.7 | 10.8 ± 6.5 | 56.3 ± 3.0 |
| LA | 33.5 ± 1.6 | 6.8 ± 3.0 | 3.7 ± 3.1 | 55.9 ± 7.7 |

[a]Data represent the mean percentage incorporation of total cellular phospholipids. Superscript roman letters indicate values that are significantly different compared to controls ([a] denotes $P < 0.05$ and [b] denotes $P < 0.02$). PC, phosphatidycholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; for other abbreviations see Table 1.

activity in this study. The CLA mixture of isomers, the pure *c*9,*t*11- and *t*12,*c*10-CLA isomers, and LA did not affect $IP_3$ in either cell line (data not shown). Total $^{14}$C-AA derivatives were increased by 28% ($P < 0.05$) in SW480 cells treated with LA only, whereas none of the CLA isomers had any effect on the total level of $^{14}$C-AA derivatives released by cells (Fig. 2).

*Effect of CLA isomers on eicosanoid synthesis.* The effects of various fatty acid treatments on enzymatic conversion of AA to primary eicosanoids ($PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $LTB_4$, and 5-HPETE) and on its nonenzymatic, free radical-catalyzed conversion to 8-epi-$PGF_{2\alpha}$, were examined. Following incubation of MCF-7 and SW480 cells in the presence of the CLA mixture and the pure *c*9,*t*11-CLA isomer at 16 µg/mL, it was found that $^{14}$C-AA conversion to $^{14}$C-$PGE_2$ was decreased by 20–30% ($P < 0.05$) whereas conversion to $^{14}$C-$PGF_{2\alpha}$ was increased by 17–44% relative to control (Figs. 3A,B). CLA treatments had a negligible effect on $^{14}$C-$PGD_2$. The *t*10,*c*12-CLA isomer had no effect on the three PG examined in either cell line. LA significantly ($P < 0.05$) increased $^{14}$C-$PGD_2$ by 13–19% in both cell lines and increased ($P < 0.05$) $^{14}$C-$PGE_2$ by 20% in the SW480 cell line only. Incubation of cells with either the CLA mixture of isomers or the pure *c*9,*t*11- or *t*10,*c*12-CLA isomers did not alter 5-HPETE or $LTB_4$ levels in the cells (data not shown), suggesting that CLA may mediate its effect *via* the cyclooxygenase component of the AA cascade. LA significantly increased 5-HPETE levels by 27% ($P < 0.05$) in the MCF-7 cell line (data not shown) but had no effect in the SW480 cells compared with untreated controls. The *c*9,*t*11-CLA isomer significantly increased ($P < 0.02$)

**TABLE 3**
**Effect of Fatty Acid Treatments on Incorporation of $^{14}$C-AA into SW480 Phospholipid Fractions[a]**

| Fatty acids | PC | PI | PS | PE |
|---|---|---|---|---|
| Control | 32.5 ± 8.0 | 3.5 ± 0.3 | 2.5 ± 0.7 | 61.5 ± 8.5 |
| CLA mixture | 25.9 ± 1.7 | 6.7 ± 1.9 | 14.5 ± 1.2[a] | 52.9 ± 0.8 |
| *c*9,*t*11-CLA | 8.3 ± 0.2[b] | 3.7 ± 0.1 | 5.5 ± 2.2 | 82.5 ± 2.1[b] |
| *t*10,*c*12-CLA | 25.8 ± 8.2 | 10.6 ± 4.4 | 9.1 ± 3.6[b] | 54.4 ± 7.5 |
| LA | 36.7 ± 9.6 | 3.7 ± 1.1 | 3.2 ± 0.4 | 56.4 ± 8.5 |

[a]Data represent the mean percentage incorporation of total cellular phospholipids. Superscript roman letters indicate values that are significantly different compared to controls ([a] denotes $P < 0.01$ and [b] denotes $P < 0.05$). For abbreviations see Tables 1 and 2.

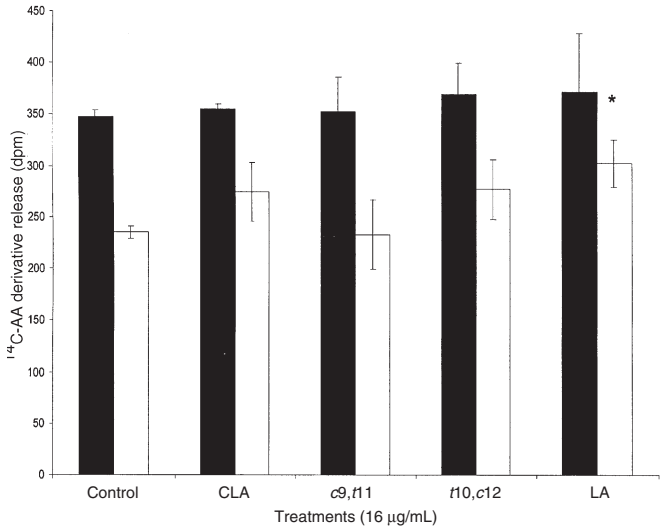


**FIG. 2.** The effect of treatments on total $^{14}$C-arachidonic acid (AA) release in MCF-7 (solid bars) and SW480 (open bars) cells. Cultures were treated with $^{14}$C-AA at 0.2 µCi for 24 h after which medium was replaced to contain either CLA mixture, LA, *c*9,*t*11-CLA, *t*10,*c*12-CLA (16 µg/mL), or ethanol (control) and then incubated for 24 h. Medium containing the released $^{14}$C-AA was removed, and an aliquot was counted by liquid scintillation. Results were expressed as mean $^{14}$C-AA released (dpm). Error bars represent standard deviation. *Values that are significantly different ($P < 0.05$) compared to controls. For abbreviations see Figure 1.

8-epi-$PGF_{2\alpha}$ in MCF-7 and SW480 cells by 38 and 48%, respectively (Figs. 3A,B). The *t*10,*c*12-CLA isomer increased ($P < 0.05$) levels of 8-epi-$PGF_{2\alpha}$ by 30% in the MCF-7 cell lines but had no effect in the SW480 cells (Fig. 3B). However, treatment of both cell lines with the mixture of CLA isomers and LA treatments had no relative effect in either cell line.

## DISCUSSION

This study shows that the MCF-7 and SW480 cell lines were sensitive to growth-inhibitory effects of not only the CLA mixture but also both the *t*10,*c*12-CLA and the *c*9,*t*11-CLA isomers following 4 d of incubation with physiological levels of CLA (5–16 µg/mL) (27). The CLA mixture of isomers at 16 µg/mL (yielding a *c*9,*t*11-CLA and *t*10,*c*12-CLA concentration of approximately 4.8 µg/mL each) was equally effective in inhibiting growth of both cell lines as the pure *c*9,*t*11-CLA and *t*10,*c*12-CLA isomer added at 16 µg/mL. This suggests that a plateau effect was reached or that one or more of the other isomers present in the mixture may be capable of altering cell viability. It is imperative, however, that more basic research be undertaken to determine the specific biological effects of other isomers present in the mixture, particularly *c*11,*t*13, which has recently been detected in natural products (36) and in liver microsomes (37). The growth-stimulatory effect of LA previously reported (27,38) was also seen in this study in the SW480 cell line treated with LA, but no effect was seen in the MCF-7 cells at the concentrations used.

This study provides an insight into the early responses of breast and colon cancer cell lines before growth is altered. Inter-

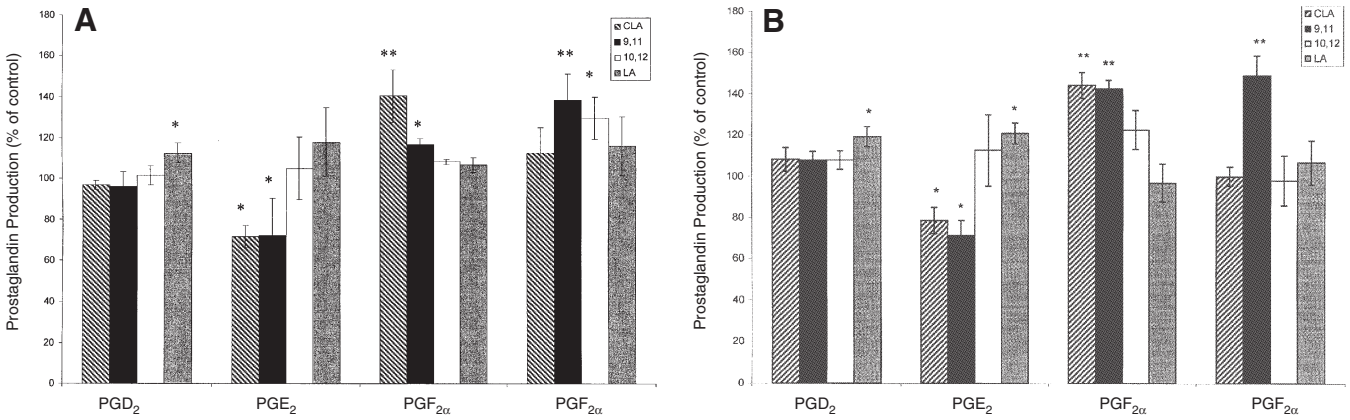


**FIG. 3.** Effect of treatments on primary prostaglandins (PG) and 8-epi-$PGF_{2\alpha}$ synthesis in (A) MCF-7 and (B) SW480 cells. Cultures were treated with 0.2 μCi/mL $^{14}$C-AA along with either the CLA mixture, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and LA (16 μg/mL) or ethanol control for 24 h. Eicosanoids were extracted from medium, and PG were separated using thin-layer chromatography and counted by liquid scintillation. Data represent the mean $^{14}$C-PG synthesis expressed as a percentage of the control, which was taken to be 100%. 8-Epi-$PGF_{2\alpha}$ levels were quantified using an enzyme immunoassay kit from Bioxytech (Bio-Stat, Stockport, United Kingdom). Error bars represent standard deviation. Asterisks indicate values that are significantly different compared to controls (**$P < 0.02$; *$P < 0.05$). For abbreviations see Figures 1 and 2.

estingly, the CLA mixture of isomers containing 4.7 μg/mL *c*9,*t*11-CLA was less effective than the pure *c*9,*t*11-CLA isomer (16 μg/mL) at redistributing AA among lipid fractions in the MCF-7 cell line and had no effect in altering AA content of individual PL of these cells. Our data demonstrate that the *c*9,*t*11-CLA isomer decreased AA uptake into PC while increasing uptake into PE in both cell lines. The decrease in uptake into PC is very significant as this is the PL preferentially hydrolyzed by $PLA_2$ to provide AA for eicosanoid synthesis (39). None of the other treatments had any effect on PS in the MCF-7 cell line, but in the SW480 cell line the CLA mixture and the *t*10,*c*12-CLA isomer both increased uptake of AA into PS. Although PS is a biosynthetic precursor of PE, it is in itself an important membrane lipid as it is an activator of membrane-associated protein kinase C (PKC), an enzyme that phosphorylates serine and threonine residues of an extremely diverse group of proteins regulating cell proliferation, activating cellular function, differentiation, and even apoptosis (40). It has been postulated that CLA may modulate PKC (41). However, activation of this enzyme is also dependent on diacylglycerol, a product of PLC activity, and $Ca^{2+}$ released from intracellular stores by $IP_3$. None of the treatments investigated altered the levels of $IP_3$ in the cells or uptake of AA into PI, suggesting that growth modulatory effects of various treatments in this study were not associated with PLC-mediated signal transduction. Other reports also indicate that physiological concentrations of CLA did not mediate changes in either PLC or PKC activity in MCF-7 cells (42) or in normal rat mammary epithelial cell organoids (43).

Interestingly, none of the CLA treatments influenced AA release from cells, yet both the CLA mixture and the *c*9,*t*11-CLA isomer decreased $^{14}$C-$PGE_2$ synthesis and increased $^{14}$C-$PGF_{2\alpha}$ in both cell lines, suggesting that a modulation of cyclooxygenase and/or downstream isomerase or reductase gene expression may be responsible. By contrast, LA stimulated $PGD_2$ production in both cell lines while stimulating $PGE_2$ production in the SW480 cell line. These changes in PG synthesis may have been responsible for the differential effects of LA and CLA treatments on growth. Levels of $LTB_4$ and 5-HPETE were not altered by any of the CLA treatments, suggesting that the anticancer effect of CLA may be mediated independently of the lipoxygenase component of the AA cascade as has been already proposed (44, 45).

A differential effect between physiological levels (0.5–5 μg/mL) of *c*9,*t*11 CLA and LA on growth of MCF-7 cells after 4 d has been reported (42). Growth inhibition by the CLA isomer was not mediated through PLC, PKC, or $PGE_2$-dependent signal transduction pathways, suggesting that another inhibitory mechanism may be involved. Because our study did show that $PGE_2$ synthesis was reduced by higher but near-physiological concentrations of CLA, it is apparent that there may be a threshold requirement for CLA and LA to affect cellular $PGE_2$ synthesis. A similar inhibitory effect of CLA on $PGE_2$ synthesis was observed in keratinocytes (17) and mouse epidermis (46). More recently CLA has been shown to inhibit prostaglandin H synthase activity in ram seminal vesicle microsomes (47).

Basu and coworkers (48) reported that CLA induced lipid peroxidation in humans by using urinary 8-iso-$PGF_{2\alpha}$ excretion as a biomarker of nonenzymatic lipid peroxidation. We showed that incubation of both cell lines with the *c*9,*t*11-CLA isomer led to significantly increased 8-epi-$PGF_{2\alpha}$ in both cell lines, while incubation with the *t*10,*c*12-CLA led to increases in 8-epi-$PGF_{2\alpha}$ levels in the MCF-7 cell line only. These isomers may be promoting nonenzymatic oxidation of AA at the expense of the formation of enzymatically derived eicosanoids. The mixture of CLA isomers (at 16 μg/mL) had no effect on 8-epi-$PGF_{2\alpha}$ levels, suggesting that a concentration of *c*9,*t*11-CLA higher than 4.7 μg/mL is needed to induce nonenzymatic oxidation of AA. A number of studies have now shown that the production of reactive oxygen species

serves to trigger an apoptotic signal transduction pathway (reviewed in Ref. 49). Further studies to investigate the effects of CLA isomers on the expression of cyclooxygenase isoforms and other signal transduction pathways are warranted to explain the potential inhibitory role of CLA on *in vitro* growth. Intervention studies have shown that increasing CLA intake led to increases in the CLA content in human milk (50), plasma (51), and adipose tissue (52). Although it is attractive to speculate that CLA may be useful in nutritional prevention of cancer in humans, evidence of beneficial effects in cancer patients receiving CLA as a dietary supplement is required. To this end, appropriate molecular and biochemical markers of both CLA nutritional status and of tumorigenesis are currently being sought.

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METHOD

# An Octaethylene Glycol Monododecyl Ether-Based Mixed Micellar Assay for Determining the Lipid Acyl Hydrolase Activity of Patatin

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**ABSTRACT:** Patatin was extracted from potato tubers (*Solanum tuberosum* L. cv. Spunta) and purified to homogeneity by ammonium sulfate salt fractionation and one sole chromatographic step. A spectrophotometric mixed micellar assay for patatin lipid acyl hydrolase (LAH) activity was designed with the detergent octaethylene glycol monododecyl ether ($C_{12}E_8$). Patatin LAH used *p*-nitrophenyl butyrate (PNP-butyrate) as substrate when solubilized in ($C_{12}E_8$) micelles. In the mixed micellar system, patatin LAH responds to the PNP-butyrate surface concentration expressed as mol% (= [PNP-butyrate]·100/([detergent] – critical micellar concentration)) and not to the molarity of PNP-butyrate. The kinetic parameters were determined; $V_{max}$ was independent of the mixed micelle concentration, as was $K_m$, when expressed as mol%. However, $K_m$ was dependent on $C_{12}E_8$ concentration when expressed in molar concentration. $C_{12}E_8$/PNP-butyrate proved to be a reliable system for assaying patatin LAH activity and is superior to the commonly used Triton X-100 and SDS methods. It permits investigation of the substrate requirements of patatin LAH activity because the concentration-independent $K_m$ can be determined both in mol% and as the absolute number of substrate molecules per micelle. In addition, the detergent did not affect the enzyme activity.

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Patatin is the trivial name given to a group of immunologically related glycoproteins with a molecular weight of 40 kDa found in practically all potato (*Solanum tuberosum* L.) cultivars thus far examined. Patatin accounts for up to 40% of the total soluble protein present in tubers (1,2) and owing to its high accumulation in the tuber, it is considered to be a storage protein (3). In several instances the storage proteins of plants have been found to have a function other than serving merely as a protein reserve. For example, several seed proteins act as proteinase inhibitors or have an antifungal or antibacterial activity (4). Patatin itself has been reported to show esterase activity with a large number of lipid substrates. By using a baculovirus system to express protein from the patatin cDNA, it has unambiguously been shown that the patatin coded by this DNA shows lipid acyl hydrolase (LAH) activity (5). The enzyme is active with phospholipids, monoacylglycerols, and *p*-nitrophenyl (PNP) esters, moderately active with galactolipids, but is apparently inactive with di- and triacylglicerols (6). This esterase activity may play a role in the plant defense mechanisms, although at present its physiological function is not understood (7).

In the literature, there is limited information on the kinetic characteristics of LAH activity of patatin, probably due to the poor solubility of its substrates in an aqueous medium. Lipids usually form several sorts of aggregates that either remain as a colloidal solution (normally turbid or opaque) or separate from the aqueous medium to form a lipid-rich phase (8). Because of the low solubility of these substances, the particular conditions, i.e., the environment in which the interaction and reaction between enzyme and substrate takes place, are unknown. It is even difficult therefore to define the concentration of a substrate because it is not homogeneously distributed in the reaction medium.

Surfactants may stabilize the lipid suspensions by forming mixed micelles, which are transparent, thermodynamically stable aggregates (8). In this way, a homogenous distribution of the lipid in the reaction medium can be achieved. Whenever the surfactant forms micelles with a stable aggregation number, it is possible to establish both the concentration of lipids relative to the concentration of surfactant and the absolute number of lipid molecules per micelle (9).

The term"surface dilution kinetics" has been widely applied to the study of kinetics of enzymes that act on lipids/interface substrates; and the theory, experimental considerations, and applications of surface dilution kinetics have been discussed (10). This kinetic model introduced the important concept that both the surface concentration of lipid and the bulk concentration in solution play critical roles in defining the kinetic parameters of lipid-dependent enzymes.

To routinely assay LAH activity, the most commonly used detergents by far have been Triton X-100 (11) or the combination of Triton X-100 and SDS (5,12–14). Although they form transparent mixed micelles with *p*-nitrophenyl esters (the most frequently used LAH substrates), they present important disadvantages; for example, they are not chemically pure substances, and thus their aggregation number is not well defined.

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Abbreviations: BSA, bovine serum albumin; CMC, critical micellar concentration; LAH, lipidacyl hydrolase; PNP, *p*-nitrophenyl; SDS, sodium dodecyl sulfate.

In the present work, a method of purifying patatin to homogeneity is presented. This procedure uses two phase-partitions, one with the detergent Triton X-114 and the other with salts, and only one chromatographic step. A system for measuring the LAH activity of patatin using the nonionic detergent octaethylene glycol monododecyl ether ($C_{12}E_8$) is also described. $C_{12}E_8$ is a chemically pure detergent, which has been well characterized physichochemically (15). It has been shown to solubilize several kinds of lipids such as palmitoyl CoA, phosphatidylglycerol and cardiolipin. López-Nicolás *et al.* (16) were able to determine lipoxygenase activity at neutral pH by using linoleic acid solubilized in $C_{12}E_8$ micelles as substrate.

The mixed micellar assay ($C_{12}E_8$/PNP butyrate) reported in this work enabled us to perform a kinetic analysis of the reaction with respect to the substrate (surface) concentration, which, until now, has not been carried out.

## EXPERIMENTAL PROCEDURES

*Plant materials.* The potatoes used, *Solanum tuberosum* L. cv. Spunta, were obtained from the local market.

*Reagents.* Triton X-114 (TX-114) was from Fluka (Madrid, Spain). Bovine serum albumin (BSA), Bradford reagent, acrylamide, bis-acrylamide, *N,N,N′,N′*-tetramethylethylenediamine, ammonium persulfate, and silver stain chemicals were obtained from Bio-Rad (Madrid, Spain). PNP butyrate, ascorbic acid, and molecular weight markers were from Sigma (Madrid, Spain). Octaethylene glycol monododecyl ether ($C_{12}E_8$) was a product from Fluka. All other chemicals were of analytical grade.

*Enzyme extraction.* After removing the peel and eyes, the potatoes (200 g) were chopped ($1 \times 1 \times 5$ cm, approximately) and homogenized with 200 mL of cold 200 mM acetate buffer pH 4.0, 20 mM EDTA, 6% (wt/vol) Triton X-114. Ascorbic acid (5 mM) was also used as an additional measure.

The homogenate was filtered through four layers of cheesecloth and kept at 4°C for 90 min to allow the precipitation of starch grains. It was then centrifuged at $100,000 \times g$ for 30 min at 4ºC. The supernatant was collected, and the concentration of TX-114 was increased by an additional 4% (wt/vol) and kept at 4°C for 15 min. This was then subjected to temperature-induced phase partitioning by warming to 35°C for 15 min (17).

The solution was then centrifuged at $10,000 \times g$ for 15 min at 25°C. The detergent-rich phase was discarded, and the clean supernatant containing patatin was collected. This solution, referred to as crude fraction, represents the starting material.

The supernatant was brought to 30% saturation with solid ammonium sulfate under continuous stirring at 4°C. After 1 h, this was centrifuged at $120,000 \times g$ for 30 min and the pellet was discarded. The ammonium sulfate concentration of the supernatant was increased to 60% and again stirred for 1 h at 4°C and centrifuged. The precipitate, which contained all the patatin lipid acylesterase activity, was redissolved in 10 mL of 10 mM phosphate buffer, pH 7.0, and dialyzed against the same buffer overnight to remove the ammonium sulfate.

*Anion exchange chromatography.* Aliquots (0.4 mL) of the enzyme extract following salt fractionation treatment were loaded onto a 1-mL Resource-Q column connected to an Äkta purifier (Pharmacia Biotech; Barcelona, Spain). The chromatography was run by the Buffer Prep method, which allows a buffer of any pH and salt concentration to be prepared on-line from four stock solutions. These four solutions were $A_1$: 0.1 M Tris; $A_2$: 0.1 M HCl; $B_1$: $H_2O$; and $B_2$: 2 M NaCl. Patatin was eluted from the column with a NaCl gradient from 0 to 1 M NaCl at 1.5 mL/min. To routinely determine LAH activity in all the collected fractions (2 mL), the assay used was that of Racusen (13), except that 2.6 mM PNP butyrate instead of PNP laurate was used as substrate.

*Affinity chromatography.* The fractions from anion exchange chromatography containing the highest esterase activity were pooled and concentrated. Aliquots of 0.2 mL were loaded onto a 1-mL HiTrap Concanavalin A column (Pharmacia Biotech) equilibrated with 20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.4, at 0.2 mL/min. Patatin was eluted from the column by a linear gradient of methyl-α-D-glucopyranoside (0–1 M) in the same buffer. Fractions (1 mL) with the highest level of LAH activity (determined as described above) were pooled, concentrated, and either used immediately or stored at –80°C.

*Protein determination.* Protein concentration was determined according to Bradford's dye-binding method (18) using BSA as a standard.

*Electrophoresis.* Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of purified proteins was run on a 12% acrylamide mini-slab according to Laemmli's method (19). Protein samples were diluted (1:4) in a denaturing sample buffer containing SDS and β-mercaptoethanol and heated at 95°C for 4 min. The gel was run at 200 V for approximately 45 min. Proteins were visualized by silver staining with a Bio-Rad kit, according to the recommended protocol. The SDS gels were scanned using micron image processing (MIP4) from Digital Image Systems (Madrid, Spain).

*Preparation of $C_{12}E_8$/PNP butyrate mixed micelles.* Mixed micelles were prepared by dissolving the detergent in 0.1 M Tris-HCl buffer, pH 8, followed by addition of the substrate, PNP butyrate dissolved in absolute ethanol. In all preparations, the final concentration of ethanol was 1% (vol/vol).

*Mixed micellar assay for lipid acylesterase activity of patatin.* Lipid acylesterase activity was assayed at 25°C by monitoring the increase in absorbance of the forming *p*-nitrophenol in a Kontron Uvikon 940 spectrophotometer at 410 nm. The reaction was started by adding 10 μL of purified patatin to 1 mL of mixed micelle preparation.

## RESULTS AND DISCUSSION

*Enzyme isolation and purification.* Patatin was extracted and purified from potato tubers (*S. tuberosum* L. cv. Spunta) as described in the Experimental Procedures section. The purifi-

**TABLE 1**
**Purification of Patatin from Potato Tuber**

| | Total protein (mg) | Total activity[a] (units) | Specific activity (units/mg) | Purification (fold) | Recovery (%) |
|---|---|---|---|---|---|
| Crude extract | 187 | 935 | 5 | 1 | 100 |
| 30–60% ammonium sulfate | 104 | 755 | 7.3 | 1.5 | 81 |
| Anion exchange chromatography | 34 | 462 | 13.6 | 2.7 | 50 |
| Affinity chromatography | 17 | 231 | 13.6 | 2.7 | 25 |

[a]Lipid acyl hydrolase activity was determined by the assay of Racusen (13), except that 2.6 mM *p*-nitrophenyl-butyrate was used as substrate.

cation stages used in the present work are summarized in Table 1. Eighty-one percent of the LAH activity of potato tubers was recovered in the 30–60% ammonium sulfate fraction. This step increased the specific activity 1.5- fold. These results were similar to those reported by Bohac (14), who obtained a 1.3-fold increase in specific activity in the 40–70% ammonium sulfate fraction from *S. tuberosum* L. cv. Superior. As Table 1 shows, there was no increase in specific activity with the additional affinity chromatographic step. The specific activity value obtained was higher than that obtained by other authors (13,14). The purification achieved in both chromatographic steps was also the same (2.7-fold), although the recovery decreased by up to 25% with the additional step. An estimate of esterase protein as a fraction of all soluble protein can be obtained by dividing the specific activity of the enzyme in the patatin fraction by its specific activity in the homogenate. Thus, 5 U/mg divided by 13.6 U/mg equals 0.37, or 37%, which is about the same as the percentage of patatin in the soluble fraction, as determined here and in earlier studies (13,20).

SDS electrophoresis revealed the purity of the enzyme fractions obtained from the different purification stages (Fig. 1). Samples subjected to anion exchange chromatography (lane 2) and to sequential anion exchange and affinity chromatography (lane 3) showed a very similar protein pattern. Apparently no further purification was achieved when the enzyme was subjected to an additional chromatographic step. This agrees with the results in Table 1, because both enzyme fractions showed the same specific activity. A closely spaced double zone was made visible by silver staining. The presence of these two patatin zones was already described by Racusen and Racusen (21), who reported the existence of two types of patatin (the low- and high-carbohydrate forms) in tuber extracts from the potato species *S. berthaultii*. The heterogeneity of patatin due to different glycosylation states is widely referenced in the bibliography (12,22–24).

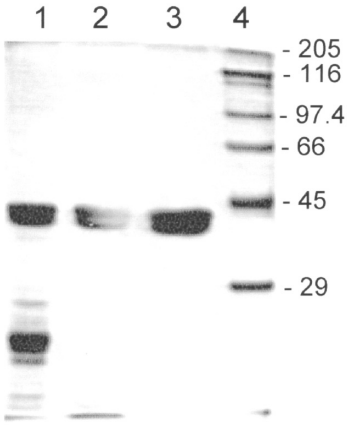


**FIG. 1.** Sequential extracts of protein purification on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, visualized with silver staining. (Lane 1) Total crude extract; (lane 2) extract from Resource-Q column; (lane 3) extract from HiTrap Con A column; (lane 4) molecular weight markers with mass indicated in kDa. Each lane contained 10 µg of protein.

The enzyme used in the kinetic experiments described below was purified up to the anion exchange chromatography step; the recovery was twice that obtained after the affinity chromatography stage (50%), and the specific activity was the same (13.6 U/mg).

*$C_{12}E_8$-based mixed micellar assay for patatin esterase activity—characteristics of the detergent.* To measure enzyme activity by a mixed micellar assay, the choice of the detergent is the first consideration, because not every surfactant may be useful. For example, the surfactant must not inhibit, inactivate, or activate the enzyme. Detergents such as SDS and Triton X-100 have been described as stimulating patatin LAH activity (11,13). A choice must also be made on the basis of the aggregation behavior of the surfactant. Although the detergent critical micellar concentration (CMC) value is flexible, it should be low. A high aggregation number ($N_{ag}$) is preferable to a low number, but it is important that both the CMC and the $N_{ag}$ are experimentally known and stable under the working conditions.

The mixed micellar system is characterized by the percentage concentration of lipid with respect to the concentration of aggregated surfactant,

$$\text{mol\%} = \frac{[\text{lipid}]}{[\text{detergent}] - \text{CMC}} \times 100, \quad [1]$$

and by the number of lipid molecules per micellar aggregate ($N_{lpm}$),

$$N_{lpm} = \frac{N_{ag} \times \text{mol\%}}{100} \quad [2]$$

A surfactant that fulfils all the preceding requirements and that is commercially available in a very pure state is $C_{12}E_8$ (CMC = 0.11 mM, $N_{ag}$ = 120–123 monomers/micelle determined between 15 and 25°C in 0.1 M NaCl) (15). According to Equations 1 and 2, 1 mol% of lipid corresponds to 1 lipid molecule per 100 $C_{12}E_8$ aggregated molecules. For instance,

in 3.11 mM $C_{12}E_8$ there are (3.11 – 0.11) = 3 mM aggregated $C_{12}E_8$ and thus 3/121.5 = 0.025 mM micelles, each containing 1.21 lipids per molecule on average.

*pH optimum.* In order to characterize patatin esterase activity in this mixed micellar assay, the effect of pH was analyzed. As shown in Figure 2, the activity varied with the pH, the optimal value being 8. This optimal pH was similar to that reported to measure esterase activity in systems using Triton X-100 and SDS as detergents (5,13,14). At acid pH values there was no detectable activity according to studies carried out on the structural stability of patatin. In a study of the pH dependence of patatin stability and conformation, Pots *et al.* (25) showed that patatin unfolds following heat or very acidic pH treatment and that, below pH 4, the tertiary stabilization of patatin decreases.

A slow rate of enzyme-independent PNP butyrate hydrolysis was observed at alkaline pH values. At pH = 8, this rate was 4% of the PNP butyrate hydrolysis in the presence of the enzyme; however, at pH = 8.5, the enzyme-independent hydrolysis was much higher, up to 18% of the enzymatic hydrolysis rate. This enzyme-independent hydrolysis rate was deducted from the patatin hydrolysis activity determined experimentally.

*Response of patatin esterase activity to the mixed micelles.* The aim of an enzymatic assay based on a mixed micellar system is to homogeneously supply a hydrophobic substrate to an enzyme able to act on it. In such a system, the variables that depend on substrate concentration and that affect the reaction rate can be controlled in a precise way (16).

The mixed micellar assay should satisfy two criteria: (i) dependence of the enzymatic activity on the mole fractions of the substrate and (ii) independence of the enzymatic activity from micelle concentration at fixed mole fractions of substrate (26). As can be seen in Figure 3, both criteria are satisfied by patatin esterase activity acting on $C_{12}E_8$/PNP butyrate mixed micelles: on the one hand, the enzyme shows no response to the overall substrate concentration but responds to the surface concentration (represented by the mole fraction); on the other hand, patatin esterase activity is independent of surfactant concentration at a fixed substrate mole fraction. For example, the overall PNP butyrate concentration at 20 mol% PNP butyrate in 4 mM $C_{12}E_8$ is half that in 8 mM $C_{12}E_8$, although the enzyme activity is the same. If there were any inhibition or inactivation, the activity would not be independent of surfactant concentration at a fixed substrate mole fraction. It might also be thought that the enzyme is already saturated in 4 mM $C_{12}E_8$ at 20 mol%, but this is not the case because an increase in substrate concentration from 20 to 40 mol% brings about a further increase in enzyme activity.

A simple explanation of these results is that the available concentration of substrate is not the overall concentration but that displayed at the surface of the mixed micelles. The constancy of the reaction rate when the mixed micelle concentration is changed confirms the hypothesis that the enzyme "feels" the amount of substrate by reference to the surface area and not the volume unit.

The usefulness of the mixed micellar assay has been demonstrated in the case of diglyceride kinase in octylglucoside/diacylglycerol mixed micelles (27). It has also proved reliable with other enzymes that act or depend on lipids, such as cholinephosphotransferase in Triton X-100/diacylglycerol/phosphatidylcholine (9) and sodium cholate/diacylglycerol (28) and glycerol-3-phosphate acyltransferase in $C_{12}E_8$/palmitoyl-CoA/car-

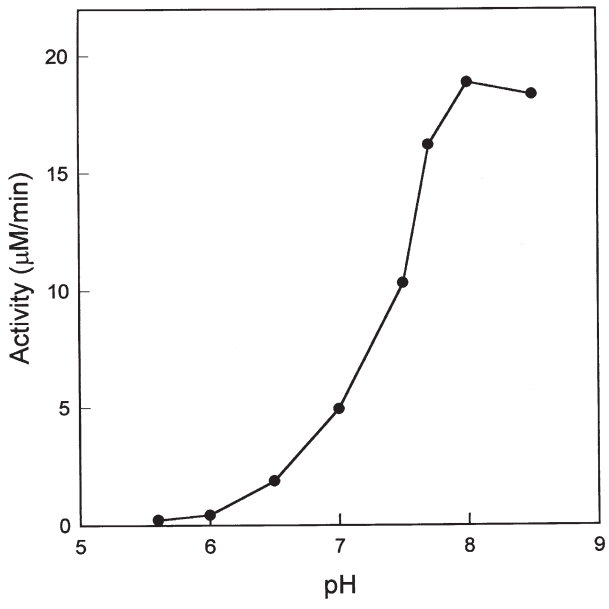


**FIG. 2.** pH response curve for lipolytic acyl-hydrolase activity in 5 mM octaethylene glycol monododecyl ether ($C_{12}E_8$)/*p*-nitrophenyl-(PNP) butyrate mixed micelles. The assay medium contained 50 mol% PNP butyrate in 0.1 M sodium phosphate buffer (pH values 5.6–7.5) or 0.1 M Tris-HCl buffer (pH 7.7–8.5). The reaction was started by the addition of purified patatin (11.5 μg/mL).

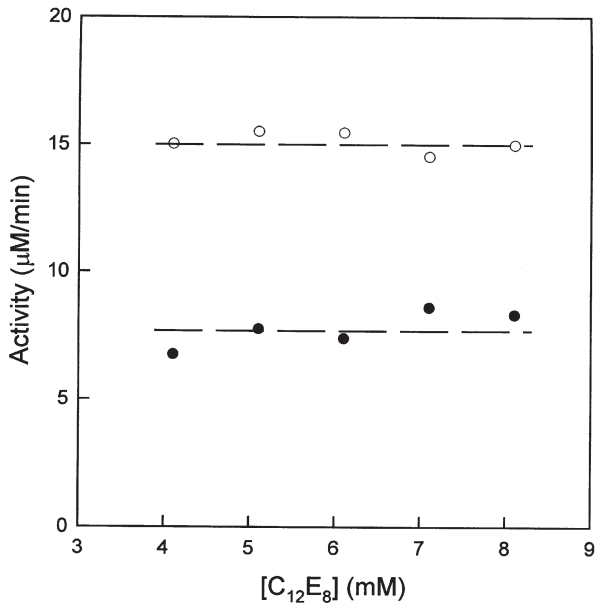


**FIG. 3.** Effect of $C_{12}E_8$ concentration on patatin lipid acyl hydrolase activity at fixed PNP butyrate mol%, (●) 20 mol% and (○) 40 mol%, in 0.1 M Tris-HCl buffer, pH 8, at 25°C. The reaction was started by the addition of purified patatin (11.5 μg/mL). For abbreviations see Figure 2.

diolipin (29). By using $C_{12}E_8$/linoleic acid, a mixed micellar assay for lipoxygenase activity was designed (16).

According to the results presented in Figure 3, the mixed micellar assay can be applied to enzymological studies of patatin esterase activity.

*Dependence of the patatin esterase activity on the substrate concentration. Evaluation of kinetic constants.* The effective substrate concentration for patatin esterase activity is that referring to the aggregated surfactant concentration expressed as mol%; therefore, we studied the dependence of enzyme activity on substrate concentration (Fig. 4). As can be seen, the enzyme showed cooperativity. The sigmoidal curves obtained at different bulk $C_{12}E_8$ concentrations were practically coincident. By fitting the data to the Hill equation, the corresponding kinetic parameters were evaluated. The results summarized in Table 2 show that $V_{max}$ was independent of surfactant concentration in every case, whereas $K_m$ varied proportionally to surfactant concentration when overall substrate concentration was taken into account ($K_m$ expressed in mol%), again demonstrating the surface dependence of the reaction. We can go further in the determination of $K_m$ by taking into account the absolute number expressed by the number of lipids per micelle ($N_{lpm}$). Since the substrate-containing surface forms discrete micelle units with a defined $N_{ag}$, the number of substrate molecules present in each micelle at each mol% of substrate can be evaluated. Through a simple calculation, it can be determined that at a $K_m$ value of 25.9 mol%, there are about 31 molecules per micelle.

To our knowledge, there are very few data concerning the evaluation of kinetic parameters for patatin esterase activity.

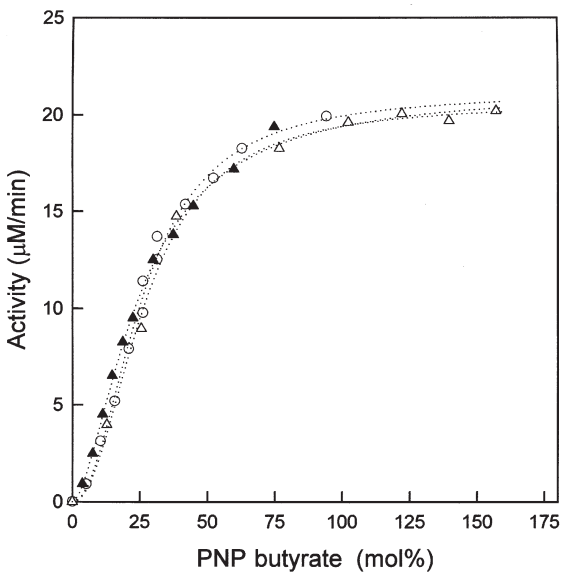


**FIG. 4.** Substrate concentration curves for patatin esterase activity at different bulk $C_{12}E_8$ concentrations, (△) 4 mM, (O) 5 mM, and (▲) 7 mM, in 0.1 M Tris-HCl buffer, pH 8. Enzyme concentration = 11.5 μg/mL. PNP butyrate surface concentration was expressed as mol% {= [PNP-butyrate]·100/([detergent] – critical micellar concentration)}. According to this expression, when mol% = 100, there is 1 molecule of PNP butyrate per 1 molecule of detergent.

**TABLE 2**
**Kinetic Constants of Patatin Lipid Acylesterase Activity in $C_{12}E_8$/*p*-Nitrophenyl-Butyrate Mixed Micelles, Evaluated at Different Bulk $C_{12}E_8$ Concentrations[a]**

| $C_{12}E_8$ (mM) | $V_{max}$ (μM/min) | $n_h$ | $K_m$ (mol%) | $K_m$ (mM) | $K_m$ ($N_{lpm}$) |
|---|---|---|---|---|---|
| 4 | 20.6 | 2.1 | 27 | 1.1 | 32.8 |
| 5 | 21.1 | 2.1 | 26 | 1.3 | 31.6 |
| 7 | 21.3 | 1.7 | 24.8 | 1.7 | 30.1 |
| Overall | 21 | 2 | 25.9 | — | 31.5 |

[a]$C_{12}E_8$, octaethylene glycol monododecyl ether.

Earlier attempts to determine the $K_m$ for patatin esterase activity used Triton X-100 as dispersing agent, yielding values of 0.5 and 0.7 mM with PNP palmitate and PNP stearate, respectively, as substrates in potassium phosphate buffer pH 7.5 (12). In those experimental conditions, the LAH enzyme activity was inhibited by high substrate concentrations. When 1% Triton X-100 and 0.017% SDS were used as detergents, the $K_m$ value for the substrate PNP laurate was 0.1 mM (14). As we have demonstrated that the patatin esterase reaction depends on the surface concentration of the substrate, the above-mentioned results as determined by volume unit in Triton X-100 or in Triton X-100 and SDS may be meaningless, especially if they were determined at one particular surfactant concentration.

In this work, patatin was extracted and purified from *S. tuberosum* L. cv. Spunta potato tubers. Although all tested potato cultivars and species have patatin, the total amount they contain as well as the esterase activity they show with specific substrates differs among cultivars. We used the cultivar Spunta, a variety that has not been previously used as enzyme source. The three-step purification procedure described here resulted in a 2.7-fold increase in esterase specific activity. This increase was similar to that obtained by Racusen (13). Since patatin has been estimated to be 30–40% of the total soluble tuber protein, the maximum purification can only be 3–4 times that of the crude extract. Hasson *et al.* (30) and Bohac (14) reported a 25- and 21-fold increase, respectively, in esterase specific activity; this greater increase in specific activity was attributed to the removal of an inhibitor or to a change in protein conformation.

This paper also demonstrates the applicability of a procedure that uses the nonionic detergent $C_{12}E_8$ for measuring patatin esterase activity. This mixed micellar system is superior to the assays that use Triton X-100 and SDS as surfactants because it allows the determination of the kinetic parameter $K_m$ independently of detergent concentration and, furthermore, does not affect enzyme activity.

## ACKNOWLEDGMENTS

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# *Trans*- and *cis*-Octadecenoic Acid Isomers in the Hump and Milk Lipids from *Camelus dromedarius*

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**ABSTRACT**: The distribution profiles of individual *trans*- as well as *cis*-18:1 isomers from the fat prepared from the hump adipose tissue and the milk from *Camelus dromedarius* (the single-humped Arabian species) are described. Gas–liquid chromatography on two capillary columns with different polarities and lengths were used for this purpose in combination with argentation thin-layer chromatography. A comparison of the profiles established is made with that of true ruminant fats. In the fats from the dromedarius as well as from true ruminants, the *trans*-18:1 isomers have their ethylenic bonds in all positions between Δ4 and Δ16. The prominent *trans* isomer is the 11-18:1 (vaccenic) acid in all species, and the complete distribution profiles are quite similar. Concerning the *cis* isomers, the prominent isomer is oleic acid, followed by *cis*-vaccenic acid, as in true ruminant fats. Other *cis* isomers encompass the Δ6-8 and the Δ12 to Δ15 isomers. Camelidae (suborder *Tylopoda*) and Bovidae (suborder *Ruminantia*) have evolved independently since the Eocene, that is for approximately 50 million years. Despite this considerable period, and the profound differences in anatomy, morphology, physiology, ecological and dietary habits between the extant species of these suborders, the rumen microflora has continued to synthesize the same *trans*- and *cis*-octadecenoic acid isomers, in comparable proportions, at least as deduced from their composition profiles. We conclude that the *trans*-18:1 acid profile is not intrinsically species-dependent, but it can be affected by the nature and the proportions of dietary unsaturated fatty acids that themselves depend on the feed, and that may be species-specific.

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Among artiodactyls, true ruminants, or Ruminantia, and Tylopoda, with the single family Camelidae, have a multi-compartmentalized stomach. In species from the first suborder, the stomach encompasses the rumen, the reticulum, the omassum, and the abomassum. The omassum is lacking, however, in Tylopoda. The rumen microflora is well known to lipid researchers for its biohydrogenation activity on dietary polyunsaturated fatty acids. The acids transform into stearic acid, leading in particular to a great number of intermediary metabolites with *trans* ethylenic bonds that are absorbed and appear in organ lipids, adipose tissues, and milk fat. The main intermediates found in ruminant fats are *trans*-octadecenoic acids, though many other minor *cis*-octadecenoic acids and geometrical and positional isomers of hexadecenoic and octadecadienoic acids also are present.

Among true ruminants, the *trans*-octadecenoic acid distribution profile in triacylglycerols is rather well documented (1), though mostly limited to a few common Bovidae, *Bos taurus* in particular, and to a lesser extent, to *Ovis* and *Capra*. Most data relate to milk because of its economical and dietary importance. Sketchy data on total *trans*-18:1 acids also are available for the meat from *Bison bison* (buffalo) and *Rangifer tarandus* (reindeer) (1) and on total *trans*-unsaturated acids for the fat from some Cervidae, e.g., *Dama dama* (fallow deer) and *Cervus unicolor* (sambur) (2).

A perusal of the available literature shows that no Tylopoda species have yet been examined with regard to the presence of *trans*-fatty acids in their tissues, and therefore their distribution profiles are unknown. This suborder presents only two genera, *Camelus* and *Lama*. Ruminantia and Tylopoda were quite well differentiated during the Eocene (*ca.* 50 million years), and their evolution was mostly accomplished in distinct geographical areas (New World and Old World, respectively). It was thus interesting to investigate the *trans*-fatty acid profile in lipids of a Tylopoda species, and we chose *Camelus dromedarius* from North Africa. We report here on the *trans*- and *cis*-octadecenoic acid profiles of lipids from hump adipose tissue and milk of this species. A comparison between lipids from the dromedarius and true ruminants shows that there are no gross differences between the two suborders Tylopoda and Ruminantia, thus excluding any effect of the species on the *trans*-octadecenoic acid profile.

## EXPERIMENTAL PROCEDURES

*Samples and standards.* Fatty tissues from the humps of six animals were obtained from a slaughterhouse in South Morocco immediately after the animals were killed. The milk was obtained from three lactating females. The tissues were transported to the laboratory at 4°C. Hump adipose tissue and milk samples were frozen at –30°C until analyzed.

*Fat extraction.* Fat was extracted from each individual sample mainly according to Folch *et al.* (3). Representative portions of the adipose tissue were minced with scisors and homogenized in methanol with an Ultra-Turrax T25 (Janke & Kunkel GmbH & Co. KG, Staufen, Germany) before adding chloroform and performing a second dispersion with the

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Abbreviations: Ag-TLC, argentation thin-layer chromatography, FAME, fatty acid methyl ester; GLC, gas–liquid chromatography.

Ultra-Turrax. Aliquots of milk were treated in the same manner. After removal of the solvents in a rotary evaporator at 45°C, the fat was weighed and transferred into 5-mL vials and stored at 4°C until used.

*Fatty acid methyl ester (FAME) preparation*. FAME were prepared by vigorously shaking for 1 min a mixture of 3 mL hexane, 1 droplet of melted fat, and 300 µL of a 0.5 N sodium methoxide solution in methanol. After centrifugation, the supernatant was directly used for gas–liquid chromatography (GLC) analysis. For argentation thin-layer chromatography (Ag-TLC), equal amounts of the six FAME solutions were mixed. Data reported here are thus for composite samples.

*Fractionation of FAME by Ag-TLC*. FAME were fractionated according to the number and geometry of double bonds by TLC on silica gel plates impregnated with $AgNO_3$. The plates were prepared by immersion in a 5% solution of $AgNO_3$ in acetonitrile as described by Wolff *et al.* (4). The developing solvent was the mixture hexane/diethyl ether (90:10, vol/vol). At the end of the chromatographic runs, the plates were briefly air-dried, lightly sprayed with a solution of 2′,7′-dichlorofluorescein, and viewed under ultraviolet light (234 nm). The *trans*- and *cis*-monoenoic acid bands were scraped off separately and eluted using a biphasic solvent system (4). The extracts from each fraction were dried under a light stream of $N_2$ and the residues were dissolved in an appropriate volume of *n*-heptane or hexane for further GLC analysis.

*Analysis of FAME by GLC*. The total fatty acid composition covering fatty acids in the range 4:0 to 24:0, including polyunsaturated fatty acids, was determined by GLC analysis of the methyl esters on a CP 9001 chromatograph (Chrompack, Middelburg, The Netherlands) equipped with a split injector (split ratio, 1:50) and a flame-ionization detector, using a 25-m capillary CP-Wax 58 CB column (0.25 mm i.d., 0.20 µm df; Chrompack). The operating conditions were as follows: $H_2$ as the carrier gas at an inlet pressure of 40 kPa; injector and detector temperature, 265°C; and oven program of 50°C for 1 min, then 5°C/min to 225°C, then 15 min isothermal, and finally 5°C/min to 260°C. Calibration of the individual fatty acids was achieved using a standard of methyl esters of all major fatty acids in bovine milk fat.

Analyses of the *cis*- and *trans*-18:1 isomers in total FAME as well as in 18:1 isomer fractions isolated by Ag-TLC were performed using a gas chromatograph CP 9000 (Chrompack) with a split injector, a flame-ionization detector, and a fused-silica capillary column (100 m × 0.25 mm i.d.) coated with 0.20 µm CP-Sil 88 (Chrompack) under the following conditions: $H_2$ as the carrier gas; injector temperature 255°C; and detector temperature 280°C. The unfractionated FAME were analyzed isothermally at 172°C with a column head pressure of 160 kPa (split ratio, 1:50). Monoenoic TLC fractions were analyzed isothermally at a lower temperature, 120°C, with a column head pressure of 220 kPa (split ratio, 1:25) (5). Under these conditions, elution of *cis*- as well as *trans*-18:1 isomers was achieved in approximately 3.5 h.

The calibration of positional isomers of *trans*-18:1 acids present in the *trans*-monoenoate fraction was achieved with the isomer *trans*-Δ11 18:1 that had been quantitated by stearic acid in the isothermal chromatography of the unfractionated FAME on the CP-Sil 88 column. Before, stearic acid was determined by total FAME analysis on the CP-Wax 58 CB column. Further, *cis*-18:1 isomers found in the *cis*-monoenoate fraction were calibrated by *cis* 9-18:1 acid that could be determined in the isothermal chromatogram of unfractionated FAME (5).

Identification of individual isomeric *trans* octadecenoates was achieved by comparison of retention times with FAME standards of the 18:1 isomers Δ6, Δ7, Δ9, Δ11, Δ12, Δ13, and Δ15 (Sigma, St. Louis, MO). *Trans*-18:1 acids isolated from butterfat were used as a secondary standard (6). Integration and quantitation were accomplished with an HP 3365 II ChemStation system (Hewlett-Packard, Palo Alto, PA). Calibration of GLC data included the conversion from FAME to free fatty acids. Thus, results expressing absolute concentrations are given as g/100 g of total fatty acids.

## RESULTS AND DISCUSSION

Quantitative data for the absolute amounts of *trans*- and *cis*-18:1 acid isomers are displayed in Table 1. Total *trans*-18:1 isomer content in hump fat (5.5%) is not significantly different from that found in beef tallow (range, 3.6–6.2%) (7). This also holds for milk fat (4.0%). It resembles that of cow milk fat during pasture feeding (4.3%) (1). The percentages of each individual isomer relative to their respective totals are described as bar graphs in Figure 1. There are no gross differences between either *cis*- or *trans*-18:1 isomers in milk and hump lipids, which would indicate that there are no discriminations in the deposition of 18:1 isomers between adipose tissue and milk fats.

**TABLE 1**
***Trans*- and *cis*-18:1 Isomers in the Hump and Milk Fat from the Dromedarius (g/100 g of total fatty acids)**

| Isomer[a] | Hump fat | Milk fat |
|---|---|---|
| *t*4 | 0.04 | 0.04 |
| *t*5 | 0.03 | 0.02 |
| *t*6-8 | 0.03 | 0.07 |
| *t*9 | 0.05 | 0.17 |
| *t*10 | 0.14 | 0.29 |
| *t*11 | 1.81 | 1.54 |
| *t*12 | 0.69 | 0.34 |
| *t*13 | 0.87 | 0.53 |
| *t*14 | 0.80 | 0.41 |
| *t*15 | 0.50 | 0.29 |
| *t*16 | 0.55 | 0.34 |
| Total | 5.51 | 4.04 |
| *c*6-8 | 0.06 | 0.07 |
| *c*9 | 16.49 | 22.57 |
| *c*11 | 1.43 | 1.40 |
| *c*12 | 0.17 | 0.37 |
| *c*13 | 0.08 | 0.10 |
| *c*14 | 0.03 | 0.08 |
| *c*15 | 0.13 | 0.18 |
| Total | 18.39 | 24.77 |

[a]*t, trans; c, cis.*

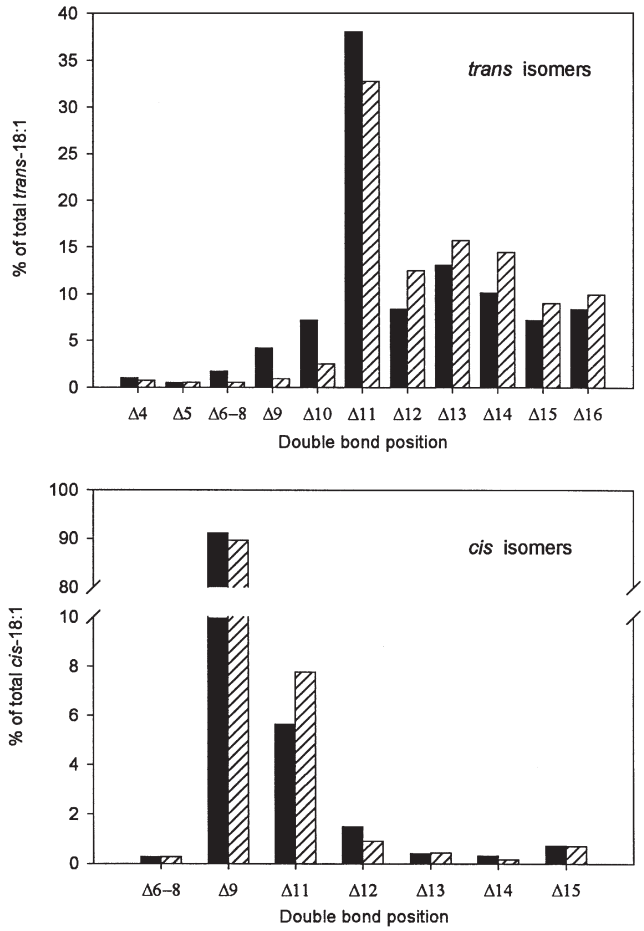


**FIG. 1.** Relative *trans*- and *cis*-18:1 isomeric profile derived from fat of dromedarius milk (black bars) and hump (hatched bars) fat.

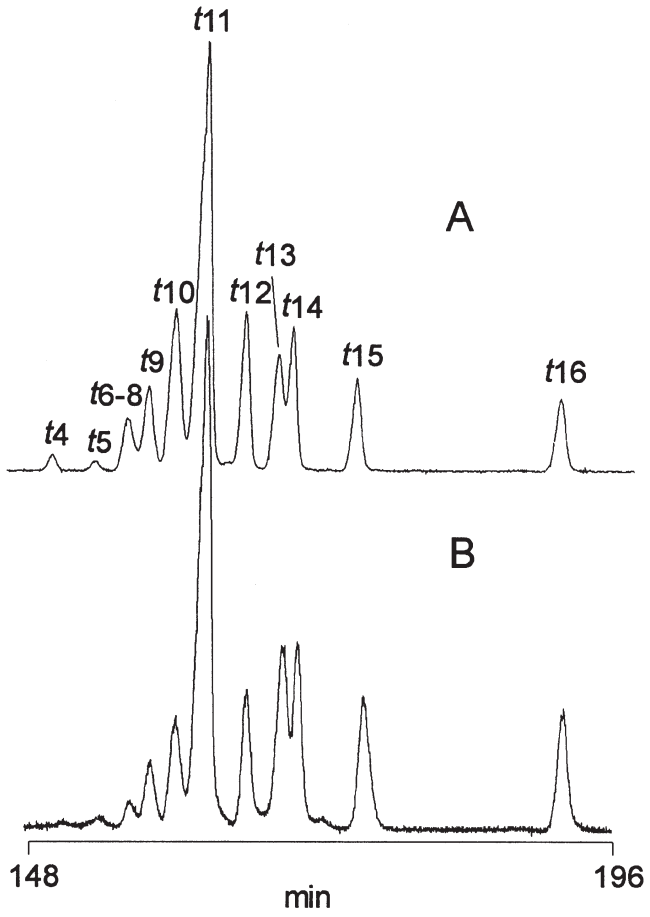


**FIG. 2.** Partial chromatograms of *trans*-18:1 acid methyl esters isolated by argentation thin-layer chromatography from dromedarius (A) and bovine (B) milk fat (see text for analytical conditions).

As found in beef tallow, meat, or milk; ewe milk or sheep meat; and goat milk (1,5), the ethylenic bonds in *trans*-octadecenoic isomers span from position Δ4 to Δ16 in dromedarius hump and milk fats. The major isomer is *trans*-11 18:1 (vaccenic) acid: 33% of total *trans*-18:1 isomers is in hump fat and 38% in milk fat. From comparison of data displayed in Table 1 with data for true ruminants (1,5), it is apparent that no gross differences exist between true ruminants and dromedarius fats, an observation that can be made by simple visual observation of the chromatograms in Figure 2. It is worthwhile to note that the *trans*-Δ16 isomer content, the latest-eluting component of *trans*-18:1 isomers, and possibly derived from dietary α-linolenic acid, is similar for the milk of dromedarius (8.4%) and other ruminants (7–10% of total *trans*-18:1 acids) (1). Also, it should be emphasized that the *trans*-18:1 acid isomer profile is also variable in bovine milk, though the general patterns remain generally comparable (1,5), provided no experimental dietary manipulations are made. The same observations can be made for *cis*-18:1 isomers.

Despite the fact that the dromedarius is not a true ruminant, biohydrogenation by its rumen microflora leads to the same *trans*- and *cis*-18:1 isomers in approximately the same proportions as in true ruminants (e.g., cattle). Thus, it can be deduced that the microflora has undergone little or no evolution during the past 50 million years, whereas the animals themselves differ considerably.

An important point is that *trans*-fatty acids are not limited to ruminants among mammals. They were shown a long time ago—in 1955—also to occur in the depot fat from marsupials, the wallaby (*Thylogale eugene*) and the quokka (*Setonix brachyurus*), a member of the kangaroo family (2), in exceptionally high amounts (approximately 20% of total fatty acids). The latter species at least would have a ruminant-like digestion, as its stomach contains a microbial flora more or less resembling that of ruminants. The *trans*-18:1 as well as the *cis*-18:1 isomers have been characterized and their profiles determined in subcutaneous fat of the monogastric xylophage rodent *Castor canadensis* (Canadian beaver) (8). They were shown to be rather similar to those of bovine milk fat (9), with vaccenic acid being the prominent isomer among *trans*-18:1 isomers. Other isomers included the Δ9 to Δ16 positional isomers.

Though the aim of the present study was not to assess the rumenic (*cis*-9,*trans*-11 18:2) acid content of hump and milk fats, the following values were determined: hump, 0.51%; and milk, 0.74%. The value for hump fat is slightly lower than

for edible beef tallow [0.60–1.67% (7)], whereas the value for milk fat lies well within the range determined for cow milk fat, 0.10–1.89% (10). Other data for ruminant milk fats are limited to ewe and goat milk fats: 0.77–1.04%, and 0.27–0.69%, respectively (11). The beaver would also contain rumenic acid in its subcutaneous fat (8), but there are no data available for marsupials.

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# Transforming Growth Factor Beta in Human Milk Does Not Change in Response to Modest Intakes of Docosahexaenoic Acid

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**ABSTRACT:** Long-chain polyunsaturated fatty acids have been associated with aspects of immune regulation including cytokine production. The purpose of this study was to investigate the effect of maternal dietary supplementation with tuna oil, rich in docosahexaenoic acid (DHA), on the concentration of transforming growth factor beta 1 (TGFβ1) and TGFβ2 in breast milk. In this randomized, dietary intervention trial, mothers of term infants consumed a daily supplement of 2000 mg oil containing either placebo ($n$ = 40), 300 mg DHA ($n$ = 40), or 600 mg DHA ($n$ = 40). The DHA increase in milk and plasma was proportional to dietary DHA. There was no relationship between milk DHA status and TGFβ1 and TGFβ2 levels.

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The cytokine family present in the largest amounts in human breast milk is transforming growth factor β (TGFβ), primarily represented by the isoforms TGFβ1 and TGFβ2 (1,2). The primary role of TGFβ in milk is thought to be immunosuppression, as TGFβ "knockout" mouse pups born to heterozygous mothers develop an excessive inflammatory response after weaning, leading to death at 3–5 wk of age (3,4). Other potentially relevant functions that may be of benefit to the breast-fed infant include stimulation of IgA isotype switching in B cells (5), maintenance of epithelial barrier function in the gut (6,7), and involvement in the induction of oral tolerance (8,9).

The inclusion of fish meals in the diet or the consumption of fish oil supplements, both rich in n-3 long-chain polyunsaturated fatty acids (LC-PUFA), leads to changes in tissue composition of n-6 and n-3 fatty acids, which in turn have been associated with immune regulation (10,11). There are conflicting data on the effect of changing the n-6/n-3 ratio on the production of TGFβ. Animal models of autoimmunity have shown that fish oil feeding is associated with significantly higher TGFβ mRNA expression and protein levels (12,13), whereas data obtained from experiments using the spontaneously hypertensive rat model describe a decrease in expression of TGFβ mRNA following supplementation with n-3 PUFA (14,15).

In this component of a larger randomized, dietary intervention trial (16), we examined the effect of supplementation with tuna oil, rich in the n-3 LCPUFA docosahexaenoic acid (DHA), on the concentration of TGFβ1 and TGFβ2 in milk from lactating women at 5 wk postpartum.

## SUBJECTS AND METHODS

One hundred twenty women, who had no history of inflammatory disorders, were not taking anti-inflammatory medication, and had delivered full-term singleton infants and intended to breast-feed, were enrolled in this double-blind, randomized, prospective study (16). The study was approved by the Flinders Clinical Research Ethics Committee, and written informed consent was obtained from all participants. Because primary outcome variables related to breast milk cytokines, women who ceased lactating by the time of sample collection were excluded. Mothers were randomly allocated to one of three groups assigned to take either (i) placebo ($n$ = 40), (ii) 300 mg DHA/d and 70 mg eicosapentaenoic acid (EPA)/d from tuna oil ($n$ = 40, LoDHA), or (iii) 600 mg DHA/d and 140 mg EPA/d from tuna oil ($n$ = 40, HiDHA). The placebo capsules (a highly monounsaturated sunflower seed oil) were identical in appearance to the tuna oil capsules and contained no DHA and no EPA. All capsules were provided by Clover Corporation Pty. Ltd. (Altona North, Victoria, Australia).

In the fourth week of the study mothers were asked to express a small sample (2 mL) of breast milk daily for 5 d. Samples were frozen and retained for fatty acid analysis as described elsewhere (16). At 5 wk postpartum (after 4 wk of dietary supplementation), a breast milk sample (30–50 mL) expressed on the morning of the appointment and peripheral blood (20 mL) were collected. The fresh 50-mL milk samples were centrifuged at 890 × $g$ for 30 min at 4°C, and the aqueous fraction was frozen at –80°C for later analysis of TGFβ1 and TGFβ2 using double antibody sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (1). The limit of detection for each ELISA was 16 pg/mL, the intra-assay precision was 4%, and the interassay precision was 17%. Samples were analyzed in duplicate, and all analy-

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; DHA, docosahexaenoic acid; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acid; TGFβ, transforming growth factor beta.

ses were completed within 3 mo of sample collection. Peripheral venous blood (1 mL) was placed into lithium heparin for separation of plasma and erythrocytes, and each component was retained for fatty acid analysis.

One-way analysis of variance (ANOVA) and *post-hoc* analysis by Newman-Keuls test were used to determine differences in plasma and breast milk fatty acids between diet groups. Because the distributions of the cytokine values were asymmetric, data were log-transformed, and significant differences between dietary groups were calculated by Kruskal-Wallis ANOVA. Data are expressed as medians (5th–95th percentiles) or as individual data points. Associations between fatty acid levels and cytokine concentration were tested using Spearman's rank correlation coefficient. All analyses were completed using SPSS for Windows 10.0 (SPSS Inc., Chicago, IL).

## RESULTS

Of those who were enrolled, 27/40 Placebo, 26/40 LoDHA, and 29/40 HiDHA were still breast-feeding their infants at the 5 wk appointment, and 93% of these mothers were providing >80% of feeds as breast-feeds. Peripheral blood (82/82) and breast milk (81/82 samples for fatty acid analysis and 80/82 fresh samples) were collected from lactating mothers on day 35 (33.6–36.1) (mean, 95% confidence interval). All 50-mL expressions were completed in the morning, and a majority of mothers expressed before a feed (67%) and from a single breast only (62%).

Compliance with the dietary intervention was confirmed by significant increases in n-3 PUFA (16). DHA increased in plasma and milk in a linear manner in response to dietary DHA whereas EPA concentration increased at the highest intake only (HiDHA, 600 mg DHA + 140 mg EPA/d). Tuna oil consumption caused a marginal decrease in plasma arachidonic acid (AA) levels, but changes were not significant in milk.

TGFβ1 and TGFβ2 were detected in all samples collected in the study. There was a wide range of concentrations in each dietary group with TGFβ2 present in two- to threefold higher amounts than TGFβ1 (Table 1). There was no difference in mean rank concentration between the dietary groups for either of the cytokines measured. In addition, there were no correlations between human milk DHA (Fig. 1), EPA, or n-3 PUFA and cytokine concentrations; however, there was a positive correlation between the two isoforms of TGFβ in the aqueous phase of human milk ($r = 0.51$, $P < 0.001$).

**TABLE 1**
**TGFβ1 and TGFβ2 in the Aqueous Phase of Human Milk Samples Collected After 4 wk of Dietary Supplementation[a]**

| | Placebo ($n = 26$) | LoDHA ($n = 25$) | HiDHA ($n = 29$) |
|---|---|---|---|
| TGFβ1 (pg/mL) | | | |
| Median | 638 | 555 | 692 |
| 5th–95th% | 301–3024 | 240–1301 | 248–1881 |
| TGFβ2 (pg/mL) | | | |
| Median | 808 | 1188 | 1045 |
| 5th–95th% | 147–11691 | 121–12424 | 388–7334 |

[a]LoDHA, 300 mg docosahexaenoic acid (DHA)/d and 70 mg eicosapentaenoic acid (EPA)/d from tuna oil; HiDHA, 600 mg DHA/d and 140 mg EPA/d from tuna oil. TGFβ, transforming growth factor beta.

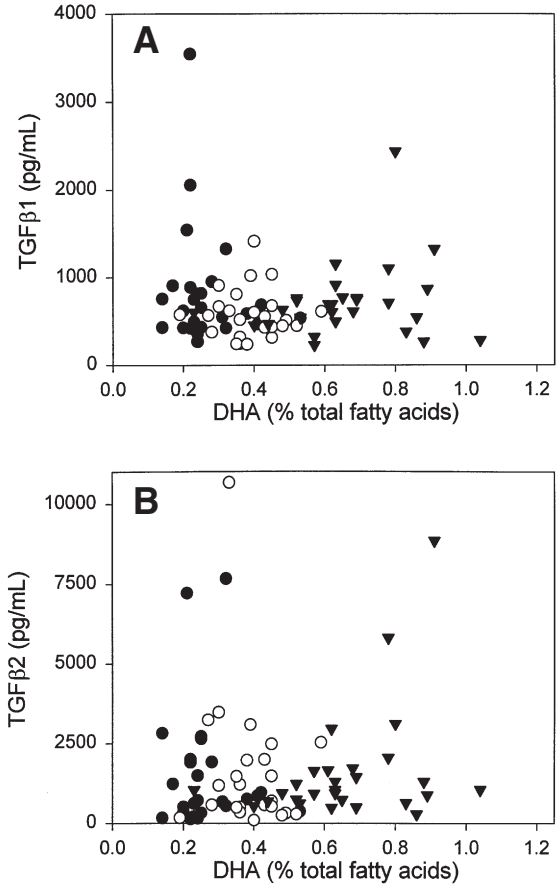


**FIG. 1.** Aqueous phase cytokines transforming growth factor beta 1 (TGFβ1) (A) and TGFβ2 (B) plotted as a function of human milk docosahexaenoic acid (DHA) after 4 wk of dietary supplementation. Placebo ●, $n = 26$; LoDHA ○, 300 mg DHA/d and 70 mg eicosapentaenoic acid (EPA)/d from tuna oil, $n = 25$; HiDHA ▼, 600 mg DHA/d and 140 mg EPA/d from tuna oil, $n = 28$.

## DISCUSSION

Very few human studies have reported the effects of dietary LCPUFA on TGFβ production, a known immunomodulatory cytokine present in high concentrations in human milk throughout lactation. Consumption of n-3 PUFA results in decreased AA levels in cell membranes and increased levels of EPA and DHA, fatty acids that competitively inhibit the conversion of AA to its metabolites, such as prostaglandin $E_2$ and leukotriene $B_4$ (10,17). One mechanism by which n-3 fatty acids may act is suggested by studies that have shown that different chemical inhibitors of AA metabolism can potentiate the effects of TGFβ1 on cellular differentiation and proliferation (18,19). Thus it is possible that a lack of some AA-derived eicosanoids modulates TGFβ effects.

In this study, we could not detect a difference in human milk TGFβ concentration at 5 wk postpartum despite increasing

milk and plasma DHA and, to a lesser extent, EPA levels following dietary supplementation with tuna oil. At this level of supplementation, there was no change in the concentration of AA in human milk. Tuna oil is rich in DHA (~30%) but contains little EPA (~6%) and it may be the latter fatty acid that plays a key role in the regulation of inflammatory mediators such as TGFβ. The wide range of concentrations of both isotypes of TGFβ detected in the milk samples collected in this study is comparable to that described in our previous study (1). Given this, and the relatively low doses of DHA and EPA in the supplement, it is not surprising that we were unable to detect any effects of diet on the cytokines in question, should they exist.

A number of commercial nutritional supplements are designed to meet the needs of pregnant and lactating women for additional n-3 PUFA as recommended by at least one nutrition committee (20). The appearance of specific supplements such as these and increasing community awareness of the benefits of including fish in the diet will result in lactating mothers choosing to increase their n-3 PUFA intake. The levels of human milk DHA described in this study cover the range expected in mothers following healthy diets that include fish. The results of this study suggest that the consumption of moderate doses of DHA (equivalent to consuming 4–5 meals of salmon per week), although increasing DHA, does not appear to cause changes in the level of TGFβ1 and TGFβ2 in human milk.

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# Influence of Dietary Supplementation with Long-Chain n-3 or n-6 Polyunsaturated Fatty Acids on Blood Inflammatory Cell Populations and Functions and on Plasma Soluble Adhesion Molecules in Healthy Adults

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**ABSTRACT:** Greatly increasing the amounts of flaxseed oil [rich in α-linolenic acid (ALNA)] or fish oil (FO); [rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] in the diet can decrease inflammatory cell functions and so might impair host defense. The objective of this study was to determine the effect of dietary supplementation with moderate levels of ALNA, γ-linolenic acid (GLA), arachidonic acid (ARA), DHA, or FO on inflammatory cell numbers and functions and on circulating levels of soluble adhesion molecules. Healthy subjects aged 55 to 75 yr consumed nine capsules per day for 12 wk. The capsules contained placebo oil (an 80:20 mix of palm and sunflowerseed oils) or blends of placebo oil with oils rich in ALNA, GLA, ARA, or DHA or FO. Subjects in these groups consumed 2 g ALNA; approximately 700 mg GLA, ARA, or DHA; or 1 g EPA plus DHA (720 mg EPA + 280 mg DHA) daily from the capsules. Total fat intake from the capsules was 4 g per day. None of the treatments affected inflammatory cell numbers in the bloodstream; neutrophil and monocyte phagocytosis or respiratory burst in response to *E. coli*; production of tumor necrosis factor-α, interleukin-1β, and interleukin-6 in response to bacterial lipopolysaccharide; or plasma concentrations of soluble intercellular adhesion molecule-1. In contrast, the ALNA and FO treatments decreased the plasma concentrations of soluble vascular cell adhesion molecule-1 (16 and 28% decrease, respectively) and soluble E-selectin (23 and 17% decrease, respectively). It is concluded that, in contrast to previous reports using higher amounts of these fatty acids, a moderate increase in consumption of long-chain n-6 or n-3 polyunsaturated fatty acids does not significantly affect inflammatory cell numbers or neutrophil and monocyte responses in humans and so would not be expected to cause immune impairment. Furthermore, we conclude that moderate levels of ALNA and FO, which could be incorporated into the diet, can decrease some markers of endothelial activation and that this mechanism of action may contribute to the reported health benefits of n-3 fatty acids.

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Inflammatory cells such as neutrophils, monocytes, and macrophages form part of the innate immune response that is responsible for the early host response to invading bacteria (1). Bacterial cell wall components such as lipopolysaccharide (LPS) stimulate the production of cytokines by monocytes and macrophages. These cytokines include tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6, which act to induce upregulation of adhesion molecules on vascular endothelial cells, so facilitating the adhesion of neutrophils and monocytes at the sites of infection, which is followed by migration, local accumulation, and activation of inflammatory cells (1). The inflammatory cells recognize bacteria in a nonspecific way and act to destroy them by phagocytosis and/or by production of superoxide and related reactive oxygen species (1). This production of reactive oxygen species is referred to as respiratory burst. The inflammatory cytokines also provide one link between inflammatory cells and specific immunity because they can stimulate T and B lymphocytes (1). Among the adhesion molecules upregulated on the endothelial cell surface in response to cytokines and other inflammatory stimuli, such as LPS and reactive oxygen species, are vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and intercellular adhesion molecule-1 (ICAM-1), although the latter is also expressed on monocytes, macrophages, and lymphocytes (2). These adhesion molecules can be shed from the cell surface and appear in the circulation as soluble forms (sVCAM-1, sE-selection, and sICAM-1, respectively) (2). The plasma concentrations of these molecules are elevated during inflammatory conditions (2), increase with age (3–5), are higher in individuals with cardiovascular disease (3,5,6), and are predictive of future myocardial infarction (7).

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Abbreviations: ALNA, α-linolenic acid; ANOVA, analysis of variance; ARA, arachidonic acid; DGLA, di-homo-γ-linolenic acid; DHA, docosahexaenoic acid; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; FO, fish oil; GLA, γ-linolenic acid; ICAM, intercellular adhesion molecule; IL, interleukin; LPS, lipopolysaccharide; MFI, median fluorescence intensity; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid; RPMI, Roswell Park Memorial Institute; s, soluble; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

There is now much evidence that the activity of inflammatory cells and the expression of adhesion molecules can be influenced by the availability of various fatty acids; differential effects of n-6 and n-3 polyunsaturated fatty acids (PUFA) have been identified in cell culture studies. Linoleic acid (18:2n-6) and arachidonic acid (ARA; 20:4n-6) enhanced superoxide release from neutrophils and macrophages (8–11), whereas eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) either inhibited superoxide production (12) or were less potent stimulators than ARA (11). ARA increased IL-1β production by a monocytic cell line (13) and by human monocytes (14). In contrast, EPA and DHA inhibited production of IL-1β and of TNF-α by human monocytes (14,15). EPA and DHA diminished expression of some adhesion molecules on the surface of cultured human endothelial cells (16–19), monocytes (20), and lymphocytes (17). The EPA- and DHA-induced decrease in adhesion molecule expression was accompanied by decreased binding of lymphocytes and monocytes to endothelial cells (17,18). In contrast, several n-6 PUFA [linoleic acid, γ-linolenic acid (GLA; 18:3n-6), di-homo-γ-linolenic acid (DGLA; 20:3n-6), and ARA] promoted neutrophil adhesion to endothelial cells (21). These studies suggest that ARA, and perhaps other n-6 PUFA, enhance inflammatory cell responses whereas the n-3 PUFA, EPA and DHA, diminish these responses. Animal feeding studies support the idea that n-3 PUFA diminish inflammatory cell responses. Feeding fish oil (FO), which contains EPA and DHA, to laboratory animals decreased superoxide production by neutrophils (22,23) and macrophages (24–26) and decreased hydrogen peroxide production by macrophages (25,27). FO feeding also decreased *ex vivo* production of TNF-α, IL-1β, and IL-6 by LPS-stimulated rodent macrophages (28–31) and monocytes (32).

A number of studies have investigated the influence of supplementing the diet of healthy human volunteers with encapsulated FO on inflammatory cell functions. FO decreased superoxide production by human neutrophils (33–35) and monocytes (36), and decreased neutrophil binding to endothelial cells (37). These studies provided between 3.1 and 9.6 g EPA plus DHA per day and were of 3 to 6 wk in duration. One study reported that increasing the amount of α-linolenic acid (ALNA; 18:3n-3) in the human diet to about 14 g per day for 4 wk resulted in a significant decrease in TNF-α and IL-1β production by LPS-stimulated mononuclear cells (38). Supplementation of the diet of healthy humans with FO providing between 2.4 and 5 g EPA plus DHA per day for 4 to 24 wk has been reported to decrease the production of TNF-α, IL-1β, and IL-6 by LPS-stimulated mononuclear cells (38–41).

Thus, there is a wealth of data from *in vitro*, animal feeding, and human supplementation studies that suggests that n-3 PUFA diminish inflammatory cell functions. There is also a limited amount of data, mainly from *in vitro* studies, that suggests that n-6 PUFA enhance inflammatory cell functions. Most often the idea that n-3 PUFA diminish inflammatory cell functions is interpreted in a favorable way, with the conclusion that n-3 PUFA are anti-inflammatory and so will be beneficial to health. However, because inflammatory cells are key components of the early host response to invading pathogens and because they produce mediators that promote the specific immune response, a reduction in inflammatory cell functions could compromise host defense. Indeed, compared with ARA, EPA and DHA decreased the ability of cultured murine macrophages to phagocytose (42), whereas dietary FO diminished rat macrophage phagocytosis (25).

There are recommendations to increase the intake of n-3 PUFA in adults because of their beneficial health effects, especially with respect to cardiovascular disease (43–45). However, the studies described above suggest that potentially detrimental immunological effects can occur at high n-3 PUFA intakes. Infants require ARA and DHA for optimal mental and visual development (46), and a case has been made to include these fatty acids in infant formulae. However, the immunological impact of these fatty acids in humans is not clear. Kelley *et al.* (47,48) reported that 6 g DHA per day for 12 wk decreased circulating granulocyte (and so total leukocyte) numbers and decreased the production of TNF-α and IL-1β by LPS-stimulated mononuclear cells from healthy adults, suggesting that a high level of this fatty acid diminishes some inflammatory cell numbers and responses. The same workers earlier demonstrated that 1.5 g ARA per day increased circulating granulocyte numbers (49).

As indicated previously, many of the studies in humans have been perfomed using large doses of n-3 PUFA (14 g ALNA or >2.4 g EPA plus DHA per day). The habitual intake of ALNA among adults in the United Kingdom is 1 to 2 g per day, whereas that of EPA plus DHA is <150 mg per day (44). Thus, the amounts of these fatty acids provided in the supplementation studies performed to date are greatly in excess of habitual intakes and greatly in excess of intakes that are recommended or that could be achieved in most individuals through dietary change. It is not clear what influence lower levels of these or other polyunsaturated fatty acids have on human inflammatory cell functions. Therefore, the current study investigated the effect of moderate supplementation of the diet of healthy subjects with encapsulated oil blends rich in ALNA, GLA, ARA, DHA, or EPA on circulating leukocyte numbers, *ex vivo* neutrophil and monocyte phagocytosis and respiratory burst, *ex vivo* production of inflammatory cytokines, and circulating levels of soluble adhesion molecules as an *in vivo* indicator of endothelial inflammation. The results presented here form part of a larger study, from which some findings have already been published (50,51).

## MATERIALS AND METHODS

*Materials.* Phosphate-buffered saline (PBS) tablets were obtained from Unipath Ltd. (Basingstoke, United Kingdom). Histopaque, bovine serum albumin (fatty acid-free), HEPES-buffered Roswell Park Memorial Institute (RPMI) medium, glutamine, antibiotics (penicillin and streptomycin), *Escherichia coli* 0111:B4 LPS, and propidium iodide were purchased

**TABLE 1**
**Characteristics of the Treatment Groups**

| Treatment[b] | Number of subjects | Males/females | No. of smokers | Age[a] (yr) | Body mass index[a] (kg/m$^2$) |
|---|---|---|---|---|---|
| Placebo | 8 | 5:3 | 1 | 62 (56–69) | 25.1 (21.3–29.7) |
| ALNA | 8 | 4:4 | 0 | 66 (60–74) | 25.5 (22.2–28.0) |
| GLA | 7 | 3:4 | 0 | 64 (55–71) | 23.3 (18.6–27.1) |
| ARA | 8 | 4:4 | 0 | 61 (56–70) | 24.1 (21.3–27.3) |
| DHA | 8 | 5:3 | 0 | 65 (58–71) | 23.5 (19.6–28.4) |
| FO | 7[c] | 3:4 | 0 | 62 (60–68) | 26.7 (22.8–31.1) |

[a]Values for age and body mass index are mean values with ranges shown in parentheses.
[b]Abbreviations: ALNA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; FO, fish oil; GLA, γ-linolenic acid.
[c]Excludes values for one female subject who dropped out during the study.

from Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom). Fluorescein isothiocyanate (FITC)-labeled mouse antihuman CD45 (clone Immu19.2) in combination with phycoerythrin-labeled mouse antihuman CD14 (clone RMO52) was purchased from Coulter Corp. (Hialeah, FL). Fluorescence-activated cell sorter (FACS)–lysing solution was purchased from Becton Dickinson (Mountain View, CA). Kits for measurement of phagocytosis and respiratory burst in whole blood (PHAGOTEST® and BURSTTEST®, respectively) were purchased from Orpegen Pharma (Heidelberg, Germany). EASIA™ cytokine enzyme-linked immunosorbent assay (ELISA) kits and Cytoscreen™ sICAM-1 and sVCAM-1 ELISA kits were obtained from BioSource Europe SA (Nivelles, Belgium). Quantikine™ sE-selectin ELISA kits were obtained from R & D Systems Europe Ltd. (Abingdon, Oxon., United Kingdom).

*Subjects and study design.* Ethical permission for all procedures involving human volunteers was obtained from the Central Oxford Research Ethics Committee (COREC No. 96.182). All volunteers completed a health and lifestyle questionnaire prior to entering the study, and physician's consent for inclusion in the study was obtained. Volunteers were excluded if they were taking any prescribed medication; had diagnosed hypercholesterolemia, hypertriglyceridemia, coronary heart disease, diabetes, or a chronic inflammatory disease; took aspirin regularly; were vegetarian; consumed FO, evening primrose oil, or vitamin capsules; were blood donors; had undergone recent weight loss; or smoked more than 10 cigarettes per day. The characteristics of the 46 subjects who completed the study are given in Table 1; mean age and body mass index did not differ among the treatment groups. All subjects were Caucasian and free-living; all lived in their own homes and none was disabled or immobile in any way. All female subjects were postmenopausal and none was taking hormone-replacement therapy. None of the subjects participated in intense or vigorous exercise. Subject height was measured to the nearest millimeter and weight to the nearest kilogram.

Forty-eight subjects were randomly allocated to receive one of the six types of encapsulated oil blends in a double-blind fashion ($n$ = 8 per treatment group); these blends are referred to as placebo, ALNA, GLA, ARA, DHA, and FO. The placebo was an 80:20 mixture of palm and sunflowerseed oils (both supplied by Loders Croklaan, Wormerveer, The Netherlands). The fatty acid composition of this blend closely resembles the fatty acid composition of the average British diet (43). The ALNA blend was a 50:13:37 mixture of palm, sunflowerseed, and super refined flaxseed oils (flaxseed oil supplied by Loders Croklaan). The GLA blend was a 21:5:74 mixture of palm oil, sunflower seed oil, and a GLA-rich triacylglycerol (supplied by Scotia Pharmaceuticals, Stirling, United Kingdom). The ARA and DHA blends were 43:11:46 mixtures of palm oil, sunflower seed oil, and ARASCO® or DHASCO®, respectively (ARASCO® and DHASCO® supplied by Martek Corp., Columbia, MD). The FO used was a Chilean FO (supplied by Loders Croklaan) containing 26 g EPA plus DHA per 100 g total fatty acids. The fatty acid composition of the oil blends (determined by gas chromatography on total lipid extracts) is shown in Table 2. The capsules were gelatin-coated, nontransparent, and green colored. Each capsule contained 445 mg of the oil blend, and subjects con-

**TABLE 2**
**Fatty Acid Composition of Oil Blends Used in the Study**

| | Fatty acid[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | Myristic | Palmitic | Palmitoleic | Stearic | Oleic | Linoleic | GLA | ALNA | ARA | EPA | DHA |
| Placebo | 0 | 37.4 | 0 | 4.0 | 36.0 | 22.7 | 0 | 0.2 | 0 | 0 | 0 |
| ALNA | 0 | 9.9 | 0 | 2.8 | 18.0 | 15.8 | 0 | 53.5 | 0 | 0 | 0 |
| GLA | 0 | 9.6 | 0 | 1.1 | 10.4 | 58.6 | 20.3 | 0.3 | 0 | 0 | 0 |
| ARA | 0 | 25.1 | 0 | 4.9 | 34.2 | 16.3 | 1.5 | 0.2 | 17.9 | 0 | 0 |
| DHA | 8.0 | 24.5 | 1.1 | 2.4 | 29.8 | 13.4 | 0 | 0.3 | 0 | 0 | 19.1 |
| FO | 10.7 | 19.1 | 9.8 | 3.2 | 11.9 | 1.2 | 0.5 | 0.9 | 1.4 | 18.8 | 7.4 |

[a]g/100 g total fatty acids; EPA, eicosapentaenoic acid. For other abbreviations see Table 1.

sumed nine capsules per day (i.e., 4 g encapsulated oil per day) for 12 wk. Therefore, subjects in the ALNA group consumed an extra 2.0 g ALNA per day; subjects in the GLA, ARA, and DHA groups consumed an extra 700 mg (approximately) GLA, ARA, or DHA per day, respectively; and subjects in the FO group consumed an extra 1 g EPA plus DHA per day (720 mg EPA + 280 mg DHA). All capsules contained 300 μg α-tocopherol-equivalents plus 180 μg ascorbyl-palmitate per gram of oil. Thus, all subjects consumed an extra 1.2 mg α-tocopherol per day.

The capsules were provided to the subjects in blister packs (nine capsules per pack) with seven blister packs per box along with clear instructions of how they should be administered (three capsules three times daily immediately prior to breakfast, lunch, and dinner); during the supplementation period subjects received fresh blister packs of capsules every 4 wk.

Two female subjects dropped out of the study once it was underway, one from the GLA group, due to illness, and one from the FO group, due to inability to comply because of stomach upsets.

A supplementation period of 12 wk was selected because almost all previous studies that reported effects of dietary PUFA on human inflammatory cell numbers or functions were of a duration of 3 to 12 wk (33–41,47–49). Blood was sampled immediately prior to beginning supplementation, every 4 wk during supplementation, and after a 4-wk washout period. Throughout this manuscript week 0 represents the baseline measurements, weeks 4, 8, and 12 represent the supplementation period, and week 16 represents the end of the washout period. Blood samples were collected into heparinized vacutainer tubes between 0800 and 1000 after a fast of at least 12 h. All treatment groups completed the study in parallel. The study ran from June 1997 (early summer) to December 1997 (early winter).

Compliance was assessed biochemically by determining the fatty acid composition of plasma and mononuclear cell phospholipids (see 50,51).

*Analysis of leukocyte numbers and subpopulations.* Leukocyte numbers and subsets were analyzed in the blood samples collected at weeks 0 (baseline), 12 (end of supplementation), and 16 (washout).

To determine total leukocyte numbers, whole blood (40 μL) was incubated with 2 mL Becton Dickinson FACS-lysing solution for 30 min to lyse the erythrocytes and fix the leukocytes. The leukocytes were then stained with propidium iodide (10 μL of a 1 mg/mL solution) and counted in a Coulter XL/MCL flow cytometer (Coulter Corp., Hialeah, FL) using a 60-μL volume stop setting.

For the determination of leukocyte subsets, whole blood (100 μL) was incubated with a combination of fluorescently labeled monoclonal antibodies to CD45 and CD14 (20 μL) for 40 min at 12°C. Erythrocytes were then lysed and leukocytes fixed with 3 mL Becton Dickinson FACS-lysing solution for 10 min. Leukocytes were collected by centrifugation ($250 \times g$, 5 min), resuspended in 3 mL PBS, and then centrifuged again. Finally, they were resuspended in 1 mL PBS and analyzed in a Coulter XL/MCL flow cytometer (Coulter Corp.). Fluorescence data were collected on $10^4$ cells and were analyzed using System II software. Neutrophils, eosinophils, and basophils were distinguished by side scatter properties after staining with anti-CD45; lymphocytes were distinguished as $CD45^+CD14^-$ cells; and monocytes were distinguished by side scatter properties after staining with anti-CD14.

*Measurement of leukocyte phagocytic activity.* Phagocytosis of neutrophils and monocytes was determined using the blood samples collected at weeks 0 (baseline), 12 (end of supplementation), and 16 (washout); PHAGOTEST® kits were used. A sample of whole blood was cooled on ice for 10 min. Prior to use in the PHAGOTEST® assay the cooled blood was vortexed. Aliquots (100 μL) were then incubated on ice (control) or in a preheated water bath at 37°C for 10 min with opsonized FITC-labeled *E. coli* (20 μL). The reaction was then stopped by adding ice-cold quenching solution (100 μL). The erythrocytes were then lysed, the leukocytes fixed, and the DNA stained according to the manufacturer's instructions. Cell preparations were then analyzed by flow cytometry in a Coulter XL/MCL flow cytometer (Coulter Corp.). Fluorescence data were collected on $10^4$ cells and were analyzed using System II software. Neutrophils and monocytes were identified by forward and side scatter. Both the percentage of neutrophils or monocytes having taken up *E. coli* (% positive; determined by subtracting the % positive cells in the control tube incubated on ice from the % positive cells in the tube incubated at 37°C) and the median fluorescence intensity (MFI) (a measure of extent of phagocytosis per leukocyte) were determined.

*Measurement of leukocyte respiratory burst.* Respiratory burst of neutrophils and monocytes was determined using the blood samples collected at weeks 0 (baseline), 12 (end of supplementation), and 16 (washout); BURSTTEST® kits were used. A sample of whole blood was cooled on ice for 10 min. Prior to use in the BURSTTEST® assay, the blood was vortexed. Aliquots (100 μL) were then incubated in a preheated water bath at 37°C for 10 min with opsonized *E. coli* or washing solution as control (20 μL in each case). The samples were then incubated for a further 10 min at 37°C with a solution (20 μL) containing the fluorogenic substrate dihydrorhodamine 123. The erythrocytes were then lysed, the leukocytes fixed, and the DNA stained according to the manufacturer's instructions. Cell preparations were then analyzed by flow cytometry in a Coulter XL/MCL flow cytometer (Coulter Corp.). Fluorescence data were collected on $10^4$ cells and were analyzed using System II software. Neutrophils and monocytes were identified by forward and side scatter. The concentration of *E. coli* used resulted in 100% of neutrophils and >85% of monocytes in all samples undergoing respiratory burst. Therefore, only the MFI values (a measure of respiratory burst activity per leukocyte) are reported here.

*Preparation of peripheral blood mononuclear cell (PBMC).* Blood was diluted 1:1 with PBS, layered onto Histopaque (density 1.077 g/L; ratio of diluted blood to Histopaque, 4:3), and centrifuged for 15 min at $800 \times g$ at 20°C. The PBMC were collected from the interphase, washed

once with PBS, resuspended in 2.5 mL PBS, and layered onto 5 mL Histopaque. They were centrifuged once more to achieve a lower degree of erythrocyte contamination, washed with PBS, and finally resuspended.

*Measurement of the production of proinflammatory cytokines by PBMC cultures.* Proinflammatory cytokine production by LPS-stimulated PBMC cultures was determined at all time points (i.e., weeks 0, 4, 8, 12, and 16). PBMC ($2 \times 10^6$) were cultured for 24 h in HEPES-buffered RPMI medium, supplemented with 2 mmol/L glutamine, 2.5% (vol/vol) autologous plasma, antibiotics, and 15 μg/mL LPS; the final culture volume was 2 mL. At the end of the incubation, the plates were centrifuged and the culture medium collected and frozen in aliquots. The concentrations of TNF-α, IL-1β, and IL-6 were measured by specific EASIA™ ELISA. Limits of detection for these assays were 3 pg/mL (TNF-α), 2 pg/mL (IL-1β), and 2 pg/mL (IL-6) (data supplied by the manufacturer of the kits). The inter- and intra-assay coefficients of variation were <10% for all cytokine ELISA.

*Measurement of plasma soluble adhesion molecule concentrations.* Plasma sICAM-1, sVCAM-1, and sE-selectin concentrations were measured only in plasma collected at weeks 0 (baseline) and 12 (end of supplementation). Soluble adhesion molecule concentrations were measured using commercially available ELISA kits. Limits of detection were 0.04 ng/mL (sICAM-1), 0.5 ng/mL (sVCAM-1), and 0.1 ng/mL (sE-selectin) (data supplied by the manufacturers of the kits). Inter- and intra-assay coefficients of variation were both <5% for sICAM and sVCAM-1 assay kits; the interassay coefficient of variation for the sE-selectin assay kit was <10%, whereas the intra-assay coefficient of variation was <5%.

*Statistical analysis.* Sample size (i.e., number of subjects per treatment group) was calculated on the basis of measurements made previously in our laboratory using the same methods as those employed in this study and of existing data from the literature. It was determined that a sample size of 8 would detect a difference in cytokine production of ≥25% at $P \leq 0.05$ with 80% power.

Unless otherwise indicated, results are expressed as mean ± standard error of the mean for seven or eight subjects per treatment group. Statistical significance of treatment, time, and the interaction of treatment and time were determined using two-factor repeat-measures analysis of variance (ANOVA). Where the interaction between treatment and time was significant, differences among treatment groups at a given time were examined by one-factor ANOVA using Bonferroni's correction for multiple comparisons, and differences within a treatment group between time points were examined by one-factor ANOVA (if data for more than two time points were available) or by paired Student's *t*-test (if data for only two time points were available). The effects of treatment on absolute and percentage changes during treatment (i.e., week 12 minus week 0) were analyzed by one-factor ANOVA using Bonferroni's correction for multiple comparisons. All statistical tests were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL), and a value of $P < 0.05$ was taken to indicate statistical significance.

## RESULTS

*Fatty acid compositions of plasma and PBMC phospholipids.* The fatty acid compositions of plasma and PBMC phospholipids from this study have been published elsewhere (50,51), and will be summarized here only briefly. The fatty acid compositions of plasma and PBMC phospholipids were not affected by the placebo or ALNA treatments. However, supplementation of the diet with GLA resulted in an increase in the proportion of DGLA (63% increase in plasma phospholipids and 45% increase in PBMC phospholipids at week 12). Supplementation with ARA resulted in an 85% increase in the proportion of ARA in plasma phospholipids and a 15% increase in the proportion of ARA in PBMC phospholipids. Supplementation with DHA resulted in an increase in the proportion of DHA in plasma and PBMC phospholipids (91 and 80% increases at week 12, respectively). Finally, supplementation with FO caused increases in the proportions of EPA (230% increase at week 12) and DHA (33% increase at week 12) in plasma phospholipids. Likewise, FO supplementation increased the proportions of EPA and DHA in PBMC phospholipids (220 and 16% increases, respectively).

*Effect of treatments on leukocyte numbers and subsets.* The number of total leukocytes and of neutrophils, eosinophils, basophils, lymphocytes, and monocytes did not differ among the treatment groups at baseline (Table 3). There were no significant effects of treatment or time on the numbers of eosinophils or lymphocytes (Table 3). There was a significant effect of treatment, but not time, on the numbers of total leukocytes and monocytes ($P$ for effect of treatment = 0.001 and 0.007, respectively), but there were no treatment × time interactions (Table 3). There were significant effects of both treatment and time on the number of neutrophils ($P = 0.001$ and 0.042, respectively) but there was no treatment × time interaction (Table 3). There were no differences among the treatment groups with respect to the absolute changes (i.e., week 12 − week 0) in circulating cell numbers (Table 3).

*Effect of treatments on phagocytosis.* The percentage of neutrophils and monocytes carrying out phagocytosis of *E. coli* (approximately 80% of neutrophils and approximately 70% of monocytes) and the activity of those cells (MFI) did not differ among the treatment groups at baseline or at the end of supplementation (data not shown). Likewise, there were no differences among the treatment groups with respect to the absolute changes (i.e., week 12 − week 0) in neutrophil and monocyte phagocytosis (data not shown).

*Effect of treatments on respiratory burst.* The respiratory burst of neutrophils and monocytes in response to *E. coli* (expressed as MFI) did not differ among the treatment groups at baseline or at the end of supplementation (data not shown). There were no differences among the treatment groups with respect to the absolute changes (i.e., week 12 − week 0) in respiratory burst (data not shown).

*Effect of treatments on proinflammatory cytokine production.* Production of TNF-α, IL-1β, and IL-6 by PBMC stimulated with 15 μg/mL LPS did not differ among the treatment

**TABLE 3**
**Circulating Leukocyte Populations in the Different Treatment Groups[a]**

| Treatment | | Leukocytes | Neutrophils[b] | Eosinophils[b] | Basophils[b] | Lymphocytes[c] | Monocytes[d] |
|---|---|---|---|---|---|---|---|
| Placebo | Week 0 | 5225 ± 457 | 2754 ± 283 | 179 ± 86 | 66 ± 13 | 1664 ± 235 | 388 ± 35 |
| | Week 12 | 4763 ± 439 | 2698 ± 309 | 189 ± 40 | 38 ± 5 | 1393 ± 195 | 327 ± 26 |
| | Week 12 – week 0 | –462 ± 236 | –56 ± 201 | 10 ± 21 | –28 ± 14 | –289 ± 82 | –60 ± 12 |
| ALNA | Week 0 | 4614 ± 491 | 2517 ± 361 | 151 ± 24 | 57 ± 10 | 1434 ± 133 | 354 ± 58 |
| | Week 12 | 4100 ± 464 | 2218 ± 266 | 159 ± 39 | 35 ± 4 | 1291 ± 231 | 317 ± 35 |
| | Week 12 – week 0 | –514 ± 605 | –30 ± 328 | 9 ± 29 | –22 ± 12 | –145 ± 210 | –37 ± 57 |
| GLA | Week 0 | 4271 ± 507 | 2287 ± 302 | 169 ± 35 | 63 ± 12 | 1372 ± 183 | 271 ± 27 |
| | Week 12 | 4171 ± 509 | 2391 ± 324 | 155 ± 30 | 39 ± 7 | 1181 ± 151 | 278 ± 35 |
| | Week 12 – week 0 | –100 ± 611 | 104 ± 372 | –14 ± 25 | –24 ± 14 | –191 ± 200 | 7 ± 37 |
| ARA | Week 0 | 3329 ± 233 | 1704 ± 129 | 109 ± 18 | 31 ± 6 | 1130 ± 158 | 269 ± 24 |
| | Week 12 | 3471 ± 210 | 1838 ± 167 | 137 ± 28 | 21 ± 3 | 1111 ± 128 | 283 ± 23 |
| | Week 12 – week 0 | 143 ± 156 | 133 ± 113 | 28 ± 16 | –10 ± 6 | –19 ± 69 | 14 ± 17 |
| DHA | Week 0 | 3943 ± 329 | 1996 ± 120 | 108 ± 14 | 51 ± 8 | 1375 ± 233 | 304 ± 30 |
| | Week 12 | 4229 ± 410 | 2256 ± 268 | 151 ± 30 | 41 ± 8 | 1350 ± 235 | 331 ± 48 |
| | Week 12 – week 0 | 285 ± 393 | 260 ± 295 | 43 ± 25 | –10 ± 6 | –36 ± 62 | 27 ± 52 |
| FO | Week 0 | 4260 ± 360 | 2370 ± 244 | 128 ± 40 | 56 ± 14 | 1236 ± 90 | 349 ± 55 |
| | Week 12 | 4300 ± 685 | 2298 ± 587 | 151 ± 29 | 33 ± 6 | 1398 ± 101 | 306 ± 14 |
| | Week 12 – week 0 | 40 ± 868 | –72 ± 628 | 23 ± 34 | –24 ± 15 | 162 ± 187 | –44 ± 53 |

[a]Data are mean ± SEM number of cells/μL blood, $n = 7$ or 8 per treatment group. For abbreviations see Table 1.
[b]Determined from side-scatter properties after staining with anti-CD45.
[c]Calculated from percentage of total leukocytes staining $CD45^+CD14^-$.
[d]Determined from side-scatter properties after staining with anti-CD14.

groups at baseline (Table 4). There were no significant effects of treatment or of time on production of TNF-α or IL-6. There was a significant effect of time on the production of IL-1β ($P = 0.020$), but there was no effect of treatment or a significant treatment × time interaction. The absolute changes in production of these cytokines (i.e., week 12 – week 0) did not differ among the treatment groups (Table 4), and there were no differences in cytokine production among the treatment groups at the end of supplementation (Table 4).

*Effect of treatments on soluble adhesion molecule concentrations.* Plasma sICAM-1, sVCAM-1, and sE-selectin concentrations did not differ among the treatment groups at baseline (Table 5). There were no significant effects of treatment or of time on the concentrations of sICAM-1 or sE-selectin (Table 5). However, there were significant effects of time ($P = 0.038$) and treatment ($P = 0.027$) and a significant treatment × time interaction ($P = 0.014$) for sVCAM-1 concentration, and so these data were analyzed further. The concentration of sVCAM-1 was significantly lower after FO supplementation than at week 0 ($P = 0.034$). Placebo, GLA, ARA, or DHA supplementation did not affect sVCAM-1 concentration.

The absolute changes in sVCAM-1 concentration in the ALNA and FO groups were significantly different from the change in the placebo group ($P = 0.034$ and 0.019, respectively) and in the GLA group ($P = 0.027$ and 0.016, respectively). The percentage change in sVCAM-1 concentration in the FO group (–28.3 ± 8.1) was significantly different from the percentage change in the placebo group (0.9 ± 7.2; $P = 0.033$) and in the GLA group (11.9 ± 11.1; $P = 0.006$). The percentage change in sVCAM-1 concentration in the ALNA group (–15.6 ± 9.3) was significantly different from the change in the GLA group ($P = 0.046$).

The absolute change in sE-selectin concentration in the ALNA group was significantly different from that in the placebo group ($P = 0.022$; Table 5). Furthermore, the percentage change in sE-selectin concentration in the ALNA group (–22.8 ± 9.1) was significantly greater ($P = 0.035$) than that in the placebo group (–4.8 ± 3.5). Although the absolute

**TABLE 4**
**Pro-inflammatory Cytokine Production by PBMC in the Different Treatments[a]**

| | TNF-α | | | IL-1β | | | IL-6 | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | Week 0 | Week 12 | Week 12 – week 0 | Week 0 | Week 12 | Week 12 – week 0 | Week 0 | Week 12 | Week 12 – week 0 |
| Placebo | 3.46 ± 0.67 | 4.12 ± 0.48 | 0.69 ± 1.09 | 4.92 ± 1.51 | 6.86 ± 0.83 | 1.88 ± 1.22 | 14.16 ± 2.79 | 19.15 ± 3.69 | 3.59 ± 3.43 |
| ALNA | 3.89 ± 0.58 | 3.01 ± 0.54 | –0.69 ± 0.57 | 5.34 ± 0.91 | 5.26 ± 0.57 | 0.34 ± 1.24 | 17.29 ± 3.06 | 16.22 ± 2.99 | –0.52 ± 3.45 |
| GLA | 3.24 ± 0.40 | 3.72 ± 0.50 | 0.51 ± 0.62 | 3.69 ± 0.47 | 5.58 ± 0.76 | 1.19 ± 0.54 | 17.77 ± 3.47 | 15.17 ± 3.13 | –2.27 ± 3.18 |
| ARA | 3.85 ± 0.61 | 4.11 ± 0.39 | 0.50 ± 0.56 | 5.01 ± 0.98 | 6.87 ± 0.80 | 2.15 ± 0.58 | 16.92 ± 2.29 | 19.64 ± 3.11 | 3.81 ± 2.01 |
| DHA | 3.45 ± 0.71 | 3.59 ± 0.65 | 0.24 ± 0.89 | 4.88 ± 0.92 | 6.77 ± 1.14 | 2.14 ± 1.54 | 17.07 ± 3.12 | 21.95 ± 4.76 | 5.16 ± 3.18 |
| FO | 4.65 ± 0.42 | 4.04 ± 0.33 | –0.54 ± 0.67 | 6.34 ± 1.13 | 7.45 ± 0.79 | 1.35 ± 1.54 | 15.63 ± 2.16 | 15.49 ± 3.33 | –0.14 ± 2.87 |

[a]Data are mean ± SEM ng/mL, $n = 7$ or 8 per treatment group. PBMC, peripheral blood mononuclear cell; IL, interleukin; TNF, tumor necrosis factor. For other abbreviations see Table 1.

**TABLE 5**
**Plasma Soluble Adhesion Molecule Concentrations in the Different Treatment Groups[a]**

| | sICAM-1 | | | sVCAM-1 | | | sE-selectin | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | Week 0 | Week 12 | Week 12 – week 0 | Week 0 | Week 12 | Week 12 – week 0 | Week 0 | Week 12 | Week 12 – week 0 |
| Placebo | 163.2 ± 13.8 | 163.3 ± 15.8 | 9.0 ± 14.4 | 857.5 ± 94.3 | 844.1 ± 85.3 | –13.5 ± 44.8 | 55.1 ± 5.9 | 51.8 ± 5.0 | –3.3 ± 2.1 |
| ALNA | 172.8 ± 9.3 | 162.0 ± 7.5 | –10.8 ± 10.9 | 924.1 ± 80.6 | 784.0 ± 45.6 | –143.7 ± 51.6[c,d] | 57.1 ± 6.8 | 41.5 ± 4.2 | –15.6 ± 4.2[c] |
| GLA | 187.4 ± 11.7 | 173.4 ± 17.1 | –14.0 ± 18.7 | 789.9 ± 74.5 | 851.6 ± 68.9 | 67.7 ± 68.0 | 48.7 ± 5.4 | 48.5 ± 8.2 | –0.3 ± 3.9 |
| ARA | 180.0 ± 20.2 | 168.9 ± 15.4 | –11.1 ± 12.1 | 965.5 ± 103.1 | 906.9 ± 71.5 | –58.6 ± 97.7 | 38.8 ± 7.0 | 34.8 ± 4.9 | –4.0 ± 4.2 |
| DHA | 176.3 ± 6.8 | 170.8 ± 12.6 | –5.5 ± 14.1 | 998.4 ± 53.9 | 979.0 ± 57.8 | –10.8 ± 49.5 | 42.0 ± 6.2 | 45.1 ± 6.8 | 3.1 ± 2.1 |
| FO | 173.4 ± 16.7 | 163.6 ± 11.8 | –25.3 ± 19.7 | 939.9 ± 91.6 | 691.4 ± 63.8[b] | –248.5 ± 40.8[c,d] | 58.5 ± 8.2 | 50.1 ± 9.4 | –13.4 ± 9.7 |

[a]Data are mean ± SEM ng/mL, $n = 7$ or 8 per treatment group. ICAM, intercellular adhesion molecule, VCAM, vascular cell adhesion molecule. For other abbreviations see Table 1.
[b]Indicates significantly different from before supplementation.
[c]Indicates significantly different from placebo treatment.
[d]Indicates significantly different from GLA treatment.

(Table 5) and percentage (–17.0 ± 10.1) changes in sE-selectin concentration in the FO group tended to be greater than those in the placebo group ($P = 0.075$ and 0.082, respectively), these were not significantly different.

## DISCUSSION

Cell culture and animal feeding studies have shown that the long-chain n-3 PUFA found in FO decrease the production of reactive oxygen species and of inflammatory cytokines and decrease the expression of adhesion molecules (11–32). These findings suggest that FO could compromise host defense. These observations are supported by a number of studies in healthy human volunteers, which have examined the influence of long-chain n-3 PUFA on inflammatory cell functions *ex vivo* (33–41,47,48). However, these human studies have used FO providing more than 2.4 g, and up to 9.6 g, of EPA plus DHA per day. Because current habitual intakes of EPA plus DHA are <150 mg per day (44), these studies represent a 15- to 60-fold increase in EPA plus DHA consumption. Likewise, the only study in healthy humans to examine the effects of ALNA on inflammatory cytokine production used about 14 g ALNA per day (38), which is at least sevenfold higher than habitual intake of this fatty acid (44). In fact, relatively little is known about the effects of more moderate consumption of n-3 PUFA on human inflammatory cell functions. Because there are recommendations to increase intake of both ALNA and long-chain n-3 PUFA (44), it seems important to ensure that there is no adverse immunological impact of a moderate increase in the consumption of these, and other, PUFA. Therefore, in the current study the effects of moderate supplementation with ALNA or FO were investigated, and this was combined with an examination of the effects of 700 mg DHA (in the absence of EPA), GLA, or ARA per day. Habitual intakes of GLA and ARA are <20 and <200 mg per day, respectively (see 50). A supplementation period of 12 wk was selected because almost all previous studies that reported effects of dietary PUFA on human inflammatory cell numbers or functions were of a duration of 3 to 12 wk (33–41,47–49).

The effects of the dietary supplementation on inflammatory cell numbers, on phagocytosis and respiratory burst by neutrophils and monocytes in response to *E. coli*, and on production of inflammatory cytokines by monocytes stimulated with LPS are reported because these have been shown to be affected by higher levels of n-3 PUFA or ARA than used here. In addition, the concentrations of plasma soluble adhesion molecules were measured because these provide a measure of longer-term endothelial activation *in vivo*, and because cell culture studies have shown that cytokine-induced upregulation of VCAM-1, E-selectin, and ICAM-1 on endothelial cells can be inhibited by n-3 PUFA (16–19).

The supplements represented significant increases in consumption of the fatty acids under study (see 50). Compliance of subjects to the supplements was assessed by determining the fatty acid composition of plasma phospholipids; according to this measure, compliance was good (see 50).

None of the treatments altered circulating inflammatory cell numbers (or proportions), suggesting that even marked increases in consumption of the fatty acids examined will not alter the numbers and types of inflammatory cells in the circulation. However, Kelley *et al.* (47) reported that 6 g DHA per day induced a 10% decrease in circulating total leukocyte numbers, which was largely due to a 21% decrease in granulocyte numbers; the total numbers of monocytes and lymphocytes did not change nor did the proportion of leukocytes as monocytes, but the proportion of lymphocytes was increased. In the current study 700 mg DHA per day did not alter circulating leukocyte numbers or types. Combined, these observations suggest that large intakes of either DHA or of long-chain n-3 PUFA in general [but not of ALNA, because 18 g ALNA per day did not alter the numbers of granulocytes, lymphocytes, or monocytes in the circulation (52)] might affect circulating leukocyte, especially granulocyte, numbers. Most of the studies using high levels of FO have not reported leukocyte numbers, and so it is not clear whether this is an effect of both EPA and DHA or of DHA alone. In the current study 700 mg ARA per day did not alter circulating leukocyte numbers or types. In another study, 1.5 g ARA per day was found to increase total leukocyte and granulocyte numbers but not to

affect the numbers of monocytes or lymphocytes (49). Thus, 1.5 g, but not 700 mg, ARA per day can affect circulating granulocyte numbers. It is not clear how the effects of elevated consumption of DHA and ARA on circulating granulocyte numbers occur; the fatty acids could affect either the rate of appearance of the cells into the bloodstream or the rate of removal of the cells from the bloodstream or both. Whatever the mechanism(s) involved, it appears that high intakes of ARA and DHA have opposing effects (47,49) but that more moderate intakes are without effect.

None of the treatments altered phagocytosis by neutrophils or monocytes, expressed as either % of active cells or as MFI. The lack of effect of FO and DHA observed in the current study is consistent with a study that used 3.8 g of either EPA or DHA per day and found no effect on phagocytosis by monocytes (53). To our knowledge there are no previous dietary studies of the effects of GLA, ALNA, or ARA on phagocytosis by human cells. None of the treatments altered respiratory burst by neutrophils or monocytes. Previous studies in humans reporting significant inhibition of respiratory burst by FO have used ≥3.1 g (and up to 9.6 g) EPA plus DHA per day (33–36). The lack of effect of FO (and DHA) observed in the current study extends previous studies showing no effect of 0.55 g EPA plus DHA per day on reactive oxygen species production by neutrophils (35) and monocytes (54). Thus, it appears that a moderate increase in intake of n-6 or n-3 PUFA, including ARA and those found in FO, will not affect phagocytosis or respiratory burst by neutrophils or monocytes.

The only study that investigated the influence of increased consumption of ALNA on cytokine production by human cells showed a marked decrease in production of TNF-α and IL-1β by LPS-stimulated PBMC after providing human volunteers with 14 g ALNA per day (38). The current study indicates that increasing ALNA intake by 2 g per day will not affect human inflammatory cytokine production. The lack of effect of 700 mg ARA per day on cytokine production in the current study is consistent with the observations of Kelley *et al.* (55) who reported no effect of 7 wk supplementation of the diet of young males with 1.5 g ARA per day on production of TNF-α, IL-1β, or IL-6 by PBMC stimulated by LPS. Similarly, the lack of effect of 700 mg GLA per day on cytokine production in the current study is consistent with the observations of Wu *et al.* (56), who reported no effect of 8 wk supplementation of the diet of elderly subjects with 675 mg GLA (plus 650 mg ALNA) per day on production of IL-1β by PBMC stimulated by LPS or *Staphylococcus epidermidis*. Thus, even significant increases in consumption of the longer-chain n-6 PUFA do not affect the production of these cytokines, which contrasts with the effects of these fatty acids seen *in vitro* (13,14). The current study observed no effect of 700 mg DHA per day on inflammatory cytokine production. However, a much larger dose of DHA (6 g per day), as part of a low-fat diet, caused a time-dependent decrease in production of TNF-α and IL-1β by LPS-stimulated PBMC (48).

The current study observed no effect of FO providing 1 g EPA plus DHA per day on production of TNF-α, IL-1β, and IL-6 by LPS-stimulated PBMC. This observation appears to contrast with previous studies (38–41). However, these studies used larger amounts of EPA plus DHA (2.4 to 5 g per day) than used here. Some other studies using low amounts of EPA plus DHA (0.55 to 1.6 g per day) report no effects on LPS-stimulated production of TNF-α (54,57), IL-1β (54,57,58), or IL-6 (54,57). However, the current study differs from each of these earlier studies. The study of Schmidt *et al.* (54) was conducted in younger subjects (24 to 52 yr of age) than the current study and used a very low dose of FO (0.55 g EPA plus DHA per day) in a population with a higher habitual intake of n-3 PUFA than in the current study. The study of Cooper *et al.* (57) also used younger subjects (18 to 36 yr of age) and was not controlled, and the study of Molvig *et al.* (58) used much younger subjects (mean age 27 yr) than the current study. The relevance of subject age is illustrated by the study of Meydani *et al.* (40), who found that *ex vivo* production of TNF, IL-1, and IL-6 by PBMC was much more strongly affected by 2.4 g EPA plus DHA per day for up to 12 wk in older women (51 to 68 years of age) than in young women (22 to 33 yr of age). Taken together these observations suggest that low to moderate doses of EPA plus DHA do not affect inflammatory cytokine production and that the threshold to exert an effect in older subjects is somewhere between 1 and 2.4 g EPA plus DHA per day.

To our knowledge this is the first study investigating the effects of ALNA, GLA, ARA, and DHA on plasma soluble adhesion molecule concentrations. GLA, ARA, and DHA had no significant effects, but ALNA decreased the concentrations of sVCAM-1 and sE-selectin by 16 and 23%, respectively. FO significantly decreased the concentrations of sVCAM-1 and sE-selectin by 28 and 17%, respectively. Because FO providing EPA plus DHA affected the concentrations of some soluble adhesion molecules but DHA alone did not, we conclude that the effects of FO are due to EPA not DHA. Whether the effects of ALNA are due to ALNA itself or relate to its conversion to EPA are not clear from this study.

The origin and roles of soluble adhesion molecules are not well understood. VCAM-1 and E-selectin are almost exclusively expressed by activated endothelial cells (2). Thus, the most likely origin of sVCAM-1 and sE-selectin is from activated endothelial cells, and this is believed to occur as a result of proteolytic shedding from the endothelial cell surface (2). ICAM-1 is expressed at basal levels on monocytes and endothelial cells and its expression can be strongly induced on a variety of cell types, including endothelial cells and monocytes, by stimuli such as cytokines, LPS, and reactive oxygen species (2). As for sVCAM-1 and sE-selectin, the appearance of sICAM-1 is believed to be a result of shedding from the cell surface (2). Thus, increased concentrations of sICAM-1, sVCAM-1, and sE-selectin in the circulation are considered to reflect upregulation of the molecules on the cell surface, and they appear to be biomarkers of chronic inflammation (2). The ability of FO and ALNA to lower the

concentrations of sVCAM-1 and sE-selectin, but not to influence that of sICAM-1, suggests that the n-3 PUFA act *in vivo* to decrease endothelial activation rather than monocyte activation. This accords with the lack of effect of FO and ALNA on *ex vivo* monocyte functions. The different effects of FO and ALNA on the concentrations of sICAM-1 and sVCAM-1 suggest that VCAM-1 and ICAM-1 are upregulated by different mechanisms and that these are not equally sensitive to n-3 PUFA intervention. This is supported by the cell culture observations that show that cytokine-induced upregulation of VCAM-1 expression on human endothelial cells is markedly inhibited by culture in the presence of long-chain n-3 PUFA (16–19), whereas upregulation of ICAM-1 is less affected (16,19) or not affected at all (17,18) by the n-3 PUFA.

Plasma concentrations of sICAM-1, sVCAM-1, and sE-selectin are higher in individuals with cardiovascular disease than in controls (3,5,6), and there is a positive correlation between the extent of atherosclerosis and these concentrations (5). The ability of dietary n-3 PUFA to lower plasma soluble adhesion molecule concentrations indicates a novel mechanism that might contribute to the protective effects of these fatty acids upon cardiovascular and inflammatory diseases.

The current study suggests that moderate levels of a range of n-6 and n-3 PUFA do not influence circulating inflammatory cell numbers or the *ex vivo* functional responses of inflammatory cells. Thus, it appears that consumption of n-3 PUFA could be increased in line with recommendations without inducing adverse effects on the innate immune response. Furthermore, this study also suggests that increased consumption of the n-6 PUFA GLA and ARA will not have such adverse effects. However, the current study was conducted in subjects aged 55 to 74 yr and the findings should not be extrapolated to other groups such as pregnant women or infants. Furthermore, the *ex vivo* measurements reported here were made using a single concentration of *E. coli* or LPS to challenge the cells. This concentration was that which induced optimal cell responses, and it cannot be determined from this study what effect, if any, the PUFA might have on cellular responses to a suboptimal challenge. The current study also found that moderate levels of GLA, ARA, and DHA do not influence circulating adhesion molecule concentrations, suggesting that they do not influence endothelial or monocyte activation *in vivo*. In contrast, moderate levels of ALNA and FO decreased the concentrations of endothelial-derived soluble adhesion molecules, suggesting that they diminish endothelial activation *in vivo*. How this might happen is unclear, but these observations indicate a novel mechanism of action of n-3 PUFA that could contribute to human health.

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# Effects of Dietary Virgin Olive Oil Phenols on Low Density Lipoprotein Oxidation in Hyperlipidemic Patients

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**ABSTRACT:** The aim of this study was to assess the effects of the dietary intake of extra virgin olive oil on the oxidative susceptibility of low density lipoproteins (LDL) isolated from the plasma of hyperlipidemic patients. Ten patients with combined hyperlipidemia (mean plasma cholesterol 281 mg/dL, triglycerides 283 mg/dL) consumed a low-fat, low-cholesterol diet, with olive oil (20 g/d) as the only added fat, with no drug or vitamin supplementation for 6 wk. Then they were asked to replace the olive oil they usually consumed with extra virgin olive oil for 4 wk. LDL were isolated at the beginning, and after the 4 wk of dietary treatment. LDL susceptibility to $CuSO_4$-mediated oxidation was evaluated by measuring the extent of lipid peroxidation. We also determined fatty acid composition and vitamin E in plasma and LDL and plasma phenolic content. Extra virgin olive oil intake did not affect fatty acid composition of LDL but significantly reduced the copper-induced formation of LDL hydroperoxides and lipoperoxidation end products as well as the depletion of LDL linoleic and arachidonic acid. A significant increase in the lag phase of conjugated diene formation was observed after dietary treatment. These differences are statistically correlated with the increase in plasma phenolic content observed at the end of the treatment with extra virgin olive oil; they are not correlated with LDL fatty acid composition or vitamin E content, which both remained unmodified after the added fat change. This report suggests that the daily intake of extra virgin olive oil in hyperlipidemic patients could reduce the susceptibility of LDL to oxidation, not only because of its high monounsaturated fatty acid content but probably also because of the antioxidative activity of its phenolic compounds.

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The dietary intake of natural compounds with antioxidant activity may play a key role in the prevention of cardiovascular diseases and other conditions whose pathogenesis is based on oxidative processes (1). Diets rich in vegetables and fruit provide protection against such diseases, most likely owing to their high content of antioxidants, including not only vitamin E, C, and β-carotene but also phenolic compounds, a large class of molecules characterized by a number of hydroxyl groups attached to a ring structure responsible for the antioxidant activity (2). Epidemiological and experimental evidence associates the more common hyperlipidemias [high low density lipoprotein (LDL)-cholesterol levels, high triacylglycerol levels, and/or low high density lipoprotein (HDL)-cholesterol levels] with the increased risk of developing cardiovascular diseases (3).

LDL modified by oxidative processes (ox-LDL) have been suggested to be involved in the pathogenesis of atherosclerosis (4). The susceptibility of LDL to oxidation depends on several factors such as their fatty acid composition and the concentration of some antioxidant vitamins, e.g., vitamin E (5–7). Epidemiological studies positively correlated the intake of dietary phenolic compounds with a lowered risk of coronary heart disease (8,9). Experimental studies support the hypothesis that several phenolics can be absorbed through the gastrointestinal tract (10–13).

Extra virgin olive oil (EVOO), a typical component of the Mediterranean diet, has been associated with a reduced incidence of coronary heart disease. This is likely due in part to the high amount of monounsaturated fatty acids (14,15) but may also be the result of several phenolic compounds (16,17). The content of phenolic compounds in EVOO can vary greatly depending on the kind of cultivar, fruit ripening, and, above all, oil extraction process (18,19). Its mild extraction process renders EVOO richer in phenolic compounds than olive oil (OO) prepared by the standard method. This suggests the hypothesis that EVOO may exert a protective effect against oxidation of LDL similar to that of green tea and red wine (20,21).

Although the antioxidant properties of EVOO phenolic compounds have been extensively discussed, little is known about their absorption and metabolism and, to the best of our knowledge, very few data are available on *in vivo* studies in humans (22). The aim of this study was thus to assess the *in vivo* effects of dietary EVOO on the oxidizability of LDL isolated from hyperlipidemic patients.

## SUBJECTS AND METHODS

The study was performed with free-living patients, who were given specific dietary advice. Informed consent was obtained

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Abbreviations: BHT, butylated hydroxytoluene; DAD, diode array detector; EVOO, extra virgin olive oil; FAME, fatty acid methyl ester; HDL, high density lipoprotein; 4-HNE, 4-hydroxy-2(*E*)-nonenal; HPLC, high-performance liquid chromatography; HRGC, high-resolution gas chromatography; LDL, low density lipoprotein; MDA, malondialdehyde; OO, olive oil; ox-LDL, oxidized LDL; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid; SEM, standard error of the mean; SPE, solid phase extraction.

from 10 patients (8 males and 2 females, aged 42–67) with combined hyperlipidemia, who were nonsmokers and leading a sedentary life (Table 1). For a period of at least 6 wk before the beginning of EVOO consumption, the patients were trained individually by a dietitian to achieve or maintain an acceptable body weight and to consume a diet low in total fat (<30% energy) and saturated fat (<10% energy) with a daily cholesterol intake below 300 mg. Recommendations adhered to the dietary goals established by the American Heart Association Step I diet (23). OO (20 g/d) was the only added fat in meals. After 6 wk, OO was replaced by the same amount of EVOO containing 238 ± 20 mg/kg of phenolics. OO and EVOO were purchased by us and were, consequently, the same for all the subjects. The oils derived from the same production and were prepared according to our request so that vitamin E content and fatty acid composition were the same in both of them. Moreover, all these parameters were controlled experimentally in our laboratory, giving the following values: vitamin E (expressed as mg α-tocopherol/kg, calculated using activity coefficients for individual tocopherols), 189 ± 7 (mean ± SD, $n = 5$) in OO and 192 ± 10 in EVOO; and total phenol compounds (expressed as tyrosol concentration, mg/kg), 11 ± 5 in OO and 238 ± 20 in EVOO. The treatment with EVOO lasted 4 wk.

The dietary intake was assessed at the beginning and at the end of the EVOO treatment by means of a 7-d food record: Patients were asked to record detailed descriptions of all foods and beverages consumed (ingredients, methods of preparation, cooking). None of the patients had coronary artery disease, hypertension, diabetes, or obesity. All subjects had normal fasting glucose levels, and they exhibited normal liver, renal, and thyroid functions. No patient used drugs with documented lipid-modifying effects such as diuretics, beta-blockers, corticosteroids, sex steroids, or antifungal agents. The Local Ethics Committee approved the experimental design.

*Blood sampling and laboratory analysis.* Blood was taken at the end of 6 wk of OO consumption and after 4 wk of EVOO consumption. After an overnight fast, blood was collected by venipuncture and divided into two tubes. One of them, containing $Na_2$ EDTA (1 mmol/L), was used for plasma isolation, and the other one to prepare serum for specific determinations. Serum total cholesterol, HDL-cholesterol, and triacylglycerol were determined enzymatically with commercial kits (Boehringer Diagnostica). LDL-cholesterol was determined according to Friedewald's formula.

*LDL isolation and oxidation.* LDL, isolated from plasma by discontinuous density gradient ultracentrifugation (24) and extensively dialyzed against phosphate-buffered saline (PBS) at 4°C, were diluted in PBS to 0.2 g protein/L and incubated with 10 μmol/L $CuSO_4$ for 5 h at 37°C. Oxidation was stopped by refrigeration and the addition of 20 μmol/L butylated hydroxy toluene (BHT) and 100 μmol/L EDTA. The extent of LDL oxidation was evaluated on the basis of the increase in hydroperoxides and end products of lipid peroxidation and the decrease in polyunsaturated fatty acids (PUFA) content. These parameters were determined in triplicate for each patient before and after EVOO treatment, both on native and oxidized LDL.

*Hydroperoxide determination.* The hydroperoxide content in the LDL fraction was determined by iodometric titration according to the method proposed by El-Saadani *et al.* (25), except that reaction time was changed from 30 to 60 min (26). Results were expressed as μmol/mg of LDL protein using $\varepsilon_{mM} = 24.6$.

*Malondialdehyde (MDA) and 4-hydroxy-2 (*E*)-nonenal (4-HNE) determinations.* End products of lipid peroxidation were measured as MDA plus 4-HNE content using a commercial kit based on a colorimetric assay (LPO-586; Bioxytech, Oxis International Inc., Portland, OR). Results were expressed as μmol/mg of LDL protein using tetramethoxypropane as standard.

*Fatty acid analysis.* Fatty acid composition was determined, before and after oxidation, in lipid extracts of plasma and LDL isolated from the patients before and after EVOO consumption. Lipids were extracted from 0.5 mL plasma using 20 mL of chloroform/methanol mixture (2:1 vol/vol). The lipid extracts, washed with 0.88% KCl (1 mL), were completely evaporated under a gentle flow of nitrogen and dissolved in *n*-hexane. An aliquot of the extracted lipids was transesterified by adding an equal volume of 3 N methanolic hydrogen chloride followed by incubation for 12 h at 60°C. Fatty acid methyl esters (FAME) were separated and quantified by high-resolution gas chromatography (HRGC). A PerkinElmer 8320 gas chromatograph (Norwalk, CT) was used, equipped with a split (50:1) injector, a flame-ionization detector, and a 30 m × 0.25 mm i.d. Omegawax 250 capillary column (Supelchem, Milano, Italy). The fatty acid composition of lipids was estimated as percentage of total fatty acids.

*LDL oxidation kinetics.* The kinetics of LDL oxidation was measured by spectrophotometrically monitoring the increase in absorbance at 234 nm due to conjugated diene formation

**TABLE 1**
**Plasma Lipid Concentration and Body Mass Index of Hyperlipidemic Patients[a] Before and After Extra Virgin Olive Oil Consumption**

| Parameter[b] | Before | After | Δ (%) | *P* | 95% CI |
|---|---|---|---|---|---|
| Total cholesterol (mg/100 mL) | 281.4 ± 45.2 | 272.9 ± 38.2 | −3.0 | 0.4 | −47.8 + 30.8 |
| LDL-cholesterol (mg/100 mL) | 172.4 ± 23.0 | 165.9 ± 23.1 | −3.8 | 0.6 | −31.2 + 18.2 |
| HDL-cholesterol (mg/100mL) | 46.6 ± 11.2 | 41.3 ± 9.1 | −5.3 | 0.5 | −11.9 + 7.3 |
| Triacylglycerols (mg/100mL) | 283.1 ± 121.0 | 293.7 ± 105.0 | −3.7 | 0.2 | −95.8 + 117.0 |
| Body mass index (kg/m$^2$) | 25.1 ± 2.3 | 25.0 ± 2.3 | −0.1 | 0.9 | −2.2 + 2.1 |

[a]Values were measured in triplicate before and after extra virgin olive oil consumption in 10 hyperlipidemic patients.
[b]Values are expressed as mean ± standard deviation. LDL, low density lipoprotein; HDL, high density lipoprotein.

**TABLE 2**
**Analytical Performance of HPLC–DAD[a] Method for the Quantification of Phenolic Compounds in Human Plasma**

| Phenol | LOD[b] (ng mL$^{-1}$) | Repeatability[c] RSD (%) | Accuracy[d] Spiked (ng mL$^{-1}$) | Accuracy[d] Average recovery (%) |
|---|---|---|---|---|
| Oleuropein | 30 | 2.2 | 30-50-80-100 | 79 |
| Oleuropein aglycone | 40 | 3.1 | 40-60-80-100 | 87 |
| 3,4-Dihydroxyphenylethanol | 15 | 4.9 | 15-30-50-100 | 91 |
| *p*-Hydroxyphenylethanol | 15 | 5.5 | 15-30-50-100 | 86 |
| *p*-Hydroxyphenylethanol aglycone | 30 | 3.4 | 30-50-80-100 | 92 |
| Gallic acid | 25 | 1.9 | 25-50-80-100 | 95 |
| Protocatechuic acid | 50 | 2.5 | 50-80-100-120 | 85 |
| Vanillic acid | 50 | 0.8 | 50-80-100-120 | 87 |
| Caffeic acid | 40 | 2.5 | 40-60-80-100 | 92 |
| *p*-Coumaric acid | 30 | 2.3 | 30-50-80-100 | 94 |
| Ferulic acid | 30 | 2.4 | 30-50-80-100 | 78 |
| Luteolin | 30 | 3.8 | 30-50-80-100 | 74 |
| Apigenin | 50 | 1.9 | 50-80-100-120 | 77 |

[a]HIgh-performance liquid chromatography–diode array detector.
[b]Limit of detection (LOD) calculated on the basis of the 3-σ criterion.
[c]Relative standard deviation (RSD) calculated on 10 replicates at the following concentrations: 50 ng mL$^{-1}$ for oleuropein, 3,4-dihydroxyphenylethanol, *p*-hydroxyphenylethanol, *p*-hydroxyphenylethanol aglycone, gallic acid, *p*-coumaric acid, ferulic acid, and luteolin; and 100 ng mL$^{-1}$ for oleuropein aglycone, protocatechuic acid, vanillic acid, caffeic acid, and apigenin.
[d]Performed by recoveries obtained from spiked (four levels) in-house standard (freeze-dried human plasma).

induced by 1.6 μmol/L $CuSO_4$, according to Puhl *et al*. (27). The length of the lag phase, expressed in minutes, was calculated by measuring the intercept of the slope of absorbance in the propagation phase with the extrapolated line for the slow reaction.

*Vitamin E determination.* The plasma and LDL contents of vitamin E, as α-, β-, δ-, and γ-tocopherols, were determined by extracting the analyte from the matrix and then separating it by means of high-performance liquid chromatography (HPLC) as reported by Cavina *et al*. (28). Results were expressed as mg/L for plasma determination and μg/mg protein for LDL determinations, using α-tocopherol acetate as internal standard.

*Determination of phenolic compounds.* In OO and EVOO total phenolic compounds were determined by means of the Folin-Ciocalteau reagent as reported by Swain and Goldstein (29). The concentrations of phenolics in plasma samples were determined by HPLC after extraction and purification procedures. The procedure was similar to that described by Tsimidou *et al.* (30) with some modifications in the extraction procedure and chromatographic conditions; their method refers solely to olive oil, whereas in this study the method was applied to plasma. Briefly, solid-phase extraction (SPE) cartridges (Spe-ed SPE $C_{18}$ 500 mg-6 mL, Applied Separations, Allentown, PA), previously conditioned by passing 2 mL of a *n*-hexane/ethyl ether solution through the cartridge (98:2 vol/vol), were used for the extraction and purification of the phenolic compounds. One milliter of the sample, acidified with 10 μL of phosphoric acid, was then deposited on the top of the cartridge, which was subsequently washed with 10 mL of the conditioning solution. The phenolic fraction was eluted with 8 mL of methanol, filtered through disposable Teflon filters, and evaporated in Rotavapor at 35°C. The dry residue was then dissolved in 0.5 mL of methanol, and 10 μL of this solution was injected into an HPLC chromatograph (Waters, Milford, MA) fitted with a Lichrosorb column (RP18, 250 × 4.6 mm, 5 μm) and a diode array (ultraviolet-visible) detector set at 280 nm. The eluents were (A) acetonitrile/methanol (50:50 vol/vol) and (B) phosphoric acid/water (1:99 vol/vol). The gradient was 4% A at $t$ = 0 min, 20% A at $t$ = 20 min, 50% A at $t$ = 40 min, 60% A at $t$ = 50 min, and 100% A at $t$ = 60 min. Peak identification was made on the basis of retention times and further confirmed by spectrum analysis with a diode array detector. The modified method was validated in a previous study (31) and the analytical performance is reported in Table 2.

Total phenolic content was expressed as tyrosol concentration (mg/L).

*Statistical analysis.* Results were presented as mean ± standard error of the mean. Three determinations were performed for each patient.

The statistical analysis was performed using paired Student's $t$-test. A probability value ≤0.05 was considered significant. The possible relationship between the modification in plasma phenolic and oxidative parameters was evaluated using correlation coefficients and their statistical significance. Data were processed with the Statgraphics software package (v. 7 for DOS, Manugistic, Rockville, MD).

## RESULTS

The 7-d food records assessed at the end of the study showed a high level of compliance with the dietary goals established by the American Heart Association Step I diet and no differences from the 7-d food record assessed before EVOO consumption. Mean body mass index and plasma lipid values remained stable during the study (Table 1), which confirms that

the dietary recommendations were observed during EVOO consumption. The quantitative analyses of the lipid extracts from plasma and the LDL fraction isolated from the patients did not show any significant difference in the content of the various lipid moieties and fatty acid composition after EVOO treatment. In particular, oleic, linoleic, and arachidonic acid contents in the LDL fraction were unchanged (Table 3).

In vitro *susceptibility of LDL to oxidative modification.* The dietary intake of 20 g/d EVOO by dyslipidemic patients resulted in a reduced susceptibility of LDL to oxidation induced by cupric ions. After the LDL isolated from patients were oxidized with 10 µmol/L $CuSO_4$, the amount of lipid peroxidation products, e.g., hydroperoxides and MDA + 4-HNE, was significantly lower in samples collected after EVOO consumption (Fig. 1). The hydroperoxide content, measured at the end of a 5-h oxidation period, was 0.54 ± 0.06 and 0.26 ± 0.04 µmol/mg protein LDL before and after EVOO consumption ($P < 0.01$), respectively.

The content of end products of lipid peroxidation, expressed as MDA + 4-HNE, was also reduced from 0.21 ± 0.01 to 0.17 ± 0.02 µmol/mg protein before and after EVOO treatment, respectively ($P < 0.01$).

*Fatty acid composition.* Figure 2 shows the content of linoleic and arachidonic acids determined before and after the oxidative treatment. Following EVOO consumption, a greater percentage of linoleic and arachidonic acid content remained in LDL oxidized by cupric ions. Linoleic acid content was 18.5 vs. 14.4% in LDL, oxidized after and before EVOO consumption ($P < 0.05$), respectively, showing a lower decrease with respect to the value determined before the oxidative treatment (35 vs. 50%). Arachidonic acid showed an even more marked difference. In fact, residual content of arachidonic acid was 1.9% after and only 0.9% before EVOO treatment ($P < 0.01$), corresponding to a 77 vs. 94% decrease.

*Diene formation during the oxidative treatment.* The kinetics of conjugated diene formation, continuously monitored at 234 nm, allowed us to evaluate the increased resistance of the LDL fraction to oxidation by the increase in the length of the lag phases observed after 4 wk of EVOO consumption. The mean value ± SEM of the lag phase rose from 88 ± 6 min to 101 ± 5 min ($P < 0.01$) after EVOO consumption. (The length of the lag phase was calculated from the curve of conjugated diene formation, by the intercept of the propagation phase tangent with the extrapolated line for the slow reaction.) No differences were obtained either in the maximal propagation rate or in the maximal concentrations of conjugated dienes, as shown in the plot of mean curves of LDL oxidation before and after EVOO consumption (Fig. 3).

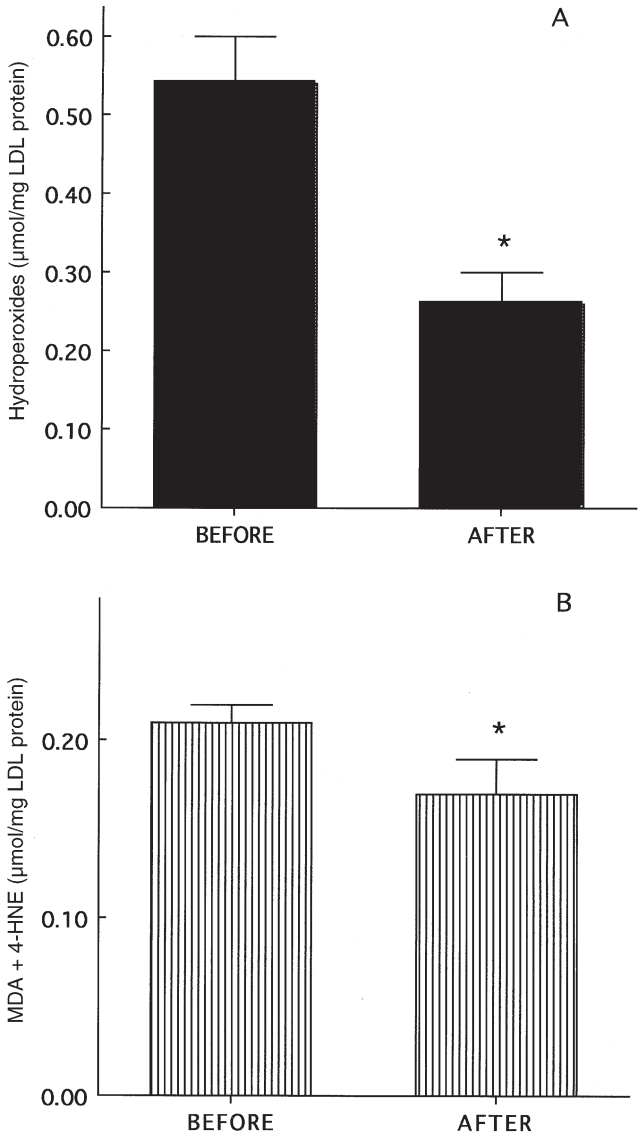


**FIG. 1.** Effect of dietary extra virgin olive oil (20 g/d) on the susceptibility of low density lipoproteins (LDL) to *in vitro* oxidation by 10 µmol/L $CuSO_4$. Plasma LDL were isolated from hyperlipidemic patients before and after 4 wk of extra virgin olive oil (EVOO) consumption. LDL oxidation was evaluated as hydroperoxide content (panel A), and as malondialdehyde (MDA) plus 4-hydroxy-2(*E*)-nonenal (4-HNE) content (panel B) by spectrophotometry. Results are given as mean (µmol/mg LDL protein) ± standard error of the mean (*$P < 0.01$).

**TABLE 3**
**Percentage of Fatty Acids in LDL[a] Fraction Before and After Extra Virgin Olive Oil Consumption**

| | Before | After |
|---|---|---|
| Palmitic acid, 16:0 | 24.4 ± 1.6 | 24.3 ± 1.5 |
| Palmitoleic acid, 16:1 | 4.4 ± 1.1 | 3.7 ± 1.0 |
| Stearic acid, 18:0 | 6.7 ± 1.3 | 5.8 ± 0.8 |
| Oleic acid, 18:1 | 27.8 ± 1.0 | 29.5 ± 0.9 |
| Linoleic acid, 18:2 | 29.5 ± 0.8 | 30.0 ± 1.7 |
| Eicosatrienoic acid, 20:3 | 1.3 ± 0.3 | 1.5 ± 0.6 |
| Arachidonic acid, 20:4 | 5.7 ± 0.5 | 6.2 ± 0.5 |

[a]Values are the percentage of the total fatty acid content measured before and after extra virgin olive oil consumption. Values are expressed as mean ± SEM. See Table 1 for abbreviation.

*Vitamin E and phenolic compounds determination.* Vitamin E content in plasma and LDL did not show significant changes associated with EVOO consumption (14.1 ± 1.0 and 16.7 ± 1.7 mg/L in plasma; 4.4 ± 0.6 and 5.0 ± 0.8 µg/mg LDL protein, before and after EVOO treatment, respectively).

The mean content ± SEM of total phenolic compounds in plasma was remarkably increased after consumption of 20 g

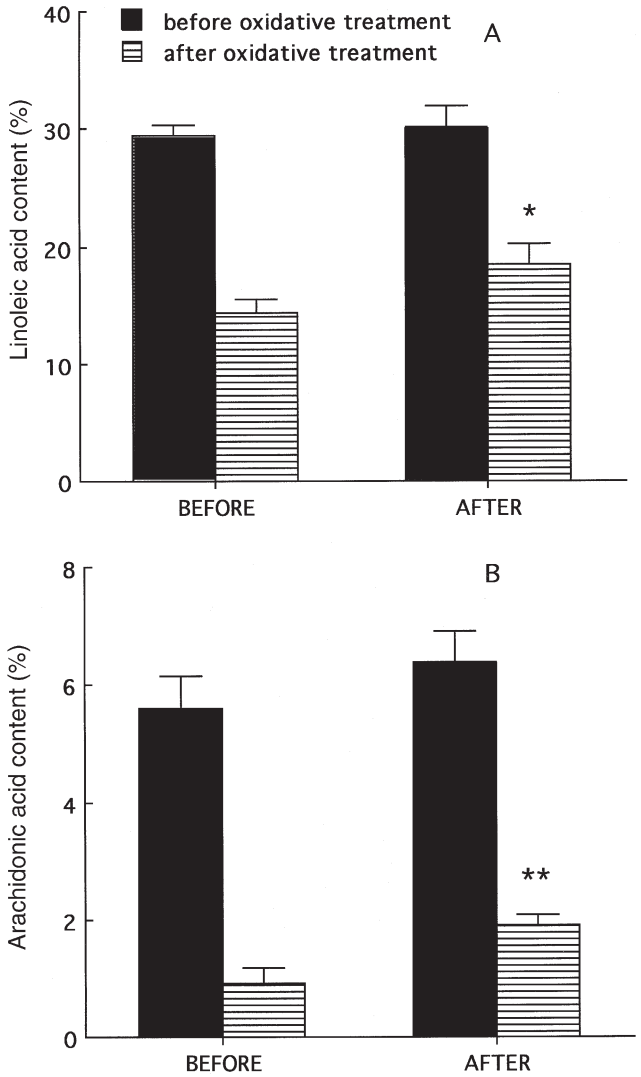


**FIG. 2.** Polyunsaturated fatty acid content of plasma LDL particles before and after oxidation by cupric ions. The figure reports the mean ± standard error of the mean of the residual percentage (% of total fatty acids) of linoleic acid (panel A) and arachidonic acid (panel B) in oxidized LDL before and after 4 wk of EVOO treatment (*$P < 0.05$, **$P < 0.01$). See Figure 1 for other abbreviations.

EVOO/d (23.0 vs. 11.3 mg/L, expressed as tyrosol concentration $P < 0.02$); in particular, oleuropein and its derivatives (oleuropein aglycone, 3,4-dihydroxyphenylethanol, *p*-hydroxyphenylethanol aglycone) increased more than 90% (data not shown). To demonstrate the phenol content differences before and after EVOO consumption, Figure 4 shows a chromatogram obtained by subtracting the chromatogram of hyperlipidemic patient plasma obtained before the treatment from that derived after 4 wk of EVOO consumption. This increase in phenol content appeared statistically correlated with the increase in the length of lag phase ($r = 0.6819$) as well as with the decreased production of hydroperoxides ($r = -0.6923$) and MDA + 4-HNE ($r = -0.8507$), and with the residual content of linoleic and arachidonic acid ($r = 0.6846$ and $r = 0.6541$, respectively) in LDL after the oxidative treatment.

## DISCUSSION

Phenolic compounds have recently been the focus of growing attention because of their antioxidative properties and their possible beneficial role in the prevention of several pathologies such as cancer and cardiovascular disease (32). The antioxidative activity of phenols seems to result from several mechanisms, such as chelation of metal ions, free radical scavenging ability, lipoperoxide chain-breaking, and antioxidant vitamin regeneration (33,34). There is some evidence that they can be directly absorbed through the small intestinal mucosa, but little is known about the efficiency of such uptake and the catabolism of phenolic compounds or their derivatives in the body (35).

This study suggests that the daily consumption of EVOO protects LDL against *ex vivo* oxidation. Like other researchers, we had previously shown the strong antioxidative power of several phenols naturally present in EVOO, capable of protecting LDL from *in vitro* oxidative modifications induced by cupric ions (36,37). The present results are consistent with our previous findings of increased resistance to oxidation of LDL isolated from rabbits fed standard diets modified by the addition of EVOO or a specific EVOO phenolic compound, namely, oleuropein (31). We also demonstrated that tyrosol, the major EVOO phenolic compound, exerted a wholly protective effect against LDL-induced injury in an *in vitro* enterocyte-like system, preventing both cytostatic and cytotoxic events (38).

The present study expanded those previous findings, suggesting that the reduced susceptibility of plasma LDL to lipid peroxidation in humans, as a result of the daily consumption of EVOO, could be mainly related to phenolic antioxidants, rather than to monounsaturated fatty acids as previously suggested (39–41). It is noteworthy that the plasma phenolic content in the patients significantly increased after the consumption of EVOO, in particular that of oleuropein and derivatives, which are compounds peculiar to olives and the most effective antioxidative phenols. This suggests that phenols are absorbed in the gut, become available in blood and are then excreted in the urine as recently reported (22). It could be hypothesized that the phenolic compounds contribute to antioxidative defenses, exerting their activity on plasma components such as LDL, as demonstrated for similar compounds contained in red wine (21).

Other mechanisms might be responsible for the increased resistance of LDL to oxidation, such as changes in the lipid composition of the lipoprotein particles or in plasma antioxidant content (42). The LDL fatty acid composition may affect LDL susceptibility to oxidative modification (43). The formation of oxidized LDL depends on their PUFA content, which is the main target of oxidative reactions, and it was shown that oleate-enriched LDL containing less PUFA were more resistant to oxidative modifications (44). Our results clearly show that the fatty acid composition of plasma LDL particles was unmodified as far as PUFA and monounsaturated fatty acids are concerned. It is worth noting that the

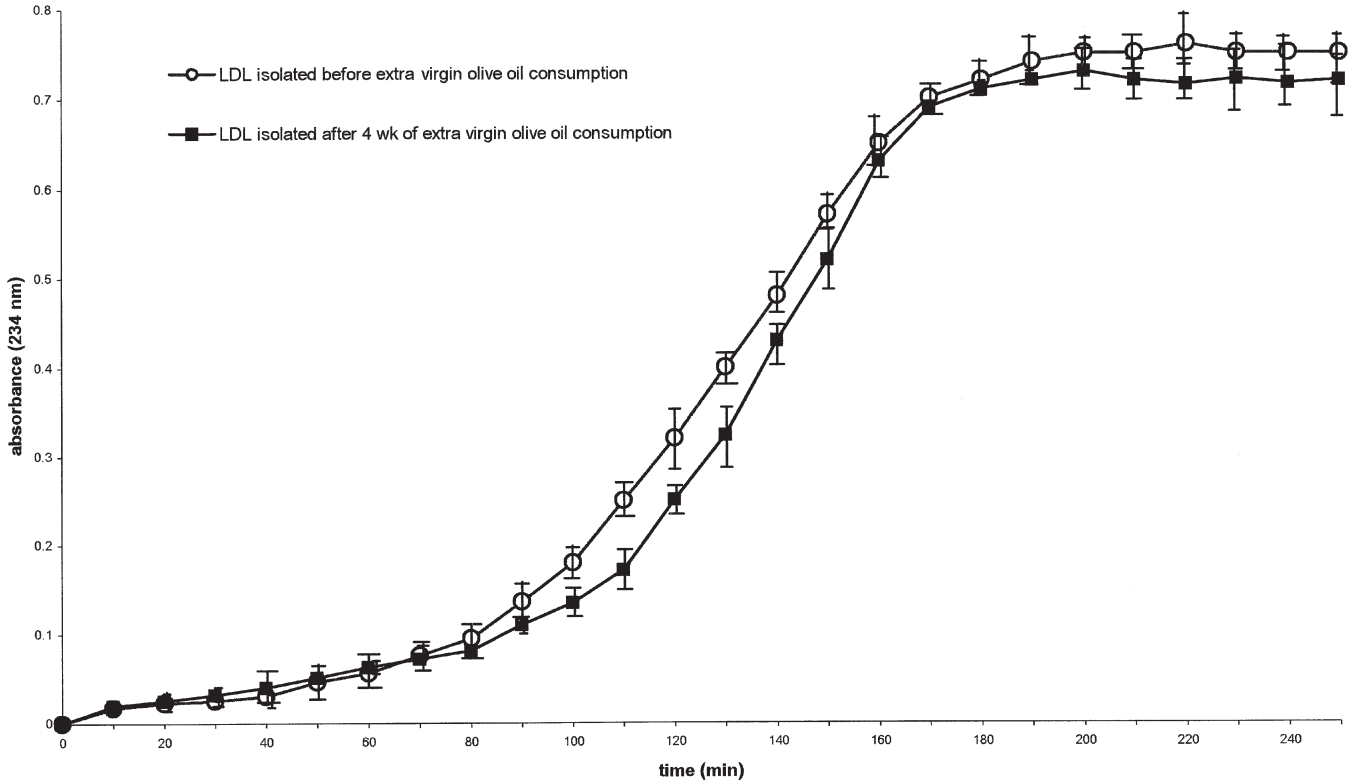


**FIG. 3.** Effect of dietary EVOO on the length of lag phase of plasma LDL. Mean curves of conjugated diene formation induced by copper in LDL isolated from patients before and after dietary intervention are presented. The change in absorbance at 234 nm was continuously monitored for 4 h. See Figure 1 for abbreviations.

intake of monounsaturated fatty acids remained unchanged throughout the study. In fact, before the beginning of the experimental period, the patients consumed OO daily, which differs from EVOO in that it features a lower amount of phenolic compounds, but the same monounsaturated fatty acid content. The progress of the conjugated diene formation curve might suggest that the additional antioxidants in the plasma of patients after the dietary treatment could be bound to LDL as reported for other phenols (21,45), delaying the propagation of the lipoperoxidation in LDL. Once these additional antioxidants were depleted, the rate of the oxidation reaction of LDL was the same at both times owing to their similar fatty acid composition. On the other hand, plasma and LDL α-tocopherol content were not significantly modified during the observation period.

Our investigation indicates that the antioxidative minor components of EVOO might play a relevant role in the prevention or attenuation of pathologies associated with redox imbalance, such as atherosclerosis and other chronic degenerative diseases.

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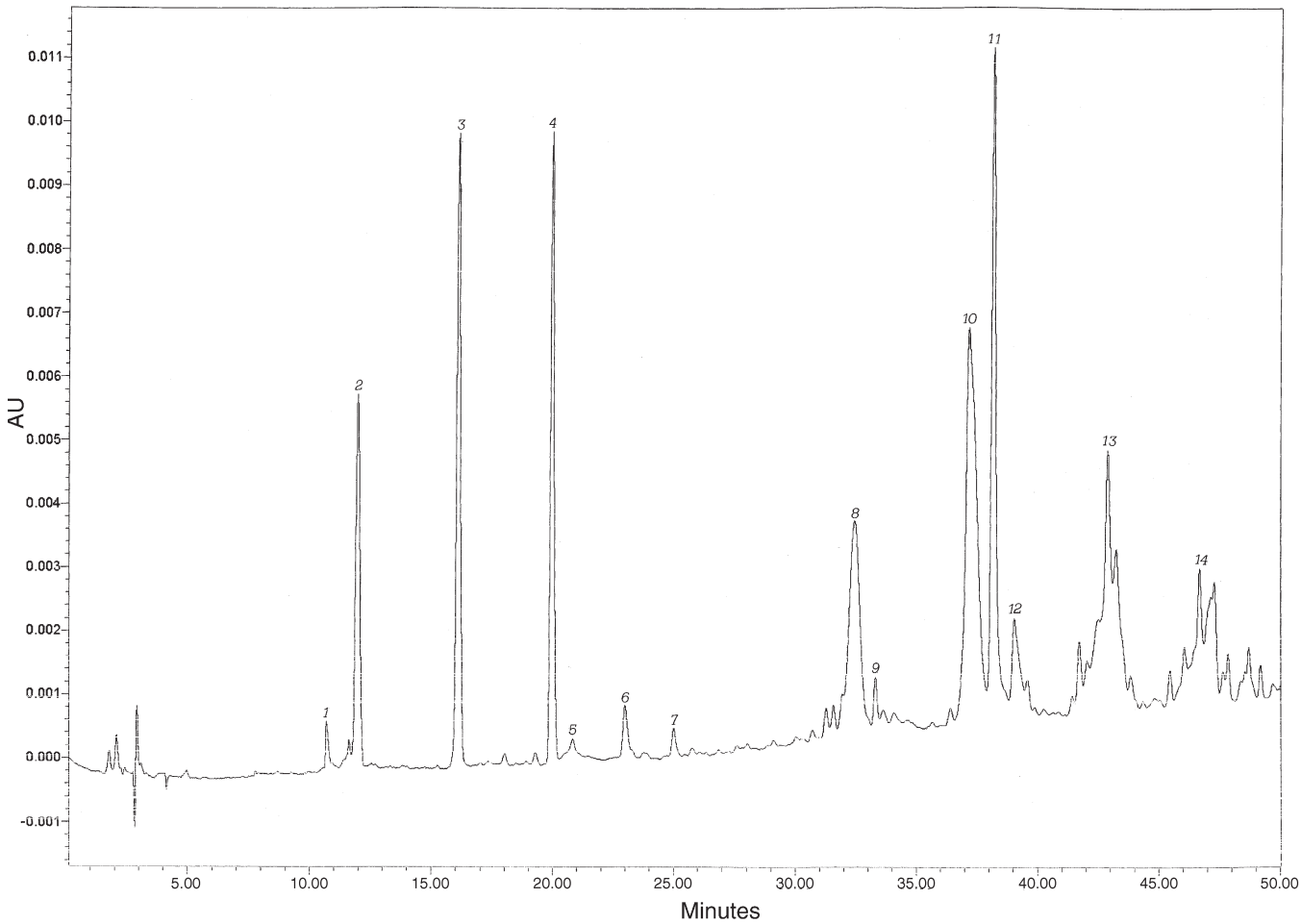


**FIG. 4.** An example of chromatogram of human plasma by hyperlipidemic patients after 4 wk of EVOO consumption. The peak pattern was obtained by subtracting the chromatogram obtained from a human plasma sample before EVOO treatment. Peak identification: (1) gallic acid; (2) 3,4-dihydroxyphenylethanol; (3) *p*-hydroxyphenylethanol; (4) syringic acid = internal standard; (5) *o*- and *p*-coumaric acids; (6) vanillic acid; (7) caffeic acid; (8) protocatechuic acid; (9) ferulic acid; (10) *p*-hydroxyphenylethanol aglycone; (11) oleuropein aglycone; (12) oleuropein; (13) luteolin; and (14) apigenin. See Figure 1 for abbreviation.


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# Lipid Remodeling in Mouse Liver and Plasma Resulting from Δ6 Fatty Acid Desaturase Inhibition

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**ABSTRACT:** Electrospray/tandem mass spectrometry was used to quantify lipid remodeling in mouse liver and plasma during inhibition of polyunsaturated fatty acid synthesis by the Δ6 fatty acid desaturase inhibitor, SC-26196. SC-26196 caused increases in linoleic acid and corresponding decreases in arachidonic acid and docosahexaenoic acid in select molecular species of phosphatidylcholine, phosphatidylethanolamine, and cholesterol esters but not in phosphatidylserine, phosphatidylinositol, or triglycerides. For linoleic acid-, arachidonic acid-, and docosahexaenoic acid-containing phospholipid species, this difference was, in part, determined by the fatty acid at the *sn*-1 position, namely, palmitic or stearic acid. An understanding of phospholipid remodeling mediated by Δ6 desaturase inhibition should aid in clarifying the contribution of arachidonic acid derived *via de novo* synthesis or obtained directly in the diet during inflammatory responses.

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Arachidonic acid (20:4n-6) is incorporated into lipoprotein particles after being synthesized *de novo* from linoleic acid (18:2n-6) in the liver (Scheme 1) or ingested directly in the diet (1–4). The arachidonic acid in lipoprotein particles is taken up by peripheral tissues and leukocytes where, upon incorporation into phospholipids and release by phospholipases, it serves as the substrate for the synthesis of proinflammatory prostaglandins, leukotrienes, and hydroperoxy or hydroxy fatty acid derivatives (5–9). Past studies have shown that arachidonic acid supplied exogenously to cells is mobilized into different lipid pools of which a select subset is utilized for eicosanoid production (10–16) or regulation of cell division (17). Understanding the remodeling of arachidonic acid in lipids would be significant because of its pivotal role in inflammation. Remodeling of docosahexaenoic acid would also be significant because of its ability to compete with and reduce the tissue level of arachidonic acid (18,19).

*In vivo* studies on the remodeling of phospholipids and the ensuing effect on the metabolism of arachidonic acid and other bioactive lipids have been limited by the lack of selective fatty acid desaturase inhibitors (20,21). This is especially true when attempting to delineate the role of polyunsaturated fatty acids (PUFA) derived from the diet vs. synthesized *de novo*. Recently, SC-26196 was described as a selective inhibitor of the Δ6 fatty acid desaturase (22). In this study, electrospray and tandem mass spectrometry (ES/MS/MS) were used to quantify changes in lipid remodeling following the dosing of mice with SC-26196. A further degree of biological sophistication was observed in that changes in the levels of linoleic and arachidonic acids occurred in specific molecular species within a given lipid class.

## MATERIALS AND METHODS

*Materials and reagents.* Authentic lipids used as standards in MS/MS methods development and as internal standards for quantification were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The vehicle for intraperitoneal (ip) dosing consisted of 0.5% methylcellulose + 0.025% Tween-20 (polyoxyethylene-sorbitan monolaurate) + 5% dimethylsulfoxide (DMSO), all purchased from Sigma Chemical Co. (St. Louis, MO). Organic solvents were Optima grade from Fisher Scientific (Pittsburgh, PA). Routine laboratory chemicals were purchased from Sigma Chemical Co. or Fisher Scientific. Sample preparation of lipids/fatty acids was done in glass test tubes having Teflon-lined caps.

*Dietary regimen and dosing.* Animal studies were approved by the Monsanto Institutional Animal Care and Use Committee and conformed to its guidelines. Female Balb/C mice were purchased at 8–10 wk of age and upon arrival were fed a standard rodent chow diet (Teklad 2215 [W] rodent diet 8640, Harlan, Madison, WI). Two days prior to initiating dosing, the mice were switched to a corn oil diet (AIN-76-based corn oil diet, DYETS, Inc., Bethlehem, PA). The switch in diets was made because approximately 0.5 mg of arachidonic acid is consumed per day in the standard chow diet by a 20-g mouse (Lab Diet™, The Richmond Standard™, Animal Diet Reference guide, PMI Feeds, Inc., St. Louis, MO). This level of arachidonic acid may be high enough to circumvent partial depletion of arachidonic acid during chronic inhibition of Δ6 desaturase activity by SC-26196. In contrast, the corn oil diet contains linoleic acid (18:2n-6) and oleic acid (18:1n-9) but not arachidonic acid (23). Furthermore, the high level of linoleic acid in the corn oil diet provides substrate for the *in vivo* synthesis of arachidonic acid, which, in the presence of SC-26196, would be inhibited.

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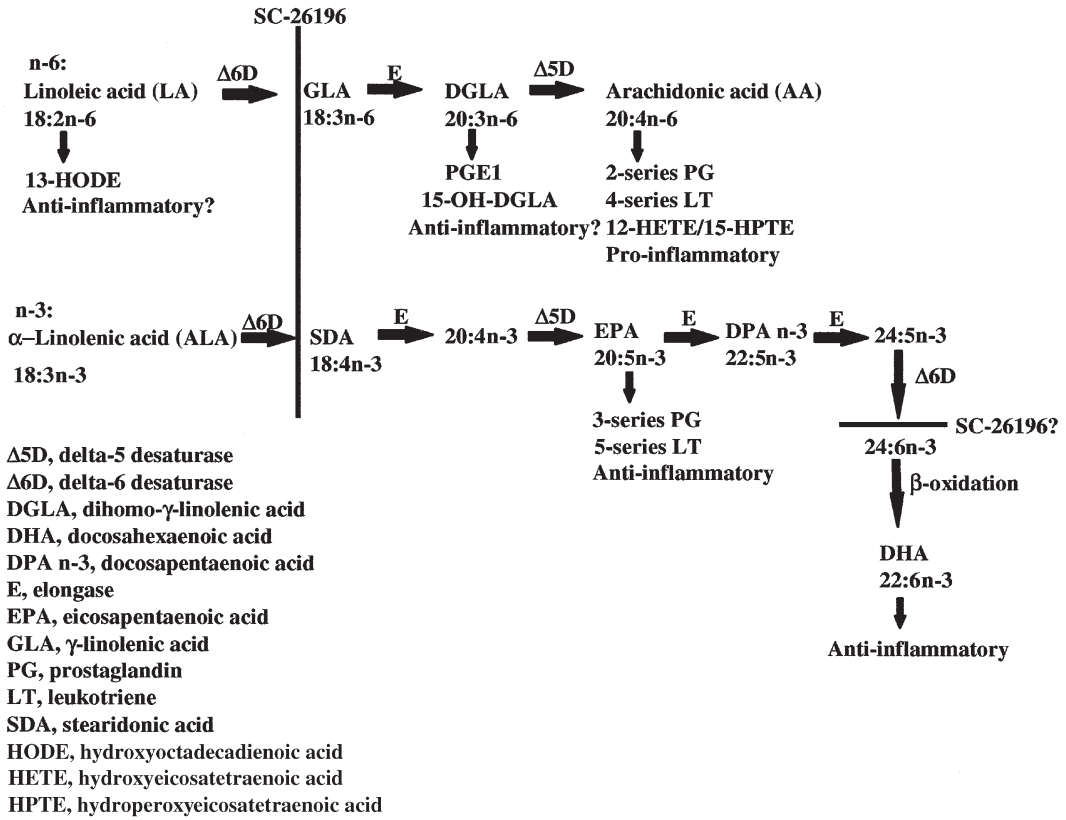
Abbreviations: DMSO, dimethylsulfoxide; ES/MS, electrospray mass spectrometry; ip, intraperitoneal; mpk, milligram per kilogram body weight; MS/MS, tandem mass spectrometry; PUFA, polyunsaturated fatty acid.




SCHEME 1

SC-26196 was prepared as a suspension in vehicle (methylcellulose/Tween-20/DMSO) by wet milling (Retsch, model MM2 wet miller, Haan, Germany). SC-26196 was administered as 0.1 mL bolus doses of 100, 30, or 10 mg/kg body weight (mpk) by ip injections twice daily (approximately 7:00 AM and 5:00 PM) for a total of 9 d. Control groups were administered vehicle using the same dosing regimen. Five mice were included per group.

*Lipid extraction.* At the end of the dosing regimen, the mice were anesthetized with $CO_2/O_2$ (80:20), blood samples were obtained by retroorbital bleeding, and plasma was prepared. The mice were then sacrificed by $CO_2$ inhalation, and liver tissue was removed and blotted. Liver and plasma samples were frozen immediately in tubes placed on dry ice and stored at −80°C until analysis.

Total lipids from liver tissue and plasma were prepared for ES/MS/MS according to a modified Bligh and Dyer procedure (24). Extraction of lipids from liver tissue was described previously (23). For extraction of lipids from plasma, 500 μL of $CHCl_3$/MeOH/$H_2O$ (1:2:0.3) was added to 25 μL plasma. The mixture was centrifuged at 10,000 × *g* for 5 min at room temperature. A second extraction was performed on the pellet by adding 300 μL of $CHCl_3$/MeOH/$H_2O$ (1:2:0.8), vortexing vigorously, and then centrifuging at 10,000 × *g* for 5 min at room temperature. The supernatant was decanted and pooled with the first supernatant. The pooled supernatants were diluted with 250 μL of chloroform, yielding a final lipid extract from 25 μL plasma solubilized in 1.1 mL of $CHCl_3$/MeOH/$H_2O$.

*ES/MS/MS.* For lipid quantification by ES/MS/MS, the liver or plasma lipid extract in $CHCl_3$/MeOH/$H_2O$ was added to an equal volume of $CHCl_3$/MeOH (1:1, vol/vol) containing 10 mM ammonium acetate and a specific concentration (10–50 μg/mL) of internal standard. Lipid extracts were introduced directly into a Sciex API III+ electrospray triple quadrupole mass spectrometer (Sciex, Inc., Thornhill, Canada) operated in the positive ion mode. Specific details of mass spectrometer operating conditions and lipid identification and quantification were published previously (23).

*Statistics.* All doses of SC-26196 and the vehicle were included in a one-way analysis of variance for each fatty acid and lipid species (Figs. 2–4). Because plasma samples were taken only from animals injected with vehicle or 100 mpk SC-26196, the focus was restricted to a comparison between the mean response for vehicle vs. 100 mpk SC-26196 in both liver and plasma samples. If the *P* value was less than 0.05 (two-tailed), the means were considered to be significantly different.

## RESULTS AND DISCUSSION

Fatty acid remodeling within particular lipid classes is critical to understanding the role of the Δ6 desaturase in inflammation because arachidonic acid is found in specific phospholipid species and can be released by phospholipases and converted to proinflammatory eicosanoids by cyclooxygenase and lipoxygenase (6,9–16,25–28). As an initial step in gaining an understanding of the role of *de novo* synthesis of PUFA in lipid remodeling, ES/MS/MS was used in a direct and

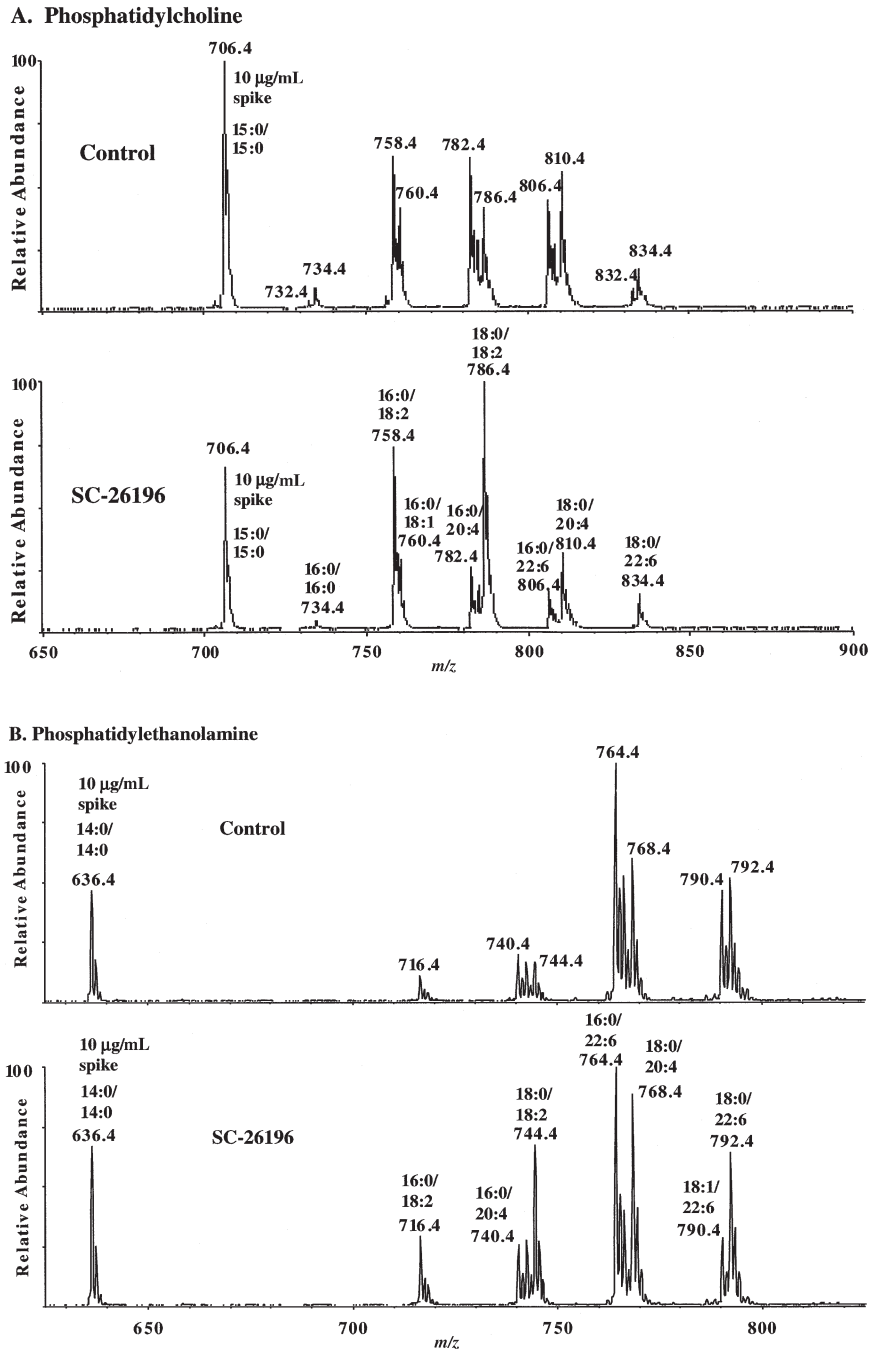



**FIG. 1.** SC-26196-mediated changes in the mass spectra of mouse liver lipids. Shown are representative electrospray mass spectra of liver phosphatidylcholine (A) and phosphatidylethanolamine (B) from a mouse injected with vehicle (control) or 100 mg/kg body weight (mpk) SC-26196 [twice daily intraperitoneal (ip) dosing for 9 d]. Liver lipids were extracted and analyzed by electrospray tandem mass spectrometry (ES/MS/MS) (see the Materials and Methods section for details). SC-26196 is a selective inhibitor of the Δ6 fatty acid desaturase.

facile way to evaluate changes resulting from inhibition of Δ6 desaturase activity by SC-26196, a selective Δ6 fatty acid desaturase inhibitor. Given that SC-26196 was not profiled for its specificity in all aspects of lipid metabolism, its precise mechanism of action *in vivo* remains uncertain.

Dosing of mice with SC-26196 caused quantitative changes in linoleic acid-, arachidonic acid-, and docosahexaenoic acid-containing phospholipid and cholesterol ester species in liver and plasma that were indicative of Δ6 desaturase inhibition, *viz.*, increased substrate (i.e., linoleic acid) and decreased products (i.e., arachidonic acid and docosahexaenoic acid) (Scheme 1, Figs. 1,2). Specifically, SC-26196 caused changes in the fatty acid composition of phosphatidylcholine, phosphatidylethanolamine, and cholesterol esters (Fig. 2) but did not change the fatty acid composition of phosphatidylinositol or phosphatidylserine (data not shown). The most significant changes were seen in linoleic acid-, arachidonic acid-, and docosahexaenoic acid-containing species (Fig. 2). In liver and plasma phosphatidylcholine, the linoleic acid-containing species increased, whereas the arachidonic

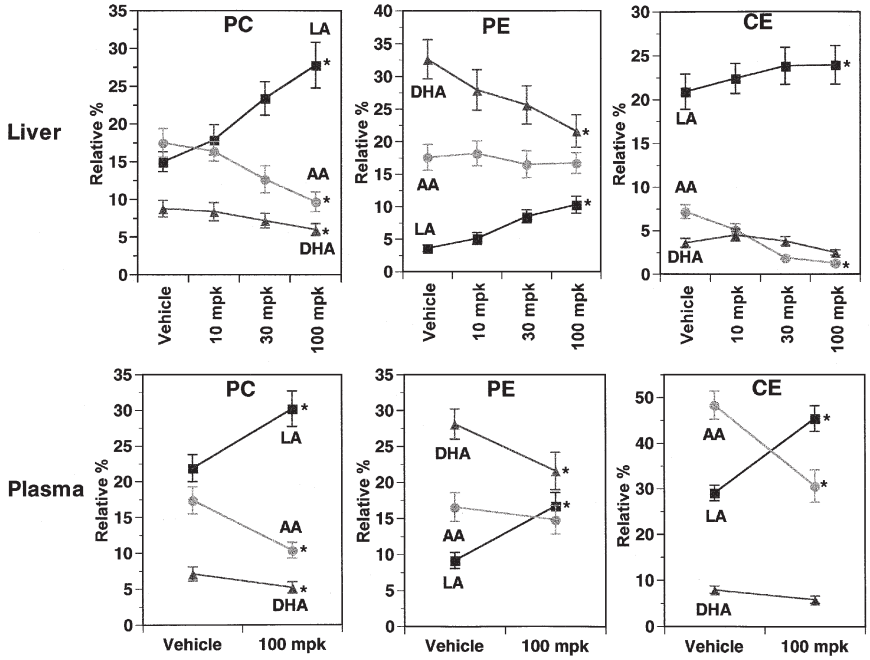



**FIG. 2.** SC-26196-mediated changes in the relative amounts of linoleic acid (LA), arachidonic acid (AA), and docosahexaenoic acid (DHA) in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cholesterol esters (CE) in mouse liver and plasma. Liver samples were taken from mice injected with vehicle (control) or 10, 30, or 100 mpk SC-26196 (twice daily ip dosing for 9 d). Plasma samples were taken from mice injected with vehicle (control) or 100 mpk SC-26196 (twice daily ip dosing for 9 d). Plasma was obtained by retroorbital bleeding. Plasma and liver lipids were extracted and analyzed by ES/MS/MS (see the Materials and Methods section for details). Results are expressed as the mean ± standard error of the mean ($n$ = 5/group). *$P < 0.05$ at 100 mpk dose. For abbreviations see Scheme 1.

acid- and docosahexaenoic acid-containing species decreased. The results were not as uniform in phosphatidylethanolamine and cholesterol esters of liver and plasma: (i) In phosphatidylethanolamine, linoleic acid-containing species increased and docosahexaenoic acid-containing species decreased, whereas arachidonic acid-containing species were unchanged, and (ii) in cholesterol esters, linoleic acid-containing species increased and arachidonic acid-containing species decreased, whereas docosahexaenoic acid-containing species were unchanged. In plasma, the most significant change in fatty acid composition of the individual lipid classes was observed in the cholesterol ester fraction in which the ratio of arachidonic acid/linoleic acid decreased from approximately 1.7 to 0.7 (Fig. 2).

Further quantitative analyses were performed on the individual fatty acid species of phosphatidylcholine and phosphatidylethanolamine in liver and plasma. A notable observation was that SC-26196 mediated changes in phosphatidylcholine and phosphatidylethanolamine lipid species that depended on the identity of the fatty acids acylated at the *sn*-1 and *sn*-2 positions. Phosphatidylcholine- and phosphatidylethanolamine-containing linoleic acid acylated at the *sn*-2 position increased in abundance more dramatically when stearic acid (18:0), as opposed to palmitic acid (16:0), was acylated at the *sn*-1 position in liver (Fig. 3) and plasma (Fig. 4). Conversely, phosphatidylcholine-containing arachidonic acid and phosphatidylcholine- and phosphatidylethanolamine-containing docosahexaenoic acid acylated at the *sn*-2 position decreased in abundance more dramatically when palmitic acid, as opposed to stearic acid, was acylated at the *sn*-1 position in liver (Fig. 3). In plasma, this difference was only observed with phosphatidylcholine-containing docosahexaenoic acid (Fig. 4). With phosphatidylethanolamine-containing arachidonic acid species in the liver there was not a significant change when palmitic acid or stearic acid was acylated at the *sn*-1 position (Fig. 3). Instead, arachidonic acid-containing species were reduced most significantly when oleic acid (18:1n-9) was acylated at the *sn*-1 position (Fig. 3).

These data suggest that the regulation of liver fatty acid metabolism by Δ6 desaturase inhibition involves differential turnover or selective synthesis of molecular species of phosphatidylcholine, phosphatidylethanolamine, and cholesterol esters, but not phosphatidylinositol or phosphatidylserine. Arachidonic and docosahexaenoic acids appear to be remodeled similarly in liver following treatment of mice with SC-26196. This observation is consistent with the requirement for Δ6 desaturation for *de novo* synthesis of both fatty acids (Scheme 1) (23). For linoleic acid-, arachidonic acid-, and docosahexaenoic acid-containing phospholipid species, remodeling, in part, appears to be determined by the fatty acid at the *sn*-1 position, most often palmitic acid or stearic acid. The specificity of *sn*-1 and *sn*-2 positioning could regulate acylase- and transacylase-mediated lipid remodeling, which could ultimately determine the lipid species that serve as sources of arachidonic acid for eicosanoid metabolism (10–16,20). Lipid species selectivity could also explain why SC-26196 did not decrease the relative amount of arachidonic acid in liver total lipids (22) or why only a small decrease occurred in liver phospholipids and cholesterol esters (Fig. 2). Only when specific liver lipid species were quantified by ES/MS/MS, *viz.,* phosphatidylcholine, phospha-

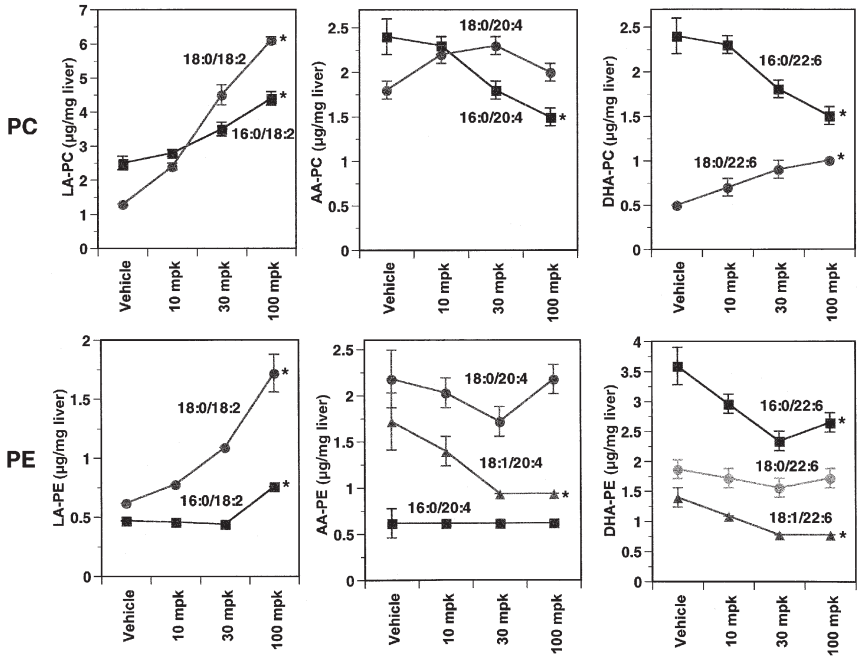



**FIG. 3.** SC-26196-mediated changes in the absolute amounts of major liver PC and PE molecular species containing LA (18:2), AA (20:4), and DHA (22:6). Liver samples were taken from mice injected with vehicle (control) or 10, 30, or 100 mpk SC-26196 (twice daily ip dosing for 9 d). Liver lipids were extracted and analyzed by ES/MS/MS (see the Materials and Methods section for details). Results are expressed as the mean ± standard error of the mean ($n$ = 5/group). *$P < 0.05$ at 100 mpk dose. Other notations: 18:0, stearic acid; 16:0, palmitic acid; and 18:1, oleic acid. For abbreviations see Figures 1 and 2.

tidylethanolamine, and cholesterol esters, did SC-26196 cause a decrease in arachidonic acid (Fig. 3).

Given that the liver is a major source of PUFA-containing lipid species transported *via* the vasculature as very low density lipoprotein and low density lipoprotein particles, it was anticipated that lipid remodeling in liver would reflect that in plasma and, generally, that was the case (Fig. 2). There were exceptions with select lipid species, *viz*., 16:0/18:2- and 18:0/20:4-phosphatidylcholine and 16:0/22:6- and 18:1/22:6-phosphatidylethanolamine (Figs. 3,4). Of the phospholipids, arachidonic acid was affected most in phosphatidylcholine (16:0/20:4) and phosphatidylethanolamine (18:1/20:4), two classes that serve as substrates for its release and metabolism to various eicosanoids (25–28). A notable caveat, however, is that lipid remodeling in liver and plasma may not be indicative of lipid remodeling in eicosanoid-producing cells (e.g., circulating leukocytes, resident macrophages, fibroblasts, and endothelial cells). Additionally, the specific arachidonic acid-containing phospholipid pool for eicosanoid biosynthesis has been difficult to define in any cell (10–16). Future work should thus address the effect of Δ6 desaturase inhibition on remodeling in eicosanoid-producing cells. Furthermore, an understanding of phospholipid remodeling in inflammatory cells mediated by Δ6 desaturase inhibition should clarify the contribution of arachidonic acid derived *via de novo* synthesis or obtained directly in the diet.

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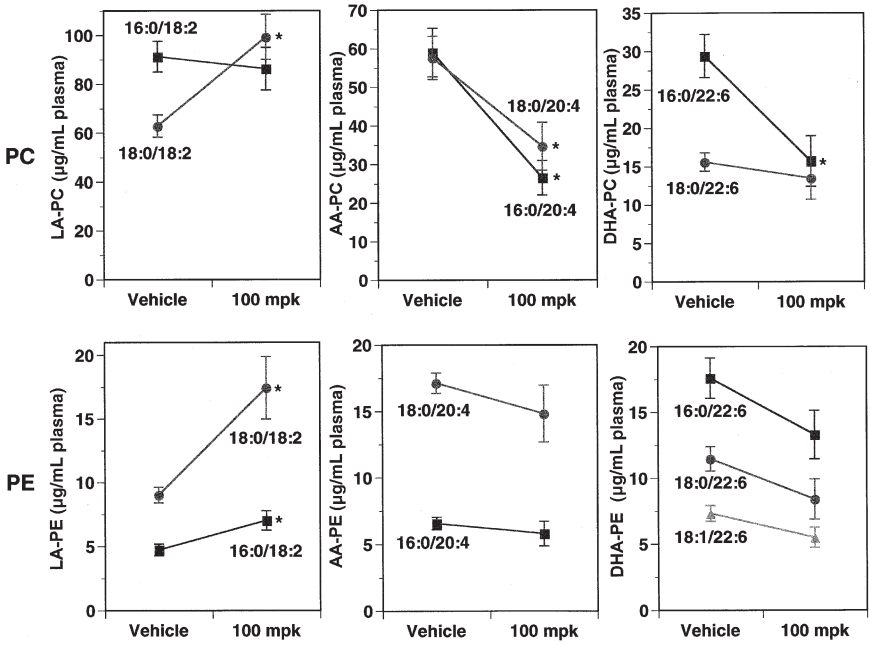


**FIG. 4.** SC-26196-mediated changes in the absolute amounts of PC and PE molecular species containing LA (18:2), AA (20:4), and DHA (22:6) in mouse plasma. Mice were injected with vehicle (control) or 100 mpk SC-26196 (twice daily ip dosing for 9 d). Plasma was obtained by retroorbital bleeding. Plasma lipids were extracted and analyzed by ES/MS/MS (see the Materials and Methods section for details). Results are expressed as the mean ± standard error of the mean ($n$ = 5/group). *$P < 0.05$ at 100 mpk dose. See Figures 1–3 for other abbreviations.

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# Soluble Fiber and Soybean Protein Reduce Atherosclerotic Lesions in Guinea Pigs. Sex and Hormonal Status Determine Lesion Extension

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**ABSTRACT:** These studies were undertaken to assess guinea pigs as potential models for early atherosclerosis development. For that purpose, male, female, and ovariectomized (to mimic menopause) guinea pigs were fed a control or a TEST diet for 12 wk. Differences between diets were the type of protein (60% casein/40% soybean vs. 100% soybean) and the type of fiber (12.5% cellulose vs. 2.5% cellulose/5% pectin/5% psyllium) for control and TEST diets, respectively. Diet had no effect on plasma cholesterol or triacylglycerol (TAG) concentrations; however, there were significant effects related to sex/hormonal status. Ovariectomized guinea pigs had higher plasma cholesterol and TAG concentrations than males or females ($P < 0.01$). In contrast to effects on plasma lipids, hepatic cholesterol and TAG were 50% lower in the TEST groups ($P < 0.01$) compared to controls. Low density lipoproteins (LDL) from guinea pigs fed the TEST diet had a lower number of cholesteryl ester (CE) molecules and a smaller diameter than LDL from controls. Atherosclerotic lesions were modulated by both diet ($P < 0.0001$) and sex ($P < 0.0001$). Guinea pigs fed the TEST diet had 25% less lesion extension whereas males had 20% larger occlusion of the arteries compared to both female and ovariectomized guinea pigs. Significant positive correlations were found between LDL CE and atherosclerotic lesions ($r = 0.495$, $P < 0.05$) and LDL size and fatty streak area ($r = 0.56$, $P < 0.01$). In addition, females fed the TEST diet had the lowest plasma and hepatic cholesterol concentrations, the smallest LDL particles, and the least atherosclerosis involvement compared to the other groups. These data indicate that dietary factors and sex/hormonal status play a role in determining plasma lipids and atherosclerosis in guinea pigs.

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Coronary heart disease (CHD) is the leading cause of death among men and women in the United States (1). Several lines of evidence indicate that elevated serum concentrations of low density lipoproteins (LDL) promote atherogenesis and increase the risk for CHD (2). Gender is a strong predictor of CHD susceptibility, and reports from the Registrar's mortality data indicate that men are more likely to develop CHD than premenopausal women (3). Postmenopausal women are at a greater risk than premenopausal women for CHD owing to detrimental changes in plasma lipids and lipoprotein levels occurring after menopause (4).

Recent reports have linked dietary soy protein and soy-based food products with a reduction in CHD and improvement in a number of related risk factors. Asian women, who consume primarily soy protein, have a lower CHD mortality than women eating a Western diet (5). Soy protein has also been shown to reduce atherosclerosis in cynomolgus monkeys (6) and rabbits (7,8). Soy protein seems to have its greatest and most consistent effect on LDL. Greaves *et al.* (9) reported that isolated soy protein containing naturally occurring isoflavones was associated with LDL cholesterol lowering in cynomolgus monkeys. Compared to a casein diet, soy protein has been shown to reduce apolipoprotein (apo) B synthesis and stimulate LDL receptors (10).

Viscous soluble fibers such as pectin or guar gum, as well as viscous nonfermentable fibers such as psyllium, lower plasma cholesterol concentrations (11). These fibers reduce cholesterol absorption in the small intestine resulting in plasma very low density lipoproteins (VLDL) and LDL cholesterol lowering in response to depletion of hepatic cholesterol and induction of apo B/E receptors (12). Soluble fibers have been shown to lower plasma cholesterol levels in humans (13) and in various animal models (11,13–16).

The present study had the following objectives: (i) to evaluate the potential use of guinea pigs as models for early atherosclerosis development, (ii) to investigate whether diets high in vegetable protein and soluble fiber reduce atherosclerotic lesions resulting from a high intake of cholesterol, and (iii) to determine whether sex and hormonal status modulate the extent of the atherosclerotic lesions. Our TEST diet (100% soybean protein, 10% soluble fiber) was used to ensure significant differences in atherosclerotic lesions between groups and to demonstrate that vegetable protein and soluble fiber have a beneficial effect on atherosclerosis development even in the presence of high levels of dietary cholesterol. In addition to lipid levels, composition and size of VLDL and LDL were determined to evaluate potential atherogenic factors within subclasses of lipoproteins. Our hypothesis was that dietary factors and sex/hormonal status would interact to determine atherosclerosis development.

Guinea pigs were used as the experimental animal model because their lipoprotein profile and response to dietary factors are similar to humans (17). We also hypothesized that guinea pigs would be a good model to evaluate fatty streak

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Abbreviations: ANOVA, analysis of variance; apo, apolipoprotein; CE, cholesteryl ester; CHD, coronary heart disease; FC, free cholesterol; LSD, least significant differences; LDL, low density lipoproteins; PL, phospholipids; TC, total cholesterol; TAG, triacylglycerol; VLDL, very low density lipoproteins.

accumulation resulting from intake of hypercholesterolemic diets. Male, female, and ovariectomized guinea pigs, a model for menopause established by our laboratory (18), were used to observe any unique gender differences in lipid metabolism and atherosclerosis development.

## MATERIALS AND METHODS

*Materials*. The source for the enzymatic assay kits for plasma free cholesterol (FC) and phospholipids (PL) was Wako Pure Chemical Industries (Osaka, Japan). Cholesterol enzymatic assay kits, cholesterol standards, triacylglycerol (TAG) enzymatic kits, TAG standards, cholesterol oxidase, and cholesterol esterase were from Boehringer Mannheim (Indianapolis, IN).

*Diets.* Control and TEST diets were prepared and pelleted by Research Diets, Inc. (New Brunswick, NJ). The TEST diet was designed to produce differences in plasma cholesterol concentrations and lipoprotein composition compared to the control diet and to determine whether these alterations in the measured parameters would relate to early atherosclerosis development. In addition, one goal was to demonstrate that high intake of dietary cholesterol in combination with vegetable protein and soluble fiber would result in a more favorable lipoprotein profile and fewer atherosclerotic lesions. Both diets had the same composition except for the type of fiber and the type of protein (Table 1). They both contained 0.33% cholesterol, equivalent to 2475 mg/d in humans (19), and the fatty acid mixture was high in lauric and myristic acids, fatty acids known to be hypercholesterolemic in guinea pigs. The fat mix was olive oil/palm kernel oil/safflower oil in a proportion 1:2:1.8 (20). The control diet contained 12.5% cellulose as the fiber source and the protein portion was made up of 60% casein and 40% soybean. The TEST diet was formulated with 2.5% cellulose, 5% pectin, and 5% psyllium as fiber sources and 100% soybean as protein source. The soybean protein used was intact, containing naturally occurring isoflavones. The control diet had 40% soybean and 60% casein because higher concentrations of casein result in the development of kwashiorkor in guinea pigs (Fernandez, M.L., and McNamara, D.J., unpublished observations).

**TABLE 1**
**Composition of Experimental Diets**

| Components | g/100 g Control | TEST |
|---|---|---|
| 40% soybean/60% casein | 23.0 | — |
| Soybean protein | — | 22.5 |
| Methionine | — | 0.5 |
| Sucrose | 25.0 | 25.0 |
| Corn starch | 15.0 | 15.0 |
| Fat mix[a] | 15.1 | 15.1 |
| Cellulose | 12.5 | 2.5 |
| Pectin | — | 5.0 |
| Psyllium | — | 5.0 |
| Mineral mix[b] | 8.2 | 8.2 |
| Vitamin mix[b] | 1.1 | 1.1 |
| Cholesterol | 0.33 | 0.33 |

[a]Fat mix contained 7.8% myristic, 23.8% lauric, 9.2% palmitic, 8.6% stearic, 19.9% oleic, 26.4% linoleic acids, and 4.3% others.
[b]Mineral and vitamin mixes were adjusted to meet National Research Council requirements for guinea pigs.

*Animals*. Forty-four male, female, or ovariectomized guinea pigs from Harlan Sprague-Dawley, Inc. (Indianapolis, IN), weighing between 300 and 400 g, were randomly assigned to either control or TEST diets. Ovariectomized female guinea pigs were incorporated to mimic menopause. The number of guinea pigs used was 14 males (8 control and 6 TEST), 13 females (7 control and 6 TEST), and 17 ovariectomized (11 control and 6 TEST). Animals were caged in pairs in a light cycle room (light from 7:00 A.M. to 7:00 P.M.). They were gradually adapted to their respective diets with nonpurified diet over a period of 7 d and then fed the experimental diet for a period of 12 wk. All experimental protocols were performed in accordance with U.S. Public Health Service and U.S. Department of Agriculture guidelines. Experiments were approved by the University of Connecticut Institutional Animal Care and Use Committee.

*Plasma cholesterol and TAG assays*. Blood samples were taken by cardiac puncture from nonfasted guinea pigs after they were anesthetized with halothane vapors. Blood was collected with a syringe containing EDTA (anticoagulant) at a concentration of 1.5 mg/mL and was centrifuged at 2000 × *g* for 20 min. Plasma was separated from red blood cells, and plasma total cholesterol (TC) and TAG concentrations were measured by enzymatic methods (21,22). High density lipoprotein cholesterol was measured after precipitation of apo B–containing lipoproteins according to Warnick *et al*. (23) with a slight modification consisting of the use of $MgCl_2$ at a concentration of 2 mol/L (24).

*Lipoprotein isolation and composition*. A preservation cocktail was added to individual plasma samples to ensure minimal changes in composition during isolation. The cocktail consisted of sodium azide ($NaN_3$) (0.1 mL per 100 mL of plasma), phenyl methyl sulfonyl fluoride (0.1 mL per 100 mL of plasma), and aprotonin (1000 IU/mL; 0.5 mL per 100 mL of plasma). VLDL and LDL were isolated by ultracentrifugation in a subset of animals (3 males, 3 females, and 5 ovariectomized per dietary group) at densities of 1.019 and 1.09 kg/L, respectively. Compositions of these fractions were determined by measuring PL (25), TAG (22), and free and total cholesterol (21). Protein was determined by a modification of the Lowry procedure (26). The number of molecules in the LDL subfraction was calculated based on one apo B for each LDL with a molecular mass of 412,000 kDa (27). The number of molecules of TAG, FC, esterified cholesterol, and PL was calculated using molecular weights of 885.4, 386.6, 645, and 734, respectively (28). LDL diameter was calculated according to Van Heek and Zilversmit (29).

*Hepatic lipids*. Samples were suspended overnight in chloroform/methanol 2:1 to extract lipids from liver. With this extract, TC and FC were determined by enzymatic methods (21). Hepatic cholesteryl ester (CE) was calculated by subtracting free from total hepatic cholesterol. Hepatic TAG were determined by an enzymatic method after blanking for glycerol (22).

*Atherosclerotic lesion assessment*. Following removal of the liver, the chest cavity of the guinea pigs was opened, exposing

the heart. The heart was perfused with 10% formalin for 30 min at a pressure that supported slow but steady flow. Hearts were removed from guinea pigs with attached ascending and thoracic aorta and covered with 10% formalin at 4°C for 24 h. Hearts were placed in a container with phosphate-buffered saline (10 mmol/L sodium phosphate, $Na_2HPO_4$), 0.15 mol/L sodium chloride, and pH adjusted to 7.4 until analysis. Prior to quantification of atherosclerosis, the aortic arch was separated from the heart above the aortic valve. Lesions were evaluated as previously reported (30,31). Briefly, the portion of the aorta located between the third neck vessel and 1 mm above the aortic valve was used. The section was cleaned of adventitia and rinsed in 60% isopropanol, and then the inner aortic surface was stained with oil red O saturated 60% with the isopropanol solution. The section was then rinsed with distilled water and the luminal surface was stained with Gill's type hematoxylin (1:20 dilution) for 1 min. The aorta was then rinsed again and opened longitudinally and mounted on a glass slide with a glass coverslip and an aqueous mounting medium. The segments were then photographed using bright field microscopy. Images from the whole mounts were captured and digitized using a Color-Space II video digitizer (Massa Micro Systems, Sunnyvale, CA). The oil red O–stained area was analyzed using a Mac II computer and Image 1.23 software. Measurements are expressed in $\mu m^2/mm^2$.

*Statistics*. Data are presented as mean ± standard deviation. Differences in plasma lipids, lipoprotein composition, hepatic lipids, and aortic fatty streaks were evaluated by two-way analysis of variance (ANOVA) with diet and gender being the two variables. Fisher's least significant differences (LSD) *post-hoc* tests were used to determine significant differences among groups. Significant correlations between variables were determined by linear regression. $P < 0.05$ was considered significant.

## RESULTS

*Preliminary study.* A preliminary study was conducted to determine the percentage of cholesterol that would most effectively develop early atherosclerosis in guinea pigs. For that purpose, male guinea pigs were used. Three guinea pigs were fed the diet described in Table 1 with 0.25% dietary cholesterol, and three were fed the same diet with 0.33% cholesterol. Results from the preliminary study to test the efficacy of the diets on atherosclerosis were: 8.08 ± 0.87 mmol/L and 6.54 ± 1.0 mmol/L for TC, 6.85 ± 2.38 μmol/g and 8.79 ± 2.74 μmol/g for hepatic FC, and 23,240 ± 5,539 $\mu m^2/mm^2$ and 12,374 ± 3,512 $\mu m^2/mm^2$ for fatty streak area of guinea pigs fed the 0.33 and 0.25% dietary cholesterol, respectively. Thus, there were no significant differences in plasma cholesterol or hepatic lipid concentrations between groups. However, the aortic fatty streak was significantly lower ($P < 0.001$) in guinea pigs fed 0.25% than in those fed 0.33% cholesterol. Based on these results, the diet containing 0.33% dietary cholesterol was used to evaluate diet and gender effects on lipoprotein metabolism and atherosclerosis development.

*Plasma and lipoprotein cholesterol and TAG concentrations.* There were no significant differences between groups in food consumption or weight gain (data not shown). Plasma TC and TAG concentrations for male, female, and ovariectomized guinea pigs fed control and TEST diets are presented in Table 2. Plasma TC concentrations were significantly higher ($P < 0.0001$) in the ovariectomized guinea pigs compared to the males and females. Surprisingly, plasma cholesterol and TAG concentrations were not different between guinea pigs fed the control or TEST diets. No interactive effect was observed for plasma cholesterol (Table 2). However, plasma TAG concentrations were higher in ovariectomized guinea pigs fed the TEST diets ($P < 0.05$), indicating that hormonal status (lack of estrogen) and dietary soluble fiber and soybean protein interacted to induce the highest plasma TAG concentrations in this group of guinea pigs.

Plasma lipoprotein cholesterol levels of male, female, and ovariectomized guinea pigs are shown in Table 3. There were no significant effects of diet or gender or an interaction on plasma VLDL or HDL cholesterol values. In contrast, plasma LDL cholesterol concentrations, similar to the observations for plasma TC, were significantly influenced by gender ($P < 0.01$), with ovariectomized guinea pigs having the highest values. No diet or interaction effect was observed for plasma LDL cholesterol (Table 3).

*Hepatic cholesterol and TAG concentrations*. There was a significant diet effect ($P < 0.001$) on hepatic TC, but there was no gender or interaction effect, as shown in Table 4. Likewise, FC and esterified cholesterol were influenced by diet ($P < 0.01$). Guinea pigs fed the TEST diet had 50% lower hepatic cholesterol concentrations than those fed the control diet. No gender or interaction effects were observed for TC, FC, or esterified cholesterol in liver (Table 4). There was a diet and a gender effect on hepatic TAG; however, there was no interaction effect as shown in Table 4. Male, female, and ovariectomized controls had an average 54% higher hepatic TAG ($P < 0.01$) than the male, female, and ovariectomized TEST guinea pigs. There was a gender effect because the males and ovariectomized guinea pigs had higher ($P < 0.01$) hepatic TAG than females.

**TABLE 2**
**Plasma Lipids in Male, Female, and Ovariectomized Guinea Pigs Fed the Control or TEST Diet[a]**

| | mmol/L | |
|---|---|---|
| Diets | Cholesterol[b] | Triacylglycerol[b] |
| Control | | |
| Male ($n = 8$) | 6.62 ± 1.86[c] | 1.09 ± 0.33[c] |
| Female ($n = 7$) | 6.78 ± 3.28[c] | 1.35 ± 0.68[c] |
| Ovariectomized ($n = 10$) | 9.75 ± 2.58[d] | 1.83 ± 0.62[d] |
| TEST | | |
| Male ($n = 6$) | 7.11 ± 0.90[c] | 1.61 ± 0.75[c] |
| Female ($n = 6$) | 6.78 ± 1.68[c] | 0.94 ± 0.44[c] |
| Ovariectomized ($n = 6$) | 10.39 ± 4.55[d] | 2.52 ± 1.65[d] |
| Two-way ANOVA | | |
| Diet effect | NS | NS |
| Group effect | $P < 0.001$ | NS |
| Interaction | NS | $P < 0.05$ |

[a]Values are expressed as mean ± standard deviation (SD) for the number of guinea pigs indicated in parentheses.
[b]Numbers in the same column with different roman superscript letters are significantly different as determined by Fisher's least significant differences (LSD) *post-hoc* test. NS, nonsignificant; ANOVA, analysis of variance.

**TABLE 3**
**Plasma Lipoprotein Cholesterol of Male, Female, and Ovariectomized Guinea Pigs Fed the Control or TEST Diet[a]**

| | mmol/L | | |
|---|---|---|---|
| Diets | VLDL-C | LDL-C | HDL-C |
| Control | | | |
| Male ($n$ = 3) | 0.32 ± 0.26 | 7.92 ± 1.40$^{c}$ | 0.30 ± 0.10 |
| Female ($n$ = 3) | 0.17 ± 0.11 | 7.06 ± 3.31$^{c,d}$ | 0.32 ± 0.17 |
| Ovariectomized ($n$ = 5) | 0.16 ± 0.06 | 10.58 ± 1.40$^{d}$ | 0.40 ± 0.20 |
| TEST | | | |
| Male ($n$ = 3) | 0.23 ± 010 | 7.17 ± 0.39$^{c,d}$ | 0.41 ± 0.30 |
| Female ($n$ = 3) | 0.37 ± 0.17 | 6.59 ± 1.50$^{c}$ | 0.34 ± 0.35 |
| Ovariectomized ($n$ = 5) | 0.18 ± 0.06 | 10.99 ± 4.1$^{d}$ | 0.36 ± 0.24 |
| Two-way ANOVA | | | |
| Diet effect | NS | NS | NS |
| Group effect | NS | $P < 0.01$ | NS |
| Interaction | NS | NS | NS |

[a]Values are expressed as mean ± SD for the number of guinea pigs indicated in parentheses. Numbers in the same column with different roman superscript letters are significantly different as determined by Fisher's LSD *post-hoc* test. VLDL-C, very low density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; for other abbreviations see Table 2.

*Lipoprotein composition*. VLDL composition data are presented in Table 5. No significant gender or interaction effects in the relative percentage of FC, CE, PL, TAG, or protein or in the CE/protein ratio of VLDL were observed. However, there were diet effects on the relative percentage of CE, PL, and CE/protein ratio. The relative percentage of CE in VLDL was higher in guinea pigs fed control diets ($P < 0.01$), whereas the relative percentage of PL was higher in guinea pigs fed the TEST diet (Table 5). The ratio of CE to protein was significantly higher in guinea pigs fed the control diet ($P < 0.05$), suggesting that VLDL particles are larger in size since they have a higher core-to-surface ratio (Table 5). Fisher's LSD *post-hoc* test indicated that female guinea pigs fed the test diet had the lowest CE/protein ratio (Table 5).

LDL composition (FC, CE, PL, and TAG), in terms of number of molecules and LDL diameter, is presented in Table 6. There was a significant diet effect ($P < 0.05$) on the number of molecules of CE. Guinea pigs fed the control diet had a higher number of CE molecules than those fed the TEST diet. In addition, the number of TAG molecules was lower in the TEST groups ($P < 0.05$) compared to the control groups (Table 6). Diet and gender effects were observed when LDL diameter was evaluated. Guinea pigs fed the control diet had significantly larger ($P < 0.001$) LDL diameters than the groups fed the TEST diet. Females also had significantly smaller ($P < 0.05$) LDL diameters when compared to male or ovariectomized groups (Table 6).

*Aortic fatty streak*. Aortic fatty streak values were influenced by both diet and gender as shown in Table 7. Two-way ANOVA indicated that there was a significant diet effect ($P < 0.0001$), as fatty streaks in the control guinea pigs were larger than in those fed the TEST diet. A significant gender effect ($P < 0.01$) was also observed. Male animals had larger fatty streaks than female or ovariectomized animals. Female TEST guinea pigs presented less ($P < 0.01$) aortic fatty streak than any other group. Ovariectomized guinea pigs fed the TEST diet were different only from male control animals (Table 7).

To further assess whether LDL composition affected the deposition of CE for foam cell formation, correlations between LDL composition and size and atherosclerosis were evaluated. There was a positive correlation ($r = 0.495$, $P < 0.05$) between number of CE molecules in LDL and fatty streak area as shown in Figure 1. The size or diameter of the LDL particle was also correlated ($r = 0.56$, $P < 0.05$) with fatty streak area. Larger LDL particles appeared to be more atherogenic as depicted in Figure 2.

## DISCUSSION

These studies suggest that guinea pigs develop atherosclerosis with hypercholesterolemic diets and that the extension of the atherosclerotic lesion is closely associated with sex and hormonal status. This is an important finding because animal models for lipid metabolism need to respond to lipid accumulation in plasma by similar physiological and pathological

**TABLE 4**
**Hepatic Total (TC), Free (FC), and Esterified Cholesterol (EC) and Triacylglycerols (TAG) of Male, Female, and Ovariectomized Guinea Pigs Fed the Control or TEST Diet[a]**

| | μmol/g | | | |
|---|---|---|---|---|
| Diets | TC | FC | EC | TAG |
| Control | | | | |
| Male ($n$ = 8) | 13.2 ± 3.4$^{c}$ | 9.8 ± 3.1$^{c}$ | 3.6 ± 1.5$^{c}$ | 91.0 ± 38.4$^{c,d}$ |
| Female ($n$ = 7) | 12.4 ± 5.4$^{c}$ | 8.3 ± 3.9$^{c}$ | 4.1 ± 2.8$^{c}$ | 76.5 ± 31.9$^{d}$ |
| Ovariectomized ($n$ = 10) | 11.7 ± 3.3$^{c}$ | 8.2 ± 3.7$^{c}$ | 3.5 ± 1.7$^{c}$ | 115.3 ± 34.8$^{c}$ |
| TEST | | | | |
| Male ($n$ = 6) | 8.5 ± 4.1$^{c,d}$ | 6.5 ± 3.4$^{c,d}$ | 2.1 ± 1.0$^{d}$ | 47.3 ± 22.2$^{d,e}$ |
| Female ($n$ = 6) | 4.9 ± 2.1$^{d}$ | 3.7 ± 1.8$^{d}$ | 1.3 ± 0.8$^{d}$ | 21.1 ± 3.6$^{e}$ |
| Ovariectomized ($n$ = 6) | 9.1 ± 3.6$^{c,d}$ | 6.5 ± 3.1$^{c,d}$ | 2.8 ± 1.0$^{c,d}$ | 61.9 ± 31.3$^{d}$ |
| Two-way ANOVA | | | | |
| Diet effect | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.001$ |
| Group effect | NS | NS | NS | $P < 0.01$ |
| Interaction | NS | NS | NS | NS |

[a]Values are expressed as mean ± SD for the number of guinea pigs indicated in parentheses. Numbers in the same column with different roman superscript letters are significantly different as determined by Fisher's LSD *post-hoc* test. For other abbreviations see Table 2.

**TABLE 5**
**Relative Percentage of FC, Cholesteryl Ester (CE), Phospholipids (PL), TAG, and Protein in VLDL of Male, Female, and Ovariectomized Guinea Pigs Fed the Control or TEST Diet[a]**

| | g/100 g | | | | | |
|---|---|---|---|---|---|---|
| Diets | FC | CE | PL | TAG | Protein | CE/Prot |
| Control | | | | | | |
| Male ($n = 3$) | 9.1 ± 8.3 | 11.2 ± 9.2[c] | 7.9 ± 3.7[c] | 57.6 ± 17.6 | 14.2 ± 7.3 | 0.76 ± 0.73[c] |
| Female ($n = 3$) | 5.7 ± 4.7 | 9.6 ± 9.1[c] | 14.0 ± 4.5[c,d] | 55.3 ± 10.4 | 15.5 ± 4.7 | 0.55 ± 0.37[c,d] |
| Ovariectomized ($n = 5$) | 8.3 ± 3.2 | 3.3 ± 2.1[d] | 14.8 ± 4.7[d] | 59.2 ± 10.2 | 14.4 ± 5.3 | 0.26 ± 0.18[d] |
| TEST | | | | | | |
| Male ($n = 3$) | 7.3 ± 2.6 | 1.8 ± 2.8[d] | 16.6 ± 1.9[d,e] | 60.6 ± 1.2 | 13.6 ± 2.4 | 0.11 ± 0.17[d] |
| Female ($n = 3$) | 9.1 ± 3.6 | 2.1 ± 2.9[d] | 17.7 ± 5.8[d,e] | 47.9 ± 4.6 | 23.2 ± 2.7 | 0.09± 0.12[d,e] |
| Ovariectomized ($n = 5$) | 9.6 ± 3.4 | 1.1 ± 1.9[e] | 21.2 ± 3.5[e] | 49.9 ± 8.8 | 18.2 ± 2.8 | 0.07 ± 0.11[d,e] |
| Two-way ANOVA | | | | | | |
| Diet effect | NS | $P < 0.05$ | $P < 0.01$ | NS | NS | $P < 0.01$ |
| Group effect | NS | NS | NS | NS | NS | NS |
| Interaction | NS | NS | NS | NS | NS | NS |

[a]Values are expressed as mean ± SD for the number of guinea pigs indicated in parentheses. Numbers in the same column with different roman superscript letters are significantly different as determined by Fisher's LSD *post-hoc* test. For other abbreviations see Tables 2 and 4.

events leading to atherosclerotic lesions to make them appropriate models that mimic the human situation (32).

In addition, plasma LDL cholesterol concentrations were not related to the extent of the lesion; rather, LDL particle size and CE content appeared to be more important determinants of fatty streak formation in guinea pigs. Also, sex and hormonal status apparently play a major role in atherosclerotic lesion formation.

*Effects of diet and gender on hepatic cholesterol and TAG metabolism.* Hepatic FC and esterified cholesterol were lower in male, female, and ovariectomized guinea pigs fed the TEST diets. This hepatic cholesterol lowering could be a direct effect of soluble fiber interfering with absorption of dietary cholesterol. It could also be due to the mobilization of cholesterol from liver to synthesize bile acids to compensate for bile acid loss through interruption of the enterohepatic circulation (11). In addition, soy protein has also been shown to reduce absorption of dietary cholesterol and increase bile acid excretion; thus, the increased amount of soy protein in the TEST diet may have contributed to the lowering of cholesterol in liver (33).

These reductions in hepatic cholesterol have been related to up-regulation of apo B/E receptor activity, which in turn draws LDL cholesterol from plasma (34). However, in this study, after guinea pigs were fed the TEST and control diets for 12 weeks, no significant effects were observed on plasma cholesterol levels, which suggests that the up-regulation of hepatic apo B/E receptors is not taking place after longer feeding periods. Guinea pigs fed the TEST diet had lower hepatic TAG concentrations than animals fed the control diet. The mechanism causing decreased hepatic TAG concentrations may be related to the capacity of soluble fiber to delay their absorption in the intestinal lumen (35). Inhibition of lipogenesis in the liver may also account for the observed reduction of hepatic TAG in conjunction with delayed intraluminal absorption (36).

In addition, in rats a higher concentration of hepatic cholesterol has been correlated with higher concentration of hepatic TAG (37). This is due to a decreased synthesis of carni-

**TABLE 6**
**LDL Number of FC, CE, PL, and TAG Molecules and LDL Diameter of Male, Female, and Ovariectomized Guinea Pigs Fed the Control or TEST Diet[a]**

| | Number of molecules | | | | |
|---|---|---|---|---|---|
| Diets | FC | CE | PL | TAG | Diameter (Å) |
| Control | | | | | |
| Male ($n = 3$) | 502 ± 240 | 1413 ± 263[c] | 178 ± 57 | 91 ± 37[c] | 217.2 ± 11.7[c] |
| Female ($n = 3$) | 536 ± 333 | 1662 ± 528[c] | 402 ± 172 | 153 ± 135[c] | 213.4 ± 34.0[c] |
| Ovariectomized ($n = 5$) | 632 ± 185 | 1801 ± 306[c] | 237 ± 226 | 108 ± 43[c] | 235.7 ± 23.5[c] |
| TEST | | | | | |
| Male ($n = 3$) | 524 ± 69 | 1405 ± 262[c] | 191 ± 75 | 61 ± 4[d] | 207.3 ± 25.5[c] |
| Female ($n = 3$) | 316 ± 138 | 970 ± 225[d] | 236 ± 64 | 43 ± 10[d] | 163.0 ± 12.5[d] |
| Ovariectomized ($n = 5$) | 492 ± 141 | 1436 ± 278[c] | 198 ± 54 | 63 ± 5[d] | 213.0 ± 10.9[c] |
| Two-way ANOVA | | | | | |
| Diet effect | NS | $P < 0.05$ | NS | $P < 0.05$ | $P < 0.001$ |
| Group effect | NS | NS | NS | NS | $P < 0.05$ |
| Interaction | NS | NS | NS | NS | NS |

[a]Values are expressed as mean ± SD for the number of guinea pigs indicated in parentheses. Numbers in the same column with different roman superscript letters are significantly different as determined by Fisher's LSD *post-hoc* test. For abbreviations see Tables 2 and 4.

**TABLE 7**
**Aortic Fatty Streak of Male, Female, and Ovariectomized Guinea Pigs Fed the Control or TEST Diet[a]**

| Diets | Fatty streak ($\mu m^2/mm^2$) |
|---|---|
| Control | |
| Male ($n$ = 8) | 25871 ± 4292[c] |
| Female ($n$ = 7) | 21486 ± 1950[d] |
| Ovariectomized ($n$ = 10) | 22503 ± 4730[c,d] |
| TEST | |
| Male ($n$ = 6) | 20705 ± 4752[d] |
| Female ($n$ = 6) | 14771 ± 2999[e] |
| Ovariectomized ($n$ = 6) | 16576 ± 2094[d,e] |
| Two-way ANOVA | |
| Diet effect | $P < 0.0001$ |
| Group effect | $P < 0.01$ |
| Interaction | NS |

[a]Values are expressed as mean ± SD for the number of guinea pigs indicated in parentheses. Numbers in the same column with different roman superscript letters are significantly different as determined by Fisher's LSD *post-hoc* test. For abbreviations see Table 2.

tine, which may result in less mobilization of fatty acids to mitochondria for β oxidation and increased fatty acid and TAG synthesis (36). In the present study, the higher hepatic cholesterol concentrations in the control groups may reflect a similar situation to that observed in the rat. The higher levels of hepatic cholesterol in control guinea pigs may affect carnitine synthesis and promote TAG accumulation in the liver. Thus, in guinea pigs fed the TEST diets, the lower concentrations of hepatic TAG could also be related to the lower concentration of hepatic cholesterol observed in the TEST groups.

*Effects of diet and gender on plasma TC and TAG levels.* Previous studies have shown that pectin and psyllium lower plasma cholesterol and TAG in guinea pigs (11,14) and other animal models (15,38). In addition, isolated soy protein lowered plasma TC in cynomolgus monkeys (9), and hamsters and guinea pigs fed soybean protein for 4 wk also had plasma cholesterol lowering (24). Contrary to these findings, in the present study, soluble fiber in conjunction with soy protein had no effect on plasma cholesterol or TAG after a longer feeding period of 12 wk. These results were unexpected and

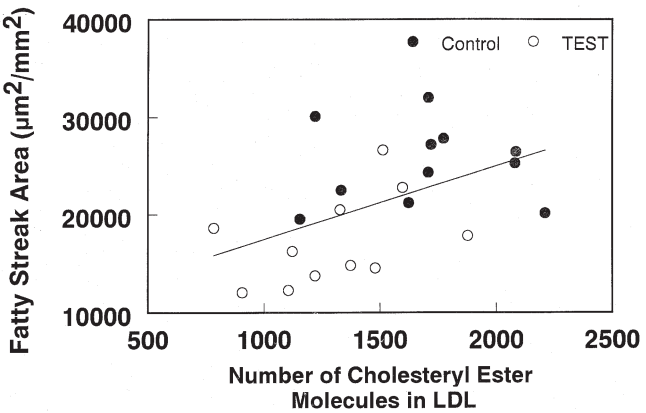


**FIG. 1.** Correlation between low density lipoprotein (LDL) number of molecules of cholesteryl ester and lesion involvement in male, female, and ovariectomized guinea pigs fed control (●) and TEST (○) diets ($r$ = 0.495, $P < 0.05$).

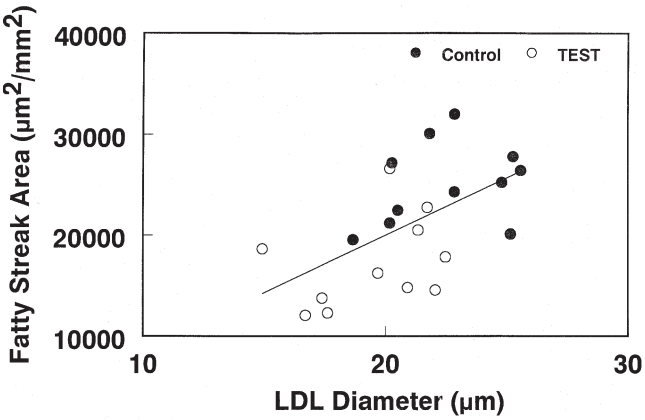


**FIG. 2.** Correlation between LDL diameter and lesion involvement in male, female, and ovariectomized guinea pigs fed control (●) and TEST (○) diets ($r$ = 0.56, $P < 0.01$). See Figure 1 for abbreviation.

can only be related to the longer feeding time in the present study compared to the earlier studies (11,14,24). Similar to the lack of correlation between plasma cholesterol and early atherosclerosis development found in the present study, Calleja *et al.* (39) also did not find changes in plasma cholesterol concentrations in apo E knockout mice eating different types of oils, whereas atherosclerotic lesions differed according to the type of fat and the sex of the animals.

In this study a group effect in TC was observed because ovariectomized guinea pigs had higher plasma cholesterol levels than males or females. Similar elevated plasma cholesterol and TAG levels have been observed in our previous study (18), and they could be attributed to the lack of estrogen. These results are in agreement with human studies that have shown postmenopausal women have significantly higher risk factors for CHD, such as higher plasma LDL cholesterol, apo B, and TAG levels (4).

Plasma TAG were highest in ovariectomized guinea pigs fed the TEST diet. It is possible that soluble fiber, similar to what has been observed in subjects treated with the bile acid-binding resin, cholestyramine (40) increased the release of VLDL from liver. Because VLDL is the major carrier of TAG in the preprandial state, an increased release of VLDL by the liver could cause an increase in plasma TAG. Vega-Lopez *et al.* (41) showed similar results in a crossover study with humans fed psyllium or control cookies for 4 wk. In this study, postmenopausal women had higher plasma TAG levels during the psyllium compared to the control period (41).

However, in female guinea pigs, plasma TAG were not increased by dietary fiber intake. These lower levels could be related to the lower hepatic TAG in females since higher hepatic triglycerides have been related to increased VLDL secretion rate (42). Endogenous estrogen may also play a role in the lowering of plasma TAG as well, although mechanisms are unclear.

*Effects of diet and gender on atherosclerosis and lipoprotein composition.* VLDL, intermediate density lipoprotein, and LDL are linked in a delipidation cascade in which TAG-rich VLDL, released from the liver, is converted into cholesterol-rich LDL. There could be a relationship between the composition of the TAG-rich VLDL being secreted by the

liver and the LDL formed in the plasma compartment. VLDL particles that are high in CE may be the precursors of LDL with a high number of CE molecules.

The TEST diet induced a lower relative percentage of CE in VLDL from male, female, and ovariectomized animals. In addition, the CE/protein ratio, which reflects the amount of esterified cholesterol compared to surface area, was higher in the control groups suggesting a larger VLDL. The VLDL particles rich in esterified cholesterol from the control guinea pigs will eventually become LDL also rich in esterified cholesterol, which are known to be more atherogenic (43). In agreement with our observations, CE-enriched VLDL have been shown to be more readily converted to LDL through the delipidation cascade in African green monkeys (44).

Epidemiologic research suggests that small, dense LDL particles may be atherogenic (45). Alternatively, there is evidence suggesting that a high-fat diet results in the predominance of larger LDL particles in humans (46) and saturated fat results in the formation of large LDL particles, which have been associated with increased atherosclerotic events (47) in nonhuman primates. In this study, intake of the control diet resulted in a higher number of CE molecules in LDL. An association between the number of CE molecules and aortic fatty streak was also observed suggesting a potential role of CE amount in early atherosclerosis development, similar to the observations in nonhuman primates (46).

In the present study, there was a reduction in LDL diameter in guinea pigs fed the TEST diet when compared to controls. In addition, an association between LDL diameter and fatty streak formation was found, implying that large LDL could be more atherogenic. Larger LDL particles were seen in the males and ovariectomized guinea pigs than the females. As a result, female TEST animals had the least amount of aortic fatty streak when compared to male and ovariectomized animals. It has also been shown in humans that large LDL particles have a reduced affinity for the LDL receptor compared with intermediate size LDL (48). This property of large LDL particles might enhance their uptake by nonreceptor-mediated pathways and thus increase their atherogenic potential (49). Interestingly, estrogen supplementation in postmenopausal women reduces levels of large LDL particles (50). The role of estrogen in reducing atherosclerosis could be related to a reduction of CE-enriched LDL particles. Honore *et al*. (51) reported that intravenous administration of genistein 30 min prior to administration of acethylcholine, a known constrictor of the artery, resulted in dilation of the artery in female monkeys. These results suggest that the higher concentrations of isoflavones present in the TEST diet might have contributed to the observed reduction in atherosclerotic lesions in guinea pigs.

In the present study we demonstrated that guinea pigs develop atherosclerosis when challenged with hypercholesterolemic diets and that sex and hormonal status mediate early lesion development. In addition, we showed a potential role of LDL composition and size in fatty streak accumulation, which is more significant than plasma LDL cholesterol concentrations. In addition, it was demonstrated that the contribution of dietary cholesterol to atherosclerosis could be greatly reduced in the presence of high levels of dietary soluble fiber and soybean protein. The influence of sex/hormonal status on lipoprotein metabolism and atherosclerotic lesions was also demonstrated. Female guinea pigs had the most beneficial responses, which might be associated with the presence of estrogen.

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# Incorporation of Dietary Linoleic and Conjugated Linoleic Acids and Related Effects on Eggs of Laying Hens

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**ABSTRACT:** In the present study, laying hens received 29 g per kg diet of a preparation containing either 70% linoleic acid (LA) or approximately the same amount of conjugated linoleic acid (CLA) in the control and experimental treatments, respectively. The CLA preparation consisted predominantly of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 fatty acid isomers as free fatty acids in a ratio of 1:1. The diets were fed for 8 wk to determine the effect of dietary CLA on quality characteristics of eggs. In addition, the fatty acid composition of liver and heart was analyzed. Performance parameters (egg weight, feed efficiency) were not significantly affected by feeding the diets supplemented with CLA. The overall amount of CLA that was incorporated into yolk was 7.95 g CLA/100 g total fatty acids, or approximately 400 mg CLA/egg. The transfer efficiency of the *cis*-9,*trans*-11 isomer was higher than that of the *trans*-10,*cis*-12 isomer; however, the transfer rate of CLA isomers into yolk and tissues was significantly lower than that of linoleic acid. Dietary CLA increased the concentration of saturated fatty acids in yolk and tissues at the expense of monounsaturated fatty acids. The proportions of myristic, palmitic, and stearic acids in yolk lipids were also changed by dietary CLA. Additionally, long-chain polyunsaturated fatty acids (arachidonic acid and docosahexaenoic acid) were decreased without changing the balance of the n-6/n-3 ratio in egg yolk. The inclusion of CLA in layer diets altered the shape of the yolk and various egg parameters (albumen height, foam index, and yolk index). The results of this study indicate that CLA induces various changes in lipid and fatty acid metabolism of laying hens and affects quality characteristics of eggs.

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Conjugated linoleic acid (CLA) is a common term for a group of dienoic isomers of linoleic acid that are present as minor constituents in certain foods. Naturally occurring CLA is produced as an intermediate product of microbial metabolism of linoleic acid in the rumen. CLA isomers are incorporated into the fat of milk and meat of ruminants, which explains their relatively high concentration in these products (about 3 to 8 mg CLA/g fat). On the other hand, foods derived from nonruminants such as poultry products have relatively low CLA levels: Edible parts of chicken and eggs, for example, contain approximately 0.9 and 0.6 mg of CLA/g fat, respectively (1). Naturally occurring CLA therefore enters human diets mainly from ruminant food fats. Animal studies have demonstrated that CLA isomers might have beneficial effects, including inhibition of carcinogenesis, modulation of the immune system, prevention of atherosclerosis, and reduction of body fat accumulation as reviewed by Belury (2) and Pariza (3). This has stimulated interest in dietary treatment with CLA and the production of foods enriched with CLA.

Ip *et al.* (4) estimated on the basis of a rat model study that, to obtain health benefits, a person weighing 70 kg should consume 3.0 g of CLA per day. The actual CLA intake, however, is less than that recommendation. In Germany, the actual CLA intake has been estimated at 0.36 g per day for women and 0.44 g per day for men (5). This raises the question as to possible ways of increasing CLA intake. In nonruminants such as pigs and chickens, the fatty acid composition of tissue lipids can be significantly altered by dietary fat; it is well known that dietary fatty acids are incorporated into tissue lipids. Enrichment of animal products with CLA is a means of raising the intake of CLA. The content of CLA in ruminant milk fat can also be enhanced [see review by Griinari and Baumann (6)], but in many regards it might be more promising to produce CLA-enriched food from broilers, pigs, and, as recently shown, from eggs. Champruspollert and Sell (7) reported that eggs from hens fed diets containing 5% CLA will contain 310 to 365 mg of CLA per egg. Consumer acceptance of fatty acid–modified eggs also depends on the quality characteristics of the treated eggs. Dietary lipids are known to affect the fatty acid composition of yolk lipids. The effect of CLA on egg quality parameters would therefore be an important consideration if eggs were to serve as a source of CLA in foods.

The objectives of this research were to determine whether dietary CLA free fatty acids consisting predominantly of two isomers could be transferred efficiently into egg yolk and to measure the effect of this CLA on egg quality parameters (yolk fatty acid composition, egg shell strength, albumen height, foam and yolk indices).

## MATERIALS AND METHODS

*Animals and diets.* A total of 60 laying hens (Lohmann Selected Leghorn, LSL), 31 wk of age, were housed individually in single-cage batteries. The birds were allocated to two

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Abbreviations: CLA, conjugated linoleic acid; FAME, fatty acid methyl ester; LA, linoleic acid; LDL, low density lipoprotein; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TAG, triacylglycerols.

groups of 30 hens each. The basal diet of the hens was supplemented with 29 g per kg of a preparation containing either predominantly linoleic acid (control group) or CLA (treatment group). The composition of the diets (g/kg) was as follows: wheat, 639; soybean meal, 200; limestone grit, 87; monocalcium phosphate, 18; soybean oil, 12; premix [containing vitamins, minerals, and amino acids according to requirements given by the National Research Council (8)], 16; LA (control diet) or CLA preparation (treatment diet), 29. Nutrients of the diets were as follows: metabolizable energy (MJ/kg), 11.9; crude protein (g/kg), 174; crude fiber (g/kg), 28; crude fat (g/kg), 57. The fat preparations containing LA or CLA were donated by TrocoCell (Hamburg, Germany). The LA preparation consisted of (in g/100 g fatty acids): palmitic acid, 7.8; stearic acid, 2.6; oleic acid, 12.8; linoleic acid, 73.4; others 3.4. The CLA preparation consisted of (in g/100 g fatty acids): palmitic acid, 7.2; stearic acid, 2.7; oleic acid, 13.2; linoleic acid, 1.1; *cis*-9,*trans*-11 CLA, 32.5; *trans*-10,*cis*-12 CLA, 32.2. The major difference between the two preparations was therefore the replacement of linoleic acid by CLA isomers, whereas concentrations of the other fatty acids were similar in both preparations. The fatty acids of both preparations were present as free fatty acids. The fatty acid composition of total lipids of the diets is shown in Table 1. The hens were given free access to food and water. Body weights, feed intake, and egg production were recorded weekly.

At the end of the 8-wk feeding trial (39 wk of age) five hens per treatment were randomly selected and slaughtered. Livers and hearts were removed and washed with saline. The organs were weighed and frozen at –20°C pending analysis. Dry matter, protein, and fat content of yolk and organs were determined, as was the fatty acid composition. Additionally, various egg characteristics, the major individual lipid fractions (triacylglycerols, phospholipids, cholesterol) and minerals were analyzed for determination of treatment effects. Egg quality was determined with reference to specific parameters (egg shell breaking strength, albumen height, yolk index, and foam index).

*Determination of crude nutrient contents.* Crude protein, crude fiber, and crude fat were analyzed according to standard procedures (9).

*Analysis of fatty acid composition.* Details of the method have been described previously (10). Total lipid was extracted from freeze-dried and finely ground animal tissues using chloroform/methanol 2:1 (vol/vol). Quantitation of fatty acids was done after preparation of fatty acid methyl esters (FAME) with trimethylsulfonium hydroxide (0.2 M in methanol; Macherey-Nagel, Düren, Germany). A Hewlett-Packard (HP) gas chromatograph, equipped with a 50 m Sil 88 capillary column (Chrompack, Middelburg, The Netherlands) of 0.25 mm i.d. and a coating thickness of 0.20 μm was used to separate FAME. Helium carrier-gas column flow rate was 0.85 mL/min. The injector temperature was maintained at 250°C. A temperature program with total run time of 68 min was used. The column temperature, after an initial isothermal period of 5 min at 60°C, was increased to 180°C in increments of 5°C/min, and was maintained at this temperature for 16 min. The temperature was increased again to 220°C and was maintained at the final temperature for 15 min. Samples (1 μL) were introduced with an automatic sampler HP 7673A. CLA *cis*-9,*trans*-11 and *trans*-10,*cis*-12 were separated as distinct peaks without any serious interference or overlaps. Fatty acid standards including CLA methyl esters and 13:0 as an internal standard were purchased from Sigma (Deisenhofen, Germany). The integration of chromatograms was performed using an HP 3365 II Chem Station. All results are given in g/100 g total fatty acids.

*Determination of total cholesterol, triacylglycerols (TAG), and phospholipids.* For the determination of yolk lipids, eggs were cooked. Lipids of cooked egg yolks were extracted using a mixture of hexane and isopropanol (3:2, vol/vol). An aliquot of the extract was pipetted into a reaction vial and the solvent evaporated under vacuum. The lipids were dissolved in a small volume of a mixture of chloroform and Triton X-100, and the solvent was evaporated again. Commercially available kit reagents for cholesterol and TAG assays were added and the concentrations of TAG and total cholesterol assayed by colorimetry (11).

*Analysis of minerals and trace elements.* To determine the concentrations of minerals in yolk, 0.5 g of dried samples were mineralized with 5 mL of 65% nitric acid in a 25-mL flask in a microwave oven at 180°C and topped up with demineralized water to 25 mL. Concentrations of minerals were determined by plasma emission spectrophotometry (inductively coupled plasma; Spectro Analytical Instruments, Klewe, Germany). Phosphorus was measured spectrophotometrically by the molybdate-vanadate method (9).

*Determination of egg quality parameters*. Eggshell breaking strength was determined on five eggs per treatment using a testing machine. Eggs were compressed between two plates with the major axis perpendicular to the compression surface. Breaking strength was defined as the force in N required to fracture the shell. To measure albumen height, the egg was cracked at room temperature on a reflector measuring plate. The height of albumen and yolk was measured in two eggs from each hen using a height gauge. The yolk index is defined as the ratio of yolk height (×100) to yolk width. The foam index was determined by pooling the whites of three eggs from each hen and beating air into 100 mL of liquid egg white for 120 s. The foam index is defined as the ratio of foam volume to volume of liquid egg white.

**TABLE 1**
**Fatty Acid Composition of Total Lipids of the Experimental Diets (g/100 g fatty acids)**

| | | LA diet | CLA diet |
|---|---|---|---|
| Palmitic acid | 16:0 | 10.1 | 9.0 |
| Stearic acid | 18:0 | 4.5 | 3.8 |
| Oleic acid | 18:1 | 22.1 | 21.2 |
| Linoleic acid | 18:2 | 55.5 | 19.0 |
| CLA isomers | 18:2 *cis*-9,*trans*-11 | —[a] | 18.1 |
| | 18:2 *trans*-10,*cis*-12 | — | 18.6 |
| | 18:2 others | — | 1.8 |
| α-Linolenic acid | 18:3 | 3.9 | 3.9 |
| Others | | 3.9 | 4.6 |

[a] —, less than 0.1 g/100 g fatty acids. LA, linoleic acid; CLA, conjugated linoleic acid.

*Calculation of transfer rates.* Transfer rates of CLA isomers and LA from diet into yolk or liver and heart were calculated as a proportion of the respective concentration ratios [as proposed by Pettersen and Opstvedt (12) for *trans* fatty acids and by Bee (13) for CLA of milk lipids in pigs]:

$$\text{transfer rate} = \frac{[\text{CLA}]/[\text{PUFA}] \text{ in the tissue}}{[\text{CLA}]/[\text{PUFA}] \text{ in the diet}} \quad [1]$$

where PUFA means polyunsaturated acids. Tissue (CLA values used in these calculations are reported in Table 7 ($n$ = 5), whereas diet values for CLA are taken from Table 1.

*Statistical analysis.* Data are presented as means ± SD. The level of significance for differences between groups was tested using either Student's *t*-test (parametric test) or the Wilcoxon-Mann-Whitney test (nonparametric test).

## RESULTS

*Animal performance data and egg quality parameters.* Performance parameters are shown in Table 2. Performance of hens was measured from 36 to 38 wk of age (test period 21 d). Body weights, egg performance (number of eggs, egg weights, and total egg mass), and feed efficiency for egg production did not differ between the two treatment groups. However, hens fed the CLA diet consumed slightly less food than those fed the LA diet. Weights of liver and heart were similar in both groups; however, the relative weight of the liver (as a percentage of body weight) was slightly higher in the hens fed the CLA diet, whereas the relative weight of heart was slightly lower than in the hens fed the LA diet (Table 3).

Eggshell stability was not significantly affected by the dietary treatment (Table 4). However, eggs from hens whose diet was supplemented with CLA had a higher yolk index and a higher foam index than eggs from hens fed the control diets. Albumen height did not differ significantly between the two groups. The concentrations of various minerals (the minerals and trace elements were identical with those shown in Table 5) in egg shells were also similar for both treatment groups (data not shown). In contrast, yolks of hens fed CLA had higher concentrations of phosphorus and zinc than yolks of hens fed the control diets (Table 5).

**TABLE 2**
**Parameters of Laying Hens Receiving a Diet Supplemented with an LA Preparation or a CLA Preparation Between the Test Period of 36 and 38 wk of Age[a]**

| | Units | LA | CLA |
|---|---|---|---|
| Initial body weight (36th wk) | kg | 1.96 ± 0.13 | 1.99 ± 0.15 |
| Final body weight (38th wk) | kg | 1.97 ± 0.18 | 2.00 ± 0.12 |
| Feed intake | kg | 2.59 ± 0.01[b] | 2.42 ± 0.03[c] |
| Eggs/hen | n | 23.9 ± 1.4 | 23.1 ± 1.6 |
| Egg weight | g | 65.6 ± 3.7 | 63.9 ± 4.2 |
| Total egg mass | g | 1568 ± 125 | 1477 ± 246 |
| Feed efficiency | kg feed/kg egg mass | 1.65 ± 0.09 | 1.64 ± 0.10 |

[a]Results are means ± standard deviations (SD) with $n$ = 27 for both groups. Means with different superscript letters differ significantly by Student´s *t*-test ($P < 0.05$). For abbreviations see Table 1.

**TABLE 3**
**Weights of Liver and Heart of Laying Hens Receiving a Diet Supplemented with an LA Preparation or a CLA Preparation at the 39th Wk of Age[a]**

| | LA | CLA |
|---|---|---|
| Liver, absolute (g) | 53.0 ± 4.7 | 58.4 ± 5.3 |
| Relative to body weight (%) | 2.62 ± 0.13[b] | 2.85 ± 0.19[c] |
| Heart, absolute (g) | 17.6 ± 1.1 | 16.6 ± 0.9 |
| Relative to body weight (%) | 0.87 ± 0.02[b] | 0.81 ± 0.06[c] |

[a]Results are means ± SD with $n$ = 5 for both groups. Means with different superscript roman letters differ significantly by Student´s *t*-test ($P < 0.05$). For abbreviations see Tables 1 and 2.

*Concentrations of lipids and protein in egg, liver, and heart.* The effect of CLA on the lipid concentrations of egg yolks is summarized in Table 6. Feeding the CLA diet reduced the concentrations of TAG, phospholipids, and total lipids; the concentration of total cholesterol, however, did not differ between the two treatments. Crude protein levels were higher in eggs from hens fed the CLA diet than in eggs from hens fed the control diet. In contrast, the concentrations of crude protein and crude fat in liver and heart did not differ between the two treatments (data not shown), but the ratio of fat to protein in both liver and heart was slightly reduced by feeding the CLA diets (liver: 0.48 ± 0.05 vs. 0.42 ± 0.03; heart: 1.40 ± 0.13 vs. 1.22 ± 0.12).

*Fatty acid composition of yolk, liver, and heart lipids.* The fatty acid composition of lipids from egg yolk, liver, and heart was significantly modified by the CLA diets (Table 7). Feeding the CLA diet caused extensive incorporation of both CLA isomers into yolk and tissue lipids. Moreover, yolk and tissue of hens fed the CLA diets had higher concentrations of saturated fatty acids (SFA) and lower concentrations of monounsaturated fatty acids (MUFA) than yolk and tissues of hens fed the control diets. The ratio of palmitic to stearic acid was reduced from 2.7 (control group) to 1.8 (treatment group) by feeding the CLA diet, and the concentration of arachidonic acid in yolk and liver was also lowered. The concentration of docosahexaenoic acid was decreased in yolk but increased in heart with the CLA diet. The ratio of n-6 to n-3 PUFA was not affected by feeding the CLA diet.

**TABLE 4**
**Parameters of Egg Quality in Laying Hens Receiving a Diet Supplemented with an LA Preparation or a CLA Preparation[a]**

| | LA | CLA |
|---|---|---|
| Breaking strength of egg shell (N)[b] | 26.39 ± 5.20 | 28.25 ± 8.44 |
| Height of egg white (mm)[c] | 5.34 ± 1.15 | 4.96 ± 0.89 |
| Yolk index[c,d] | 46.2 ± 2.8[f] | 52.1 ± 5.7[g] |
| Foam index[c,e] | 5.82 ± 1.20[f] | 6.84 ± 1.34[g] |

[a]Results are means ± SD. Means with different superscript roman letters (f, g) differ significantly by Student´s *t*-test ($P < 0.05$)
[b]The number of samples was 48 in the LA group and 46 in the CLA group.
[c]The number of samples was 30 in both groups.
[d](Height of yolk × 100)/width of yolk.
[e]Foam volume/egg white volume. For abbreviations see Tables 1 and 2.

**TABLE 5**
**Concentrations of Some Minerals (g/kg dry matter) in the Yolk of Laying Hens Receiving a Diet Supplemented with an LA preparation or a CLA Preparation[a]**

| | LA | CLA |
|---|---|---|
| Sodium | 1.34 ± 0.17 | 1.41 ± 0.19 |
| Magnesium | 0.27 ± 0.03 | 0.33 ± 0.02 |
| Phosphorus | 10.0 ± 0.07[b] | 10.5 ± 0.43[c] |
| Potassium | 2.17 ± 0.19 | 1.95 ± 0.10 |
| Calcium | 2.89 ± 0.29 | 3.25 ± 0.16 |
| Iron | 0.11 ± 0.01 | 0.14 ± 0.02 |
| Copper | 0.0036 ± 0.0007 | 0.0034 ± 0.0003 |
| Zinc | 0.075 ± 0.001[b] | 0.084 ± 0.004[c] |

[a]Results are means ± SD with $n = 3$ for both groups. Means with different superscript roman letters differ significantly by Student´s *t*-test ($P < 0.05$). For abbreviations see Tables 1 and 2.

**TABLE 6**
**Concentrations of Lipids and Crude Protein in the Yolk of Eggs from Laying Hens Receiving a Diet Supplemented with an LA Preparation or a CLA Preparation[a]**

| | LA | CLA |
|---|---|---|
| Triacylglycerols | | |
| g/yolk | 4.35 ± 0.59[b] | 3.57 ± 0.68[c] |
| mg/g dry matter | 480 ± 44[b] | 417 ± 73[c] |
| Phospholipids | | |
| g/yolk | 1.44 ± 0.18[b] | 1.33 ± 0.17[c] |
| mg/g dry matter | 159 ± 12 | 155 ± 10 |
| Total cholesterol | | |
| g/yolk | 0.198 ± 0.038 | 0.207 ± 0.094 |
| mg/g dry matter | 21.9 ± 3.4 | 24.5 ± 11.4 |
| Total lipids | | |
| g/yolk | 5.99 ± 0.76[b] | 5.11 ± 0.73[c] |
| mg/g dry matter | 611 ± 50[b] | 597 ± 72[c] |
| Crude protein | | |
| mg/g dry matter | 316 ± 12[b] | 335 ± 8[c] |

[a]Results are means ± SD with $n = 24$ for both groups. Means with different superscript roman letters differ significantly by Student´s *t*-test ($P < 0.05$). For abbreviations see Tables 1 and 2.

*Transfer efficiency of CLA isomers and LA into yolk, liver, and heart lipids.* On the basis of calculated transfer rates (Table 8), the incorporation of LA into yolk and tissues was higher than that of CLA isomers. Of the two CLA isomers, the transfer efficiency of the *cis*-9,*trans*-11 isomer was higher than that of the *trans*-10,*cis*-12 isomer. Moreover, the results indicate tissue-specific features in the CLA isomer profile. The relative transfer efficiency of the *cis*-9,*trans*-11 isomer into yolk is higher (up to 0.88) than into liver (up to 0.66) and heart (up to 0.32).

## DISCUSSION

*Chemical properties of CLA-enriched eggs.* The growing focus on the biological and health benefits of CLA has stimulated interest in producing CLA-enriched foods. The results presented here show that laying hens fed a CLA-enriched diet accumulated CLA in yolk lipids. The overall amount of CLA that was incorporated into yolk was 7.95 g CLA/100 g total fatty acids, or approximately 400 mg CLA/egg. An amount similar to that of hens fed 5% CLA (365 mg CLA/egg) was reported by Chamruspollert and Sell (7). Supplementing the diet with CLA had no adverse effects on the laying performance of hens. This is in agreement with results of other studies (7,14–16).

CLA has been reported to modulate energy metabolism and body composition. Animals that were fed diets supplemented with CLA deposited less body fat, and the ratio of fat to protein was modified in favour of the protein fraction. The effect of CLA in reducing body fat accretion was most conspicuous in growing animals such as mice (17), pigs (18), and

**TABLE 7**
**Fatty Acid Composition (g/100 g of total fatty acids) of Yolk, Liver, and Heart Total Lipids of Laying Hens Receiving a Diet Supplemented with an LA Preparation or a CLA Preparation[a]**

| | Yolk | | Liver | | Heart | |
|---|---|---|---|---|---|---|
| | LA | CLA | LA | CLA | LA | CLA |
| Total SFA | 34.35 ± 0.78[b] | 54.38 ± 4.31[c] | 33.88 ± 0.79[b] | 53.35 ± 7.99[c] | 25.00 ± 0.45[b] | 29.45 ± 1.08[c] |
| 14:0 | 0.35 ± 0.02[b] | 0.88 ± 0.19[c] | 0.44 ± 0.07[b] | 0.81 ± 0.21[c] | 0.53 ± 0.02[b] | 0.65 ± 0.05[c] |
| 16:0 | 24.83 ± 0.76[b] | 34.53 ± 2.65[c] | 23.24 ± 0.70[b] | 33.86 ± 5.70[c] | 18.33 ± 0.29[b] | 20.85 ± 0.88[c] |
| 18:0 | 9.17 ± 0.25[b] | 18.97 ± 2.12[c] | 10.21 ± 0.85[b] | 18.68 ± 2.18[c] | 6.14 ± 0.18[b] | 7.95 ± 0.21[c] |
| Total MUFA | 37.13 ± 2.48[b] | 18.93 ± 2.32[c] | 36.35 ± 3.69[b] | 19.36 ± 3.12[c] | 37.85 ± 0.73[b] | 34.19 ± 0.50[c] |
| 16:1 | 2.00 ± 0.10[b] | 0.60 ± 0.04[c] | 2.22 ± 0.53[b] | 0.85 ± 0.14[c] | 2.53 ± 0.13[b] | 2.13 ± 0.05[c] |
| 18:1 | 35.13 ± 2.40[b] | 18.33 ± 2.34[c] | 34.12 ± 3.38[b] | 18.52 ± 3.12[c] | 35.32 ± 0.67[b] | 32.06 ± 0.50[c] |
| Total PUFA | 23.25 ± 2.19 | 19.75 ± 2.10 | 25.99 ± 3.98 | 22.61 ± 4.26 | 31.65 ± 1.02 | 31.08 ± 0.95 |
| 18:2 | 19.93 ± 2.11[b] | 9.80 ± 1.35[c] | 19.98 ± 2.57[b] | 10.88 ± 2.61[c] | 27.52 ± 0.83[b] | 20.77 ± 0.83[c] |
| 18:3 | 0.57 ± 0.09[b] | 0.85 ± 0.14[c] | 0.92 ± 0.25 | 0.90 ± 0.22 | 2.31 ± 0.09 | 2.26 ± 0.03 |
| 20:4 | 2.03 ± 0.06[b] | 0.81 ± 0.06[c] | 3.33 ± 0.83[b] | 1.86 ± 0.61[c] | 1.64 ± 0.09 | 1.87 ± 0.48 |
| 20:5 | — | — | 0.31 ± 0.10 | 0.28 ± 0.05 | 0.13 ± 0.02 | 0.13 ± 0.03 |
| 22:6 | 0.66 ± 0.08[b] | 0.34 ± 0.02[c] | 1.46 ± 0.96 | 1.03 ± 0.32 | 0.17 ± 0.06[b] | 0.26 ± 0.06[c] |
| Total CLA | 0.05 ± 0.01[b] | 7.95 ± 0.90[c] | 0.24 ± 0.13[b] | 7.78 ± 1.18[c] | 0.32 ± 0.07[b] | 5.81 ± 0.31[c] |
| *Cis*-9,*trans*-11 | 0.05 ± 0.01[b] | 5.13 ± 0.55[c] | 0.20 ± 0.05[b] | 4.43 ± 0.69[c] | 0.23 ± 0.039[b] | 2.97 ± 0.22[c] |
| *Trans*-10,*cis*-12 | — | 2.500 ± 0.300 | — | 2.75 ± 0.45 | 0.09 ± 0.03[b] | 2.47 ± 0.13[c] |
| Other isomers | — | 0.32 ± 0.06 | — | 0.76 ± 0.10 | — | 0.37 ± 0.04 |

[a]Results are means ± SD with $n = 5$ for both groups. Means with different superscript roman letters differ significantly by Student´s *t*-test ($P < 0.05$). For abbreviations see Tables 1 and 2.

**TABLE 8**
**Transfer Rate of LA and CLA Isomers from the Diet into Heart, Liver, and Egg Yolk[a]**

| | LA | $CLA_{total}$ | *cis*-9,*trans*-11 | *trans*-10,*cis*-12 |
|---|---|---|---|---|
| Heart | 2.16 | 0.30 | 0.32 | 0.26 |
| Liver | 1.55 | 0.55 | 0.66 | 0.40 |
| Yolk | 1.60 | 0.64 | 0.88 | 0.42 |

[a]Details of calculations are given in the Materials and Methods section. For abbreviations see Table 1.

fast-growing chicks (19). A major objective of the present study was to investigate whether dietary CLA treatment of laying hens also influenced the fat-to-protein ratio in eggs. The results of this study demonstrate that this is the case. Feeding a diet supplemented with CLA also reduced the ratio of fat to protein in the liver and heart. Thus, a fat-to-lean partitioning effect of CLA was evident in laying hens as well.

Yolk lipid composition depends on the rate of liver lipid neosynthesis, hepatic uptake of fatty acids from the blood, and incorporation of lipid components from the diet (20). This study shows that the rate of incorporation varies for different CLA isomers. A higher transfer rate of the *cis*-9,*trans*-11 isomer compared with that of *trans*-10,*cis*-12 isomer as observed in this study was also reported for rodents (17), pigs (13,21), broilers (19), and cows (22). The efficiency with which the *cis*-9,*trans*-11 isomer is transferred from the diet into the egg seems to be similar to the rate of transfer from the diet into sow's milk (13). The calculated transfer rates show, however, that the transfer rate of both CLA isomers is much lower than that of LA. Incorporation of dietary fatty acids into tissues depends on several factors such as rate of absorption from the diet, metabolic utilization for β-oxidation, desaturation to highly unsaturated fatty acids, and incorporation into specific lipid fractions. Little is known to date about the absorption of CLA isomers from the diet, their utilization for β-oxidation, and their desaturation. The incorporation pattern of CLA isomers and LA is quite different, however. The majority of CLA is incorporated into the TAG fraction of eggs, whereas only a small proportion is incorporated into phospholipids (23). LA, on the other hand, is incorporated at similar levels into TAG and phospholipids, in particular phosphatidylcholine (24).

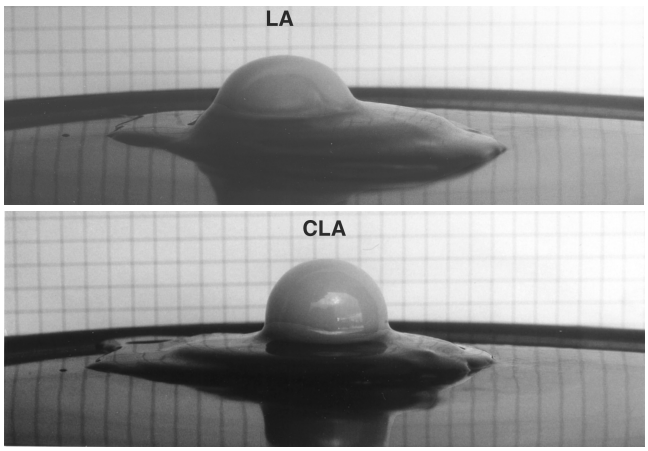


**FIG. 1.** Shape of yolks of eggs from by a laying hen receiving a diet supplemented with a linoleic acid (LA) preparation or a conjugated linoleic acid (CLA) preparation. The effect of CLA was observed after storage of eggs under refrigeration conditions (4°C).

*Effects of CLA-enriched eggs on blood lipids in humans.* This study shows that feeding CLA-supplemented diets is a means of enriching eggs with CLA. According to the literature, CLA might provide human health benefits, particularly with regard to cancer prevention. This study shows that incorporation of CLA into eggs is associated with a general alteration of the fatty acid composition of egg yolk lipids. Compared with the LA group, significantly more SFA and less MUFA were deposited in yolk lipids. Additionally, long-chain PUFA (arachidonic acid and docosahexaenoic acid) were decreased without changing the balance of the n-6/n-3 ratio in egg yolk. The increase in the SFA concentration and the decrease in PUFA in yolk lipids as a result of dietary CLA might be relevant with regard to cholesterol concentration in plasma and the low density lipoprotein (LDL) fraction in humans consuming these eggs. The ratio of PUFA to SFA decreased from 0.68 of yolk in group LA to 0.36 of yolk in group CLA. Dietary SFA are known to increase the cholesterol concentration, whereas PUFA, particularly of the n-6 type, are known to reduce it. Thus, the incorporation of CLA into egg yolk results in a fat with an increased atherogenic index. On the other hand, studies with model animals, particularly hamsters, indicate that CLA isomers could lower cholesterol concentrations in plasma and LDL, thus reducing the ratio of LDL cholesterol to high density lipoprotein–cholesterol, which is also a strong atherogenic indicator (25). It is as yet uncertain whether CLA also lowers cholesterol levels in humans. We are therefore unable to draw a firm conclusion as to whether CLA-enriched eggs would have beneficial effects on blood lipids of humans.

*Physical characteristics of CLA-enriched eggs.* The present study has demonstrated that feeding diets supplemented with CLA modifies some egg quality characteristics such as eggshell breaking strength, albumen height, foam index, and yolk index. The changes in the yolk index could be due to alterations of the lipid composition of egg yolk. The concentration of SFA in egg yolk is generally kept at a constant level, regardless of the fat in the hen's diet (26). CLA caused a marked increase of SFA in yolk at the expense of MUFA as a result of a reduced activity of Δ9 desaturase (27). This large increase in SFA and the reduction of available MUFA could be associated with alterations of physical properties, particularly the shape of the yolk. At room temperature, yolk is slightly higher in eggs of hens fed diets supplemented with CLA than in eggs of control hens. At a low temperature of 4°C, the increase in yolk height is very pronounced (Fig. 1). Feeding diets supplemented with cottonseed oil to hens also causes a marked increase in the stearic acid concentration in yolk (28). This effect might be due to reduced Δ9-desaturation caused by cyclopropene fatty acids present in cottonseed oil (29). The increased SFA concentration was also associated with alterations in the shape and physical properties of egg yolk similar to those observed with dietary CLA in the present study.

This suggests that increasing the content of SFA at the expense of MUFA affects physical properties of egg yolk.

There are potentially several different ways in which CLA can affect the physical properties of eggs. Evaluation of eggs during storage at 4°C confirmed that CLA-enriched eggs differ from typical eggs (30). Storage of CLA-enriched eggs increased the firmness and texture (elastic properties) of hard-boiled egg yolk. Also, the possibility of CLA-induced alterations of eggshell matrix proteins cannot be ruled out. Eggshell matrix proteins are thought to influence the mechanical properties of the eggshell (31). In the present study CLA treatment marginally increased eggshell stability. However, current knowledge of CLA-related effects on eggshell quality parameters is limited, and further studies on the effects of CLA isomers on physical properties of eggs are needed.

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# *Trans*-10,*Cis*-12 Conjugated Linoleic Acid Reduces Triglyceride Content While Differentially Affecting Peroxisome Proliferator Activated Receptor γ2 and aP2 Expression in 3T3-L1 Preadipocytes

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**ABSTRACT:** A series of experiments was conducted using 3T3-L1 preadipocytes as the cell model to determine: (i) whether the triglyceride (TG)-lowering effects of a crude mixture of conjugated linoleic acid (CLA) isomers were due to a specific isomer of CLA and the timing of treatment, (ii) if CLA reduced TG content by inhibiting a key regulator of adipogenesis, (iii) if CLA incorporated into either neutral lipid or phospholipid cell fractions, and (iv) whether the effects of CLA treatment were reversible. *Trans*-10,*cis*-12 CLA reduced TG content, whereas the *cis*-9,*trans*-11 isomer increased TG content compared to vehicle [bovine serum albumin (BSA)] controls. Treatment with 50 μM *trans*-10,*cis*-12 CLA during the entire 6 d of differentiation reduced TG content to a greater extent than treatment during either the first 3 d or last 3 d of differentiation. *Trans*-10,*cis*-12 CLA treatment of preadipocyte cultures for 48 h increased peroxisome proliferator activated receptor γ2 (PPARγ2) protein expression compared to cultures treated with linoleic acid (LA) or the BSA controls. CLA had no effect on adipose P2 (aP2), a fatty acid-binding protein regulated by PPARγ2. Both the *cis*-9,*trans*-11 and the *trans*-10,*cis*-12 isomers of CLA were incorporated into neutral lipids and phospholipids. However, *cis*-9,*trans*-11 CLA levels were one- to twofold higher than *trans*-10,*cis*-12 CLA levels. Moreover, *trans*-10,*cis*-12 CLA treatment reduced *cis*-11 18:1 concentrations in both neutral lipids and phospholipids while increasing *cis*-9 18:1 and 18:2 concentrations. Palmitoleic acid (16:1) levels were also lower in the neutral lipid fraction of cultures treated with *trans*-10,*cis*-12 CLA. Supplementing *trans*-10,*cis*-12 CLA-treated cultures (50 μM) with increasing levels of LA resulted in a dose-dependent increase in TG content compared to cultures treated with 50 μM CLA alone. LA supplementation also prevented some of the morphological changes associated with *trans*-10,*cis*-12 CLA treatment as seen with scanning electron microscopy. Treatment with 50 μM *trans*-10,*cis*-12 CLA for 6 d decreased PPARγ2 levels, and supplementation of CLA-treated cultures with LA increased PPARγ2 levels compared with cultures treated with CLA alone. Taken together, these data indicate that in cultures of 3T3-L1 preadipocytes: (i) *trans*-10,*cis*-12 CLA is the TG-lowering isomer of CLA, and its effects are dependent on dose, duration of treatment, and the amount of LA in the cultures; (ii) *trans*-10,*cis*-12 CLA treatment alters the monounsaturated fatty acid profile of neutral- and phospholipids of the cultures; and (iii) although acute (2-d) *trans*-10,*cis*-12 CLA treatment increased PPARγ2 protein levels, chronic (6-d) treatment decreased PPARγ2 levels.

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Conjugated linoleic acid (CLA) consists of a group of positional and geometric fatty acid isomers that are derived from linoleic acid (LA; 18:2n-6). Food sources of CLA, such as ruminant meats, pasteurized cheeses, and dairy products, are rich (~80%) in the *cis*-9,*trans*-11 isomer. Commercial sources of CLA used in many research studies contain predominantly *cis*-9,*trans*-11 (~40%) and *trans*-10,*cis*-12 (~40%) isomers. In rodents, CLA has anticarcinogenic (1), antiatherogenic (2,3), antidiabetic (4), and antiobesity properties (4–9). As an antiobesity agent, mice, pigs, and hamsters fed low levels of CLA (<1.5%, w/w) had less body fat and more lean body mass than controls (5–8,10–14). In addition, CLA treatment (3.4–6.8 g/d) for 3 mon reduced body fat mass of obese and overweight adult men and women (15). In contrast, Zambell *et al.* (16) found that CLA consumption (3 g/d mixed isomers) over 3 mon did not affect fat mass, fat-free mass, percent body fat, or body weight in normal weight human subjects.

*In vitro*, several studies have shown that treatment with a mixture of CLA isomers alters the lipid content of (pre)adipocytes (6,8,17–19). For example, Park *et al.* (6) showed that mature murine 3T3-L1 adipocytes treated with 20–200 μM of a mixture of CLA isomers for 2 d had less triglyceride (TG) content and lower lipoprotein lipase (LPL) activity compared to control cultures. Moreover, the *trans*-10,*cis*-12 isomer of CLA was determined to be the bioactive isomer that reduced LPL activity and TG content (8). However, another study (17) found that differentiating 3T3-L1 preadipocytes treated with a mixture of CLA isomers had greater rates of lipogenesis and more TG than

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; BCA, bicinchoninic acid; BSA, bovine serum albumin; CLA, conjugated linoleic acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HMDS, hexamethyldisilazane; IBMX, isobutylmethylxanthine; LA, linoleic acid; LPL, lipoprotein lipase; PBS, phosphate buffered saline; PPARγ2, peroxisome proliferator activated receptor γ2; SCD-1, stearoyl-CoA desaturase-1; SEM, scanning electron microscopy; TBP,TFII basal transcription factor; TBS-T, medium containing Tris HCl + NaCl + Tween 20; TG, triglyceride.

nontreated cultures. In contrast, Brodie *et al.* (18) demonstrated that 25–100 μM of mixed CLA isomers inhibited both proliferation and differentiation and reduced mRNA levels of peroxisome proliferator activated receptor γ2 (PPARγ2) and adipose P2 (aP2) in cultures of 3T3-L1 preadipocytes. A recent study also found that *trans*-10,*cis*-12 CLA reduced stearoyl-CoA desaturase (SCD-1) activity and mRNA levels without affecting PPARγ or aP2 mRNA, suggesting that CLA may be interfering with the desaturation of long-chain fatty acids and their subsequent esterification into TG (20).

The aforementioned studies suggest that the antiobesity actions of a crude mixture of CLA isomers may be due to the direct influence of a specific isomer of CLA (i.e., *trans*-10,*cis*-12 CLA) on preadipocyte differentiation. However, the specific mechanism behind CLA's antiadipogenic actions remains to be determined. Therefore, the purpose of this study was to investigate: (i) the effect of various doses of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers and treatment periods on TG content (Exp. 1), (ii) the influence of these two CLA isomers on the expression of a key transcription factor that regulates adipogenesis (Exp. 2), (iii) the impact of CLA on neutral lipid and phospholipid fatty acid profile (Exp. 3), and (iv) the ability of LA to block CLA's reduction of TG content in differentiating cultures of 3T3-L1 (pre)adipocytes (Exp. 4).

## MATERIALS AND METHODS.

*Cell model and culture conditions.* Postconfluent monolayers of differentiating 3T3-L1 preadipocytes were used as the cellular model for these studies. 3T3-L1 preadipocytes are a nontransformed cell line, which is a continuous substrain of Swiss albino 3T3 murine cells developed through clonal expansion (21). The cells and protocols for differentiating the cultures were generously provided by Dr. Howard Green (Harvard University, Boston, MA).

Both isomers of CLA and LA were complexed to 1 mM fatty acid–free bovine serum albumin (BSA; 1 mM BSA: 4 mM fatty acid) and added to the cultures on day 1 of differentiation (Fig. 1) as previously described (19). LA (99% pure according to the manufacturers) was obtained from Nu-Chek-Prep (Elysian, MN). The *trans*-10,*cis*-12 CLA and *cis*-9,*trans*-11 CLA isomers (98+% pure, according to the manufacturers) were obtained from Matreya Inc. (Pleasant Gap, PA). Since we did not analyze CLA and LA for their purity, we realize that other isomers could be present in each fatty acid. All treatments contained 0.2 mM α-tocopherol (Sigma Chemical Co., St. Louis, MO) to prevent lipid peroxidation as previously described (19). Media were changed at 2-d intervals and fresh fatty acids added each media change until the day of harvest.

Cells were seeded at a density of $3.3 \times 10^3/cm^2$ in 12-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM), 10% bovine calf serum, and antibiotics until confluent. Unless otherwise indicated, cells were cultured and treated in 12-well plates (Falcon Labware; Becton Dickinson & Co., Franklin Lakes, NJ). Two days postconfluence, the cells were stimulated to differentiate with DMEM containing 10% fetal bovine serum (FBS) (charcoal-stripped to remove endogenous fatty acids), 10 μg/mL insulin, 0.5 mM isobutylmethylxanthine, 0.1 μM dexamethasone, 0.2 mM α-tocopherol, and 1% antibiotics. On day 3 of differentiation, the above medium was replaced with DMEM, 10% stripped FBS, 2.5 μg/mL insulin, 0.2 mM α-tocopherol, and 1% antibiotics. From day 5 onward, medium containing DMEM, 10% stripped FBS, 0.2 mM α-tocopherol, and 1% antibiotics was used.

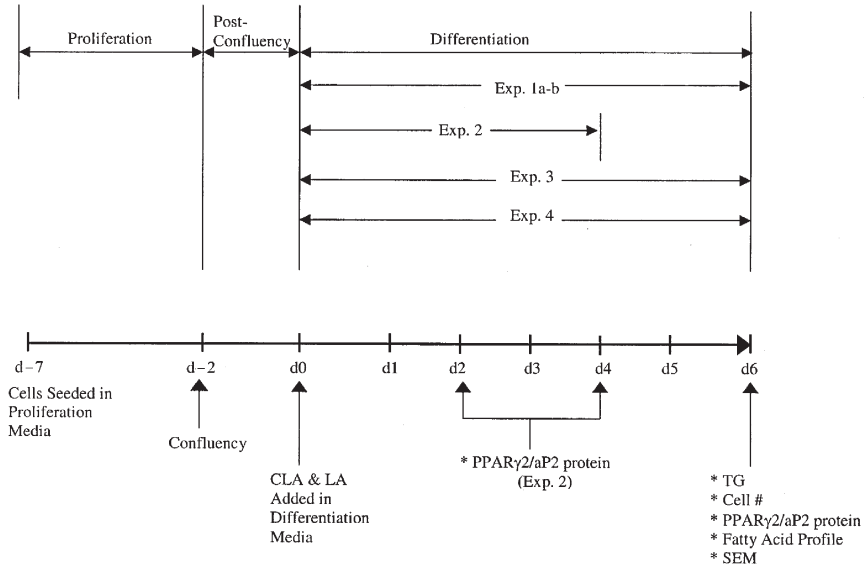


**FIG. 1.** Design of experiments. Abbreviations: CLA, conjugated linoleic acid; LA, linoleic acid; TG, triglyceride; SEM, scanning electron microscopy; PPARγ2, peroxisome proliferator activated receptor γ2; aP2, adipose P2.

*(i) TG content (Exp. 1a–b; Exp. 4a).* TG content was measured using a commercially available colorimetric kit (Sigma #339-10; Sigma Chemical Co.) modified for cell culture as previously described (22). This procedure employs enzymatic hydrolysis of glycerol and fatty acids and the glycerol is quantified spectrophotometrically at 520 nm.

*(ii) Cell number (Exp. 1a–b; Exp. 4a).* Adherent cells were harvested in a cell counting solution (25 mM glucose, 0.154 M NaCl, 0.01 M $NaH_2PO_4$ [monobasic], 5 mM 2% albumin, pH 7.4) and counted on a Coulter Multisizer IIE (Coulter Corp., Miami, FL).

*(iii) Protein content (Exp. 2; Exp. 4c).* Protein was measured using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). This assay measures the reduction of $Cu^{2+}$ to $Cu^{+}$ by protein and can be quantified spectrophotometrically at 542 nm.

*PPARγ2 and aP2 expression (Exp. 2; Exp. 4c).* PPARγ2 and aP2 protein levels were assessed 48 and 96 h following treatment with vehicle (BSA), 50 μM LA, *cis*-9,*trans*-11 CLA, or *trans*-10,*cis*-12 CLA (Exp. 2) or 6 d posttreatment with vehicle (BSA) or increasing doses of LA (0–100 μM) in the presence or absence of 50 μM *trans*-10,*cis*-12 CLA (Exp. 4c). Cultures were grown in 10-cm dishes (Falcon Labware) with $n = 3$ for each experiment unless otherwise indicated. Cells were harvested according to the directions provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Briefly, the cells were washed twice with phosphate-buffered saline (PBS) and lysed with 0.3 mL radioimmunoprecipitation assay (RIPA) lysis buffer [1 ml RIPA stock (PBS buffer at pH 7.5 containing 1% detergent NP40, 0.1% sodium dodecylsulfate, and 0.5% sodium deoxycholate) plus 30 μL/mL aprotinin, 10 μL/mL of 10 mg/mL phenylmethylsulfonyl fluoride, and 10 μL/mL of 100 mM sodium orthovanadate made fresh at each harvest]. Cells were scraped into one corner of the dish, transferred to a microfuge tube, and placed on ice for 20 min. Whole cell lysates were recovered by centrifugation at 15,000 × *g*, 4°C for 20 min, and stored at –80°C. The protein concentration was determined using the BCA assay. Twenty micrograms of protein was fractionated by electrophoresis using 12% NOVEX Tris-Glycine precast gels (Invitrogen Corp., Carlsbad, CA) for PPARγ2 and TFII basal transcription factor (TBP). TBP proteins are constitutively expressed during 3T3-L1 preadipoctye differentiation and therefore are more reliable as a loading control protein than cytoskeletal proteins that are differentially expressed during differentiation (23; Mandrup, S., University of Southern Denmark, personal communication). One microgram of protein was fractionated by electrophoresis on 16% Tris-Glycine Novex precast gels for aP2 protein expression. Separated proteins were transferred electrophoretically to Novex polyvinylidene difluoride membrane. Membranes were incubated in TBS-T (25 mM Tris-HCl at pH 8.0, 125 mM NaCl, and 0.5% Tween 20) containing 5% nonfat dry milk to block nonspecific binding. The blots were then incubated with primary antibody, a polyclonal anti-PPARγ2 (1:2500) (the generous gift of Dr. P. Pekala, East Carolina University), anti-TBP (sc-273; Santa Cruz Biotechnology) (1:500), or anti-aP2 (a generous gift of Dr. D. Bernlohr, University of Minnesota) (1:30,000) antiserum in blocking solution (TBS − T + 5% dry milk) for 1 h at room temperature. After washing twice with TBS-T for 10 min, the blots were incubated with a secondary antibody, horseradish peroxidase-conjugated rabbit antirabbit immunoglobulin G (1:2000 dilution; Santa Cruz Biotechnology) for 1 h. After washing four times with TBS-T for 5 min, the blots were developed using an enhanced chemiluminescence system (ECL Plus, Amersham, Arlington, IL) and visualized by exposure to X-ray film (ECL Hyperfilm; Amersham). The bands were quantified by densitometry using an Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA).

*Fatty acid extraction and gas chromatography analyses (Exp. 3).* Cultures were grown in 10-cm Falcon dishes ($n = 4$ per treatment). Cellular lipids were extracted with chloroform/methanol (2:1) (24). Neutral and polar lipids were separated using Sep-Pak silica cartridges (Waters Corp., Milford, MA) (25). Lipid extracts were dried under nitrogen and converted to fatty acid methyl esters using 4% HCl/methanol at 60°C for 20 min. Fatty acid methyl esters were extracted into hexane and analyzed on a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) fitted with a flame-ionization detector and 3396A integrator. A Supelcowax-10 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 200°C, increased 20°C per min, held for 50 min, increased 10°C per min to 220°C, and held for 50 min. Fatty acid methyl esters were identified by comparison with standards (Sigma Chemical Co. and Nu-Chek-Prep).

*Scanning electron microscopy (SEM)* (Exp. 4b). 3T3-L1 monolayers were grown and treated until day 6 of differentiation on inserts with 0.2-μm pore size (Falcon Labware) that fit into individual wells, where the media and solutions for processing were applied as previously described (19). Cells on inserts were fixed with 2% glutaraldehyde in 0.1 M cacodylate in 0.1 M sucrose buffer for 1 h. Cells were rinsed with cacodylate-sucrose buffer prior to postfixation for 1 h in 1% osmium tetroxide. Inserts were then rinsed with 0.1 M piperazinc-*N,N′*-bis(2-ethanesulfonic acid) buffer and refrigerated until they were processed for SEM. The inserts with attached cells were processed for SEM by chemically drying with hexamethyldisilazane (HMDS). They were dehydrated in a series of ethanol dilutions (70, 85, 95, and 100%) for 5 min each. Next, they were placed in HMDS and dried overnight under vacuum. After drying, the insert membranes with attached cells were placed on aluminum stubs and coated with gold in an SPI Module Sputter coater. Samples were examined with a JEOL JSM-35CF scanning electron microscope (Japan Electron Optics Limited, Peabody, MA). Images at 480 and 2000× magnification were recorded on Type 52 Polaroid film.

*Statistical analyses.* Data were analyzed for analysis of variance (ANOVA) using commercially available software (SuperAnova; Abacus Concepts, Inc., Berkeley CA). For experiments 1a–b, two-way ANOVA were conducted, and their interactions (fatty acid × dose in Exp. 1a; fatty acid × time

**TABLE 1**
**Fatty Acid Composition[a] of 3T3-L1 Preadipocytes Treated with Vehicle (BSA), 10–50 μM *cis*-9,*trans*-11 CLA, or 10–50 μM *trans*-10,*cis*-12 CLA for 6 d.**

| Fatty acid | BSA | 10 μM 9,11 | 25 μM 9,11 | 50 μM 9,11 | 10 μM 10,12 | 25 μM 10,12 | 50 μM 10,12 |
|---|---|---|---|---|---|---|---|
| | | | | g/100g | | | |
| Phospholipid fraction | | | | | | | |
| 16:0 | 12.15 ± 0.39$^{a}$ | 12.82 ± 0.47$^{a}$ | 11.56 ± 0.47$^{a}$ | 12.55 ± 0.21$^{a}$ | 12.17 ± 0.45$^{a}$ | 12.02 ± 0.48$^{a}$ | 12.07 ± 0.66$^{a}$ |
| 16:1 | 31.91 ± 1.50$^{a,b}$ | 30.31 ± 0.28$^{b}$ | 31.92 ± 1.73$^{a,b}$ | 32.62 ± 1.23$^{a,b}$ | 33.48 ± 1.48$^{a,b}$ | 34.07 ± 1.37$^{a}$ | 31.11 ± 1.67$^{a}$ |
| 18:0 | 4.89 ± 0.57$^{a}$ | 5.06 ± 0.54$^{a}$ | 4.28 ± .64$^{a}$ | 4.47 ± 0.65$^{a}$ | 4.71 ± 0.53$^{a}$ | 4.57 ± 0.54$^{a}$ | 4.50 ± 0.51$^{a}$ |
| 18:1 *c*9 | 7.26 ± 0.14$^{b}$ | 7.13 ± 0.18$^{b}$ | 6.76 ± 0.19$^{c}$ | 6.65 ± 0.28$^{c}$ | 7.13 ± 0.40$^{b}$ | 7.15 ± 0.35$^{b}$ | 7.99 ± 0.35$^{a}$ |
| 18:1 *c*11 | 7.97 ± 0.81$^{a}$ | 8.01 ± 0.74$^{a}$ | 7.40 ± 0.75$^{a,b}$ | 6.67 ± 0.36$^{b}$ | 7.46 ± 0.51$^{a,b}$ | 6.75 ± 0.62$^{a,b}$ | 5.14 ± 0.39$^{c}$ |
| 18:2 | 1.02 ± 0.07$^{b}$ | 1.11 ± 0.16$^{b}$ | 1.12 ± 0.08$^{b}$ | 1.12 ± 0.06$^{b}$ | 1.12 ± 0.11$^{b}$ | 1.21 ± 0.13$^{b}$ | 1.89 ± 0.29$^{a}$ |
| 18:2 *c*9,*t*11 | 0 | 0 | 0.45 ± 0.04$^{b}$ | 1.29 ± 0.07$^{a}$ | 0 | 0 | 0 |
| 18:2 *t*10,*c*12 | 0 | 0 | 0 | 0 | 0 | 0 | 0.61 ± 0.19$^{a}$ |
| 20:4 | 4.96 ± 0.44$^{a}$ | 5.61 ± 0.32$^{a}$ | 5.24 ± 0.38$^{a}$ | 4.78 ± 0.19$^{a}$ | 4.98 ± 0.57$^{a}$ | 5.21 ± 0.57$^{a}$ | 5.61 ± 0.84$^{a}$ |
| Neutral lipid fraction | | | | | | | |
| 16:0 | 15.66 ± 1.70$^{a}$ | 16.29 ± 1.79$^{a}$ | 15.33 ± 0.77$^{a}$ | 16.95 ± 1.92$^{a}$ | 15.12 ± 0.99$^{a}$ | 16.29 ± 1.38$^{a}$ | 15.18 ± 1.19$^{a}$ |
| 16:1 | 34.81 ± 0.82$^{a}$ | 33.84 ± 0.75$^{a}$ | 33.74 ± 0.65$^{a}$ | 33.70 ± 0.59$^{a}$ | 33.66 ± 1.07$^{a}$ | 34.16 ± 1.29$^{a}$ | 30.66 ± 1.85$^{b}$ |
| 18:0 | 0.77 ± 0.10$^{b}$ | 0.66 ± 0.07$^{b}$ | 0.70 ± 0.12$^{b}$ | 0.61 ± 0.12$^{b}$ | 0.71 ± 0.13$^{b}$ | 0.72 ± 0.16$^{a,b}$ | 1.00 ± 0.10$^{a}$ |
| 18:1 *c*9 | 5.15 ± 0.29$^{b}$ | 4.88 ± 0.24$^{b}$ | 4.75 ± 0.45$^{b,c}$ | 4.34 ± 0.31$^{b,c}$ | 4.74 ± 0.37$^{b,c}$ | 4.79 ± 0.57$^{b}$ | 5.72 ± 0.32$^{a}$ |
| 18:1 *c*11 | 2.84 ± 0.38$^{a}$ | 2.50 ± 0.26$^{a}$ | 2.40 ± 0.34$^{a,b}$ | 1.96 ± 0.24$^{b}$ | 2.42 ± 0.31$^{a}$ | 2.24 ± 0.42$^{a,b}$ | 2.17 ± 0.17$^{b}$ |
| 18:2 | 0.33 ± 0.04$^{b}$ | 0.32 ± 0.03$^{b}$ | 0.34 ± 0.03$^{b}$ | 0.31 ± 0.02$^{b}$ | 0.45 ± 0.08$^{b}$ | 0.35 ± 0.03$^{b}$ | 0.43 ± 0.01$^{a}$ |
| 18:2 *c*9,*t*11 | 0 | 0 | 0.49 ± 0.07$^{b}$ | 1.51 ± 0.06$^{a}$ | 0 | 0 | 0 |
| 18:2 *t*10,*c*12 | 0 | 0 | 0 | 0 | 0.25 ± 0.12$^{b}$ | 0 | 0.57 ± 0.19$^{a}$ |
| 20:4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a]Values are means ± SEM, $n = 4$. Values in a row with different roman superscripts are significantly different at $P < 0.05$. BSA, bovine serum albumin; CLA, conjugated linoleic acid; SEM, standard error of the mean.

period in Exp. 1b) were compared for significance at the $P < 0.05$ level. For experiment 2, a three-way ANOVA (fatty acid, treatment duration, replication) was conducted, and the fatty acid × treatment duration interactions were compared for significance at the $P < 0.05$ level. In experiment 3, a two-way ANOVA was conducted and their interactions (fatty acid × dose) were compared for significance at the $P < 0.05$ level. For each fatty acid, an additional analysis was computed in which the treatment sum of squares for fatty acid dose was subdivided into linear response curves and tested for significance. In experiments 4a and 4c, a two-way ANOVA (fatty acid × replication) was conducted, and the effects of fatty acid treatments were compared for significance at the $P < 0.05$ level. The means ± standard errors of the means of the treatment interactions and their statistical differences are presented in the figures and Table 1.

## RESULTS

*Experiment 1 a-b: Dose response and time periods (Figs. 2,3). (i) Dose response (Fig. 2).* The TG content of the cultures treated with 50 μM *trans*-10,*cis*-12 CLA was lower than control cultures. In contrast, the TG content was higher in cultures treated with 50 μM *cis*-9,*trans*-11 CLA compared to the BSA controls.

*(ii) Time periods (Fig. 3).* Cultures treated with *trans*-10, *cis*-12 CLA during the entire 6-d period of differentiation had 38 and 43% less TG than LA- and *cis*-9,*trans*-11 CLA-treated cultures (days 1–6), respectively. Cultures treated during only the last 3 d of differentiation (days 4–6) with *trans*-10,*cis*-12 CLA contained 13 and 18% less TG than LA- and *cis*-9, *trans*-11 CLA-treated cultures, respectively. In contrast, there was no difference in the TG content between the three fatty acid treatments during the first 3 d of differentiation.

*Experiment 2: PPARγ2 and aP2 protein expression after 48 h and 96 h of treatment (Figs. 4,5). (i) PPARγ2 protein expression.* Cultures treated with 50 μM *trans*-10,*cis*-12 CLA had 35% higher PPARγ2 protein levels than BSA controls after 48 h of treatment (Fig. 4). In contrast, *cis*-9,*trans*-11

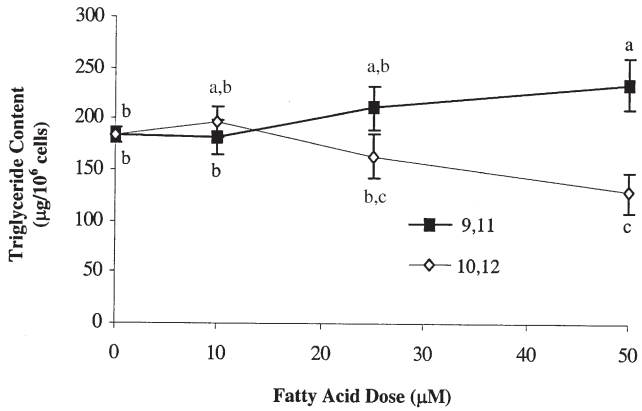


**FIG. 2.** The effect of increasing doses of *cis*-9,*trans*-11 CLA or *trans*-10, *cis*-12 CLA on TG content on day 6 of postconfluent cultures of differentiating 3T3-L1 preadipocytes (Exp. 1a). Cultures were treated continuously and harvested after 6 d of treatment. Means (±standard errors of the means; $n = 6$) not sharing a common superscript are significantly different ($P < 0.05$). For abbreviations see Figure 1.

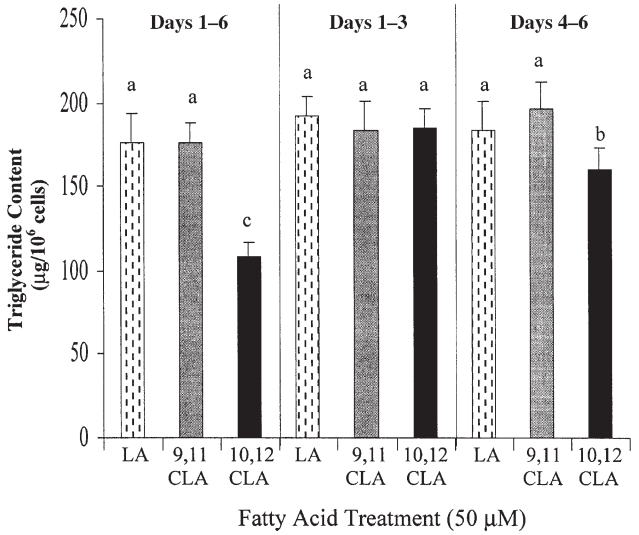


**FIG. 3.** The effect of time and fatty acid treatment [(LA, *cis*-9,*trans*-11 CLA, or *trans*-10,*cis*-12 CLA] on TG content on day 6 of post-confluent cultures of differentiating 3T3-L1 preadipocytes (Exp. 1b). Cultures were treated continuously and harvested after 6 d of treatment. Means (± standard errors of the means; $n = 6$) not sharing a common superscript are significantly different ($P < 0.05$). For abbreviations see Figure 1.

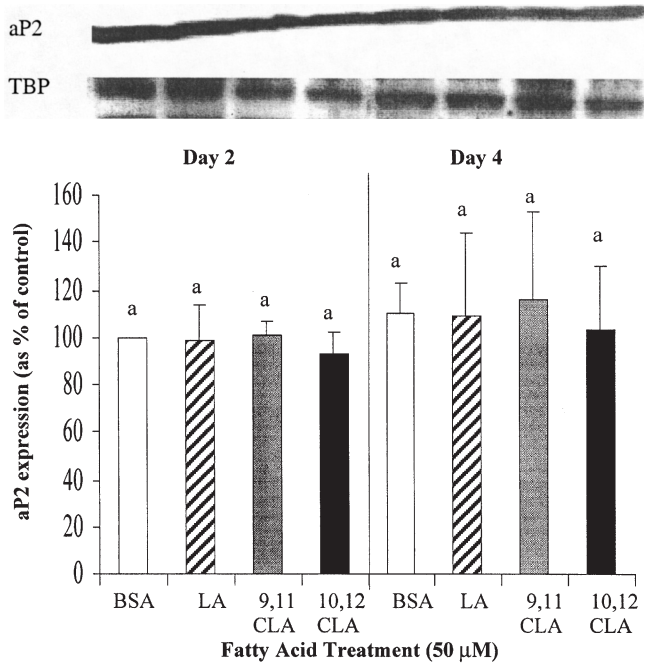


**FIG. 5.** Effect of BSA (vehicle control), 50 μM LA, *cis*-9, *trans*-11 or *trans*-10, *cis*-12 CLA for 48 or 96 h on aP2 protein expression after normalization for TBP (Exp. 2). Protein levels are expressed as a percentage of control (BSA) levels on day 2 of differentiation. Means (±standard error of the mean; $n = 3$) not sharing a common superscript are significantly different ($P < 0.05$). For other abbreviations see Figures 1 and 4.

CLA-treated cultures had similar PPARγ2 protein levels as the BSA controls, while LA-treated cells had lower PPARγ2 protein levels than BSA controls. After 96 h of treatment, there were no significant differences in PPARγ2 protein expression due to fatty acid treatment.

*(ii) aP2 protein expression.* After 48 and 96 h of treatment there was no significant difference in aP2 protein expression due to fatty acid treatment (Fig. 5).

*Experiment 3: Influence of CLA on cellular lipids (Table 1).* Both isomers of CLA were detected in the neutral and polar lipid fractions of the cultures. However, more *cis*-9, *trans*-11 CLA than *trans*-10,*cis*-12 CLA was detected in the fatty acids of each fraction. Both CLA isomers incorporated into the neutral and polar lipid fractions in approximately equal proportions. For example, cultures treated with 50 μm *cis*-9,*trans*-11 and 50 μM *trans*-10,*cis*-12 CLA isomers had 1.29 and 1.51 g/100 g *cis*-9,*trans*-11 CLA and 0.61 and 0.57 g/100 g *trans*-10,*cis*-12 CLA in their phospholipid and neutral lipid fractions, respectively. *Trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA treatment caused a dose-dependent decrease in *cis*-11 18:1 in fatty acids from both the neutral and polar lipid fractions (linear dose, $P < 0.05$). Cultures treated with 50 μM *trans*-10,*cis*-12 CLA had the lowest amount of *cis*-11 18:1 in the phospholipid fraction. In contrast, 50 μM *trans*-10,*cis*-12 CLA treated cultures had increased amounts of *cis*-9 18:1 in both the neutral and phospholipid fractions, whereas 25 and 50 μM *cis*-9,*trans*-11 supplementation reduced *cis*-9 18:1 levels in the phospholipid fraction only. Interestingly, cultures treated with 50 μM *trans*-10,*cis*-12 CLA had more LA in both the neutral and phospholipid fractions and more 18:0 in the neutral lipid fraction compared to controls.

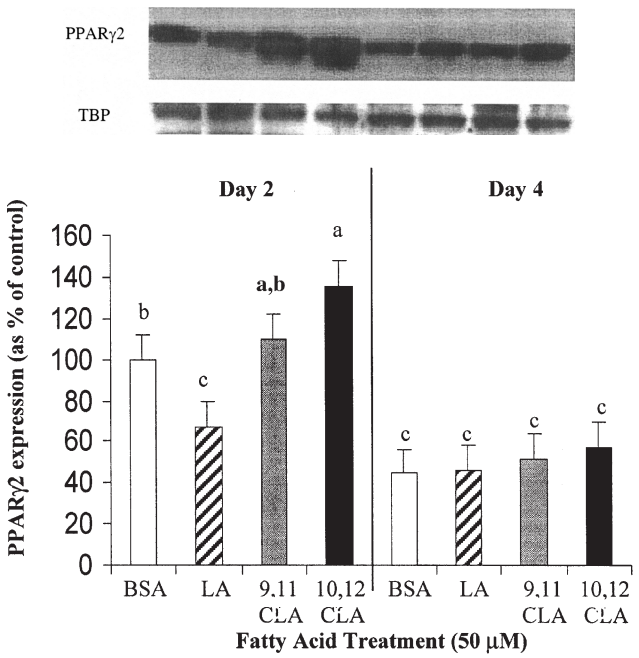


**FIG. 4.** Effect of bovine serum albumin (BSA) (vehicle control), 50 μM LA, *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA for 48 or 96 h on PPARγ2 protein expression normalized for TFII basal transcription factor (TBP) expression (Exp. 2). Protein levels are expressed as a percentage of BSA levels on day 2 of differentiation. Means (±standard errors of the means; $n = 3$) not sharing a common superscript are significantly different ($P < 0.05$). For other abbreviations, see Figure 1.

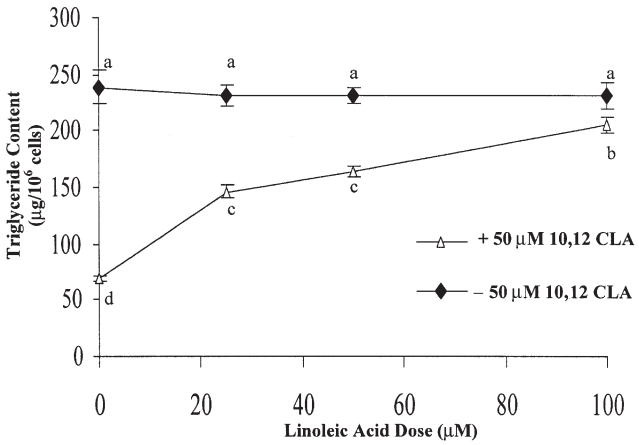


**FIG. 6.** The effect of increasing doses of LA with and without *trans*-10, *cis*-12 CLA supplementation on TG content on day 6 of postconfluent cultures of differentiating 3T3-L1 preadipocytes (Exp. 4a). Cultures were treated continuously and harvested after 6 d of treatment. Means (± standard error of the means; $n = 6$) not sharing a common superscript are significantly different ($P < 0.05$). For abbreviations see Figure 1.

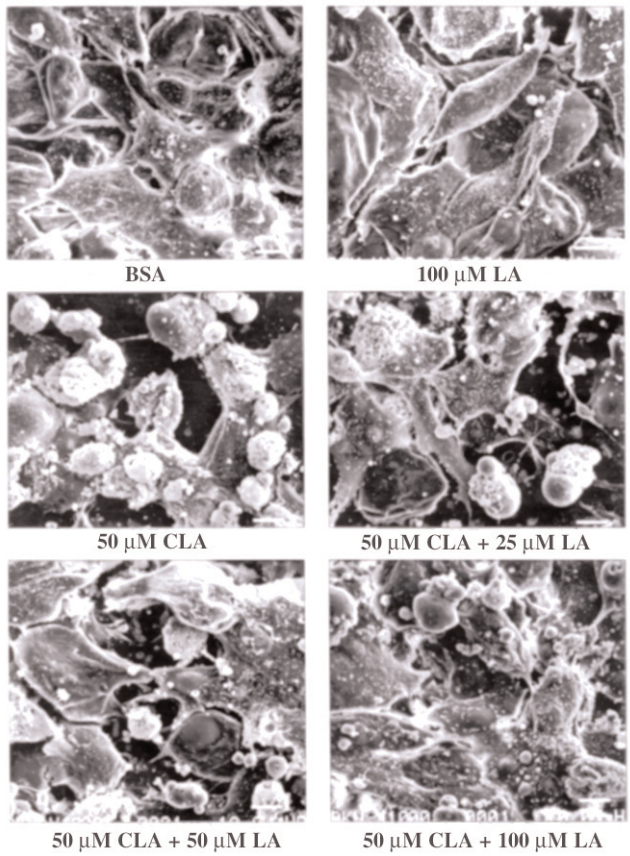


**FIG. 7.** Scanning electron micrographs of postconfluent cultures of differentiating 3T3-L1 preadipocytes treated with increasing doses of LA in the presence or absence of 50 μM *trans*-10,*cis*-12 CLA for 6 d (Exp. 4b). Magnification (480×) is the same in all photographs. Representative samples that were taken from at least two different cultures in each treatment group were similar in appearance. For abbreviations see Figures 1 and 4.

*Experiment 4a-c: trans-10,cis-12 CLA + LA supplementation (Figs. 6–9). (i) TG content*. LA supplementation of CLA-treated cultures increased TG content of the cultures in a dose-dependent manner (Fig. 6). Although this increase was significant, it did not reach the level of the BSA controls or the LA only-treated cultures.

*(ii) SEM micrographs (1000×).* SEM of the cultures revealed normal cell morphology for the BSA control cultures (Fig. 7). Cells were fibroblastic in shape with numerous microvilli and sharp-pointed lamellipodia. At 100 μM LA, some cells still retained a flattened fibroblastic appearance. However, number and length of microvilli per cell appeared decreased. In cultures treated with 50 μM of *trans*-10,*cis*-12 CLA, there were still some flattened cells, but there were large numbers of small, rounded cells that appeared lumpy in appearance. Cells that appeared to be blebbing, a characteristic of apoptotic cells, were also seen in the CLA-treated cultures. Addition of LA to cultures treated with 50 μM *trans*-10,*cis*-12 CLA seemed to offset the rounding and blebbing seen with CLA treatment. Furthermore, the number of small, rounded cells and the degree of blebbing seemed to decrease as the dose of LA increased.

*(iii) PPARγ2 and aP2 protein expression.* Cultures treated with 50 and 100 μM LA had lower PPARγ2 protein levels on day 6 of differentiation compared to BSA controls (Fig. 8). In contrast to what was observed after 48 and 96 h of treatment, treatment with 50 μM *trans*-10,*cis*-12 CLA for 6 d decreased

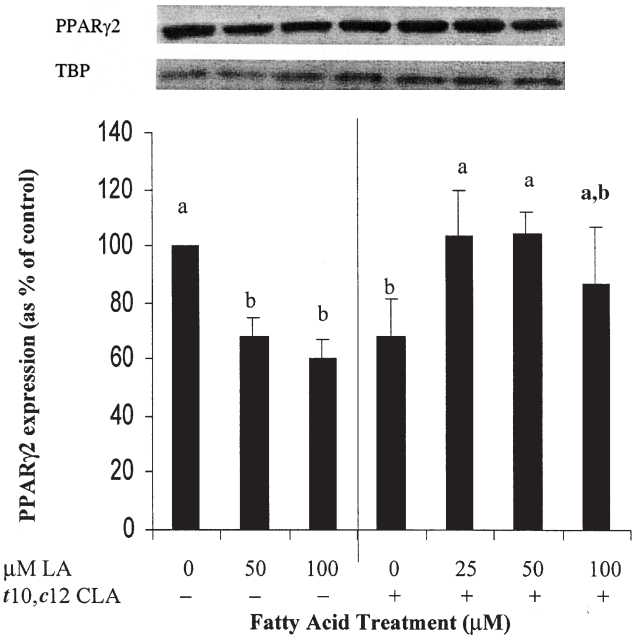


**FIG. 8.** Effect of increasing doses of LA in the presence or absence of 50 μM *trans*-10,*cis*-12 CLA on PPARγ2 protein expression normalized for TBP protein expression on day 6 of differentiation (Exp. 4c). Protein levels are expressed as a percentage of vehicle control (BSA) levels on day 6 of differentiation. Means (±standard errors of the means, $n = 2$) not sharing a common superscript are significantly different ($P < 0.05$). For abbreviations see Figures 1 and 4.

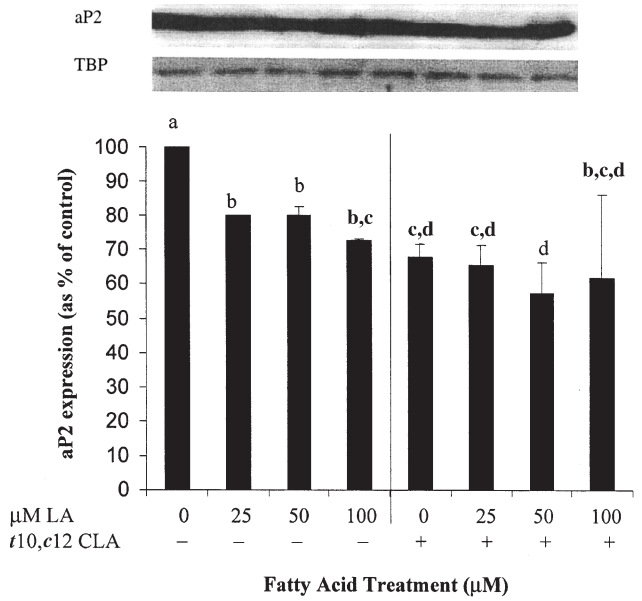


**FIG. 9.** Effect of increasing doses of LA in the presence or absence of 50 μM *trans*-10,*cis*-12 CLA on aP2 protein expression normalized for TBP protein expression on day 6 of differentiation. Protein levels are expressed as a percentage of control (BSA) levels on day 6 of differentiation. Means (±standard errors of the means, $n = 2$) not sharing a common superscript are significantly different ($P < 0.05$). For abbreviations see Figures 1 and 4.

PPARγ2 levels, and supplementation of CLA-treated cultures with 25 or 50 μM LA increased PPARγ2 expression compared to CLA alone. As with PPARγ2 expression, treatment with 25–100 μM LA reduced aP2 protein expression on day 6 of differentiation (Fig. 9). Supplementation with 50 μM *trans*-10,*cis*-12 CLA reduced aP2 protein expression, whereas addition of 25–100 μM LA to CLA-treated cultures did not affect aP2 protein expression.

## DISCUSSION

We have confirmed that *trans*-10,*cis*-12 CLA is responsible for the TG-lowering effects of a commercially available crude mixture of CLA isomers using 3T3-L1 preadipocytes as the cell model. However, the effects of *trans*-10,*cis*-12 CLA were dependent on dose, duration, and time period of treatment, as treatment throughout the first 6 d of differentiation was more effective than treatment during either the first 3 d or the last 3 d of differentiation. Our results substantiate the reports of previous research demonstrating that *trans*-10,*cis*-12 CLA is the antiadipogenic isomer of CLA. *In vivo*, ICR mice consuming 0.25% *trans*-10,*cis*-12-enriched CLA had lower body fat percentages than controls or mice fed 0.25% *cis*-9,*trans*-11-enriched CLA (8). Furthermore, Baumgard *et al.* (26) found that only the *trans*-10,*cis*-12 isomer of CLA reduced milk fat percentage and yield in Holstein cows, suggesting this CLA isomer may reduce *de novo* lipogenesis. *In vitro*, Park *et al.* (8) showed that 3T3-L1 preadipocytes treated for 48 h with *trans*-10,*cis*-12 CLA contained less intracellular TG and glycerol than *cis*-9,*trans*-11 CLA-treated cultures. More recently, Choi *et al.* (20) found that *trans*-10,*cis*-12 CLA inhibited the production of SCD-1 without reducing PPARγ2 or aP2 mRNA levels in 3T3-L1 preadipocytes.

To our knowledge, our results are the first to show that the impact of *trans*-10,*cis*-12 CLA on 3T3-L1 preadipocytes depends on the time period of treatment. Data in Figure 2 clearly show that treatment during the entire period of differentiation reduced TG content to a greater extent than treatment during the last 3 d of differentiation. However, *trans*-10,*cis*-12 CLA treatment during only the first 3 d of differentiation was not sufficient to lower TG concentrations. Park *et al.* (8) showed that 3T3-L1 preadipocytes treated with mixed isomers of CLA for 48 h on day 4 of differentiation had lower TG levels than LA controls, results that correspond to our effects seen with treatment during the last 3 d of differentiation.

In an attempt to elicit a mechanism through which *trans*-10,*cis*-12 CLA inhibits TG accumulation, we assessed the expression of PPARγ2 and aP2 protein. In contrast to our hypothesis that *trans*-10,*cis*-12 CLA decreases TG content by reducing the expression of PPARγ2, we found that *trans*-10,*cis*-12 CLA had differential effects on PPARγ2 expression throughout differentiation. Specifically, PPARγ2 expression increased on day 2 of differentiation compared to BSA controls, whereas PPARγ2 protein expression on day 4 was unchanged. aP2 protein levels were unaffected by either 2 or 4 d of CLA treatment. In contrast, the expression of PPARγ2 and aP2 proteins decreased after 6 d of treatment with *trans*-10,*cis*-12 CLA, suggesting that *trans*-10,*cis*-12 CLA has different effects on the regulation of differentiation depending on the time period of treatment.

Interestingly, LA treatment reduced PPARγ2 protein expression. In support of this unexpected result, LA has previously been suggested to inhibit preadipocyte differentiation and SCD-1 gene expression (27). Furthermore, we have consistently found that LA treatment increased cell number (data not shown), which would correlate with a suppression of PPARγ2 expression to allow clonal expansion during the early phase of differentiation (21,28).

To our knowledge, this is the first report of the impact of *trans*-10,*cis*-12 CLA treatment on PPARγ2 protein levels in 3T3-L1 preadipocytes. Brodie *et al.* (18) found that 50 μM of a crude mixture of CLA isomers reduced PPARγ mRNA expression in 3T3-L1 preadipocytes treated for 2–7 d. However, these researchers used a general probe for PPARγ, not one that was specific for PPARγ2—the isoform that is abundant in adipose tissue and generally mediates fatty acid-induced gene expression (29). Furthermore, the combination of CLA isomers found in a crude CLA mixture may have differential effects on PPARγ isoform expression. In contrast to the results of Brodie *et al.* (18), Choi *et al.* (20) reported that, although a crude mixture of CLA isomers reduced PPARγ2 mRNA expression, 45 μM *trans*-10,*cis*-12 CLA did not affect PPARγ2 mRNA levels. Furthermore, Houseknecht *et al.* (4) found that 100–200 μM of a crude mixture of CLA isomers activated the expression of PPARγ in CV-1 cells transiently transfected with a human PPARγ reporter gene construct.

We also found that *trans*-10,*cis*-12 CLA did not affect aP2 protein expression after 2 or 4 d of treatment but decreased its expression after 6 d of continuous treatment. Similar to our early results, Choi *et al.* (20) found no effect of *trans*-10,*cis*-12 CLA on aP2 mRNA levels. In contrast, *in vivo,* Houseknecht *et al.* (4) found that 1.5% (w/w) of a crude mixture of CLA isomers increased aP2 mRNA levels in prediabetic ZDF rats. However, the effect of the individual CLA isomers on aP2 expression is unknown. Furthermore, these researchers used epididymal adipose tissue that contains a mixture of stromal vascular cells, including preadipocytes and adipocytes in various stages of differentiation, making the interpretation of these results unclear.

For the lipid-lowering effects of CLA to be physiologically relevant, CLA must incorporate into cellular lipids or alter lipid composition. To this end, we found that both *trans*-10, *cis*-12 CLA and *cis*-9,*trans*-11 CLA incorporated into neutral lipid and phospholipid fractions; however, the *cis*-9,*trans*-11 CLA isomer was one- to two-fold more abundant than the *trans*-10,*cis*-12 isomer. Interestingly, Comb White Leghorn laying hens fed mixed isomers of CLA had higher levels of *cis*-9,*trans*-11 CLA than *trans*-10,*cis*-12 CLA in their egg yolks (30). In addition, albino rats fed 0.5–1.5% (w/w) of a crude mixture of CLA isomers for 60 d had almost twice the amount of *cis*-9,*trans*-11 CLA in their adipose tissue as *trans*-10,*cis*-12 CLA (31).

CLA's antiadipogenic actions have been proposed to be due to inhibited elongation and/or desaturation of saturated fatty acids such as palmitic acid and stearic acid into monounsaturated fatty acids (20). In support of this hypothesis, we found that 50 μM *trans*-10,*cis*-12 CLA-treated cultures had lower amounts of palmitoleic acid (16:1) (in the neutral lipid fraction) and *cis*-11 oleic acid (18:1) (in both the neutral and polar lipid fractions) compared to BSA controls. However, *trans*-10,*cis*-12 CLA increased the amount of *cis*-9 oleic acid as well as the level of LA (18:2). These results differ from those of Satory and Smith (17), who found that 3T3-L1 preadipocytes cultured with 5–10 mg/L of mixed isomers of CLA had increased amounts (g/100 g total fatty acid) of palmitic acid (16:0) and palmitoleic acid (16:1) and decreased stearic acid (18:0) and oleic acid (18:1) concentrations. However, these researchers treated their preadipocytes with the crude mixture of CLA isomers, dissolved their fatty acids in ethanol as the delivery vehicle, evaluated a combination of neutral and polar lipids, and used lower amounts of CLA, all of which could account for the differences from our results. More recently, Choi *et al.* (20) showed that 3T3-L1 preadipocytes treated with 45 μM *trans*-10,*cis*-12 CLA had lower levels of both 16:1 and 18:1 in their cellular lipids, similar to our results in neutral lipids where TG is stored (20). Finally, Δ-6 desaturation of linoleic acid in rat hepatic microsomes was decreased in the presence of both *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA (32). However, only the *trans*-10,*cis*-12 isomer of CLA inhibited Δ-9 desaturation of stearic acid.

CLA treatment has also been theorized to inhibit the production of adipogenic fatty acids such as arachidonic acid (AA) and its subsequent eicosanoid metabolites. A reduction in AA and other adipogenic fatty acids may decrease TG esterification, conversion into phospholipids that are critical for cellular metabolism, and/or synthesis into lipid second messengers, such as prostaglandin $J_2$ ($PGJ_2$), that may regulate adipogenesis. In contrast to this theory, our data showed no difference in AA levels in the phospholipid fraction with either *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA treatment. Our results conflict with those of Satory and Smith (17) who found that 3T3-L1 preadipocytes cultured with 5–10 mg/L of a crude mixture of CLA isomers had lower levels of AA. However, Choi *et al.* (20) found that, while mixed CLA treatment had no effect on 20:4 concentrations in 3T3-L1 preadipocytes, treatment with *trans*-10,*cis*-12 CLA increased 20:4 concentrations. Thus, our results, along with those of Choi *et al.* (20), dispute the suggestion that CLA's antiadipogenic actions may be the result of an inhibition of adipogenic fatty acid production. However, since we did not assess the production of eicosanoid metabolites and lipid second messengers such as $PGJ_2$ that may impact adipogenesis, it is still possible that *trans*-10,*cis*-12 CLA may be inhibiting TG production through these pathways.

Last, in a fourth set of experiments, we examined the impact of LA supplementation on TG content, morphology, and adipogenic protein expression of *trans*-10,*cis*-12 CLA-treated cultures. To our knowledge, we are the first to report that concurrent treatment with LA (18:2n-6) was able to prevent the TG-lowering effects of *trans*-10,*cis*-12 CLA in cell culture. Furthermore, the increase was dose-dependent and almost reached the level of LA-only supplemented cultures. These results may indicate that *trans*-10,*cis*-12 CLA's TG-lowering effects can be overcome by supplementation with polyunsaturated fatty acids, at least in cultures of preadipocytes. Further research to determine if other fatty acids have differential rescue effects dependent on structure are in progress.

In previous research, we showed that treatment with both mixed CLA isomers and *trans*-10,*cis*-12 CLA induced molecular (i.e., increased percentage of cells in the sub-$G_1$ phase) and morphological changes (i.e., nuclear condensation, cellular rounding, and membrane blebbing)—changes that are characteristic of apoptosis (19). In the current research, supplementation of *trans*-10,*cis*-12 CLA-treated cultures with LA inhibited the CLA-induced morphological changes in a dose-dependent manner (Fig. 7). In agreement with these data, CLA-treated cultures supplemented with LA have greater TG content (Fig. 6). A number of studies have also shown that CLA is capable of inducing apoptosis. For example, cells in the adipose tissue of C57BL/6J mice fed 1% (w/w) of mixed isomers of CLA underwent apoptosis (9). Additional studies in primary rat mammary cells (33) as well as NMU mammary cells (34) have also demonstrated that CLA induces apoptosis. However, the mechanism through which LA prevents the induction of apoptosis by *trans*-10,*cis*-12 CLA is unclear.

Finally, we examined PPARγ2 and aP2 protein expression after 6 d of supplementation with *trans*-10,*cis*-12 CLA and LA. Interestingly, concurrent LA and *trans*-10,*cis*-12 CLA

treatment for 6 d increased PPARγ2 protein levels compared to *trans*-10,*cis*-12 CLA treatment alone, an effect that may explain some of LA's ability to reverse CLA's antilipogenic effects. However, the mechanisms through which LA is able to reverse the decrease in PPARγ2 expression and by which LA lowers the effects of CLA remain to be determined.

We found that *trans*-10,*cis*-12 CLA is the TG-lowering isomer of CLA in 3T3-L1 preadipocytes. Furthermore, the effects of *trans*-10,*cis*-12 are time- and dose-dependent and do not appear to depend directly on a reduction in PPARγ2 or aP2 protein expression during the first 4 d of differentiation. However, after 6 d of treatment with *trans*-10,*cis*-12 CLA, PPARγ2 protein levels were lower than control cultures, suggesting that chronic treatment with CLA may reduce TG by downregulating PPARγ2 protein expression. We also discovered that *trans*-10,*cis* 12 CLA decreased the production of certain monounsaturated fatty acids such as 16:1 and *cis*-11 oleic acid, while increasing LA and *cis*-9 18:1 levels. Finally, supplementation with LA was able to prevent some, but not all, of *trans*-10,*cis*-12 CLA's effects on TG content and morphology in cultures of 3T3-L1 preadipocytes.

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# Growth Modulation of Low Density Lipoprotein Receptor Sterol Sensitivity in Cultured Human Fibroblasts

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**ABSTRACT:** Sterols regulate low density lipoprotein (LDL) receptor and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase gene expressions by end product repression. Studies on cultured cells have shown that growing cells have a higher LDL uptake than quiescent cells and that incubation of cells with growth factors or mitogenic compounds leads to sterol-resistant upregulation of LDL receptor gene expression. The recent finding that elevated LDL receptor activity in acute myelogenous leukemia cells was characterized by a decreased sensitivity to downregulation by sterols raises the possibility that the mechanism behind this is related to the cellular growth rate. By using cultured human fibroblasts as a model system we therefore studied whether growth modulation of sterol sensitivity takes place in normal actively growing cells. Judging from the ability of sterols (25-hydroxycholesterol + cholesterol) to inhibit $^{125}$I-LDL degradation, we found that the sensitivity to sterols varied markedly between cells of different densities. The lowest sensitivity to sterols and highest $^{125}$I-LDL degradation rate were found in subconfluent cells, whereas sparse and confluent cells were the most sensitive ones. In contrast to the LDL receptor, HMG-CoA reductase sterol sensitivity did not appear to be growth regulated. We conclude that growth-dependent modulation of sterol sensitivity and LDL receptor activity takes place in normal human fibroblasts. Modulation of sterol sensitivity may be an important mechanism to ensure an adequate cholesterol supply in growing cells.

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Cells can supply their cholesterol needs by receptor-mediated uptake of the major cholesterol-carrying lipoprotein in plasma, low density lipoprotein (LDL), or by *de novo* synthesis (1,2). The rate-limiting enzyme in cholesterol synthesis is the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Both pathways are regulated by the cellular cholesterol concentration *via* end product repression (2–4).

Studies on cultured cells have shown that growing cells have a higher LDL uptake than quiescent cells (3,5–7). One reason for this may be that the cellular cholesterol concentration decreases as a result of increased cholesterol requirements during cell growth, which stimulates LDL receptor activity. Another possibility is that factors involved in growth stimulation, e.g., growth factors, directly stimulate LDL receptor expression. Previous studies have shown that LDL receptor expression is enhanced upon incubation of cells with growth factors (8–10) or mitogenic substances (11). Moreover, incubation of cells with growth factors or mitogenic compounds has been demonstrated to cause a resistance to the downregulatory effect of sterols on LDL receptor expression (9,11,12). It has, however, not been demonstrated that cells indeed growth-modulate their own sterol sensitivity.

Elevated LDL receptor activity, compared to the corresponding normal cells or tissues, has been reported in several types of malignant cells, e.g., leukemic cells from patients with acute myelogenous leukemia (AML), lung cancer tissue, and colon cancer tissue (13–16). In a recent study we demonstrated that elevated LDL receptor activity, in leukemia cells from AML patients, was characterized by a decreased sensitivity to sterols (25-hydroxycholesterol + cholesterol) compared with normal mononuclear cells (17). The median sterol concentration for 50% inhibition ($IC_{50}$) of LDL receptor activity was more than five times higher for AML cells than for normal mononuclear cells. We also observed that the LDL receptor activity in these cells correlated with the white blood cell count but not with the cellular free cholesterol content (17), raising the possibility that the mechanism behind the elevated LDL receptor activity and sterol resistance is growth related.

The objective of this study was therefore to investigate whether growth modulation of the LDL receptor sterol sensitivity normally occurs in cells. We studied gene expression in cells at different growth rates and at different densities and investigated the sensitivity to sterols from the ability of 25-hydroxycholesterol and cholesterol to inhibit LDL receptor activity. As a model system we used cultured human fibroblasts.

## MATERIALS AND METHODS

*Materials.* Radiolabeled sodium iodide (Na $^{125}$I, carrier-free, pH 7–11), cytidine 5′-(*a*-$^{35}$S)thiotriphosphate (triethylammonium salt, >1000 Ci/mmol), uridine 5′-(*a*-$^{35}$S)thiotriphosphate (triethylammonium salt, >1000 Ci/mmol), 3-hydroxy-3-methyl (3-$^{14}$C) glutaryl coenzyme A (52 mCi/mmol), and D,L-(2-$^{3}$H) mevalonic acid lactone (1.26 Ci/mmol) were from Amersham Pharmacia Biotech (Uppsala, Sweden). RNase A, RNase T1, proteinase K, transfer-RNA, and salmon sperm DNA were obtained from Sigma Chemical Co. (St. Louis, MO). Formamide (p.a.) was from Merck (Darmstadt, Germany). The plasmid, pGEM4z, restriction endonucleases, and the *in vitro*

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Abbreviations: AML, acute myelogenous leukemia; FCS, fetal calf serum; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; $IC_{50}$, median sterol concentration for 50% inhibition; LDL, low density lipoprotein; LPDS, human lipoprotein-deficient serum; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

transcription kit (Riboprobe Gemini II Core System) were from Promega (Madison, WI). Sephadex G-50 columns (Nick columns) were from Amersham Pharmacia Biotech.

Lipoproteins. LDL (density 1.020–1.063 g/mL) and human lipoprotein-deficient serum (LPDS) were isolated from serum of healthy blood donors by sequential ultracentrifugation (18). $^{125}$I-LDL (specific activity (150–350 cpm/ng protein) was prepared as described by Langer *et al.* (19). The purity of LDL and LPDS preparations was checked by agarose gel electrophoresis. Less than 1% of the radioactivity in the $^{125}$I-LDL preparation was present as free iodide.

*Cell culture and incubations*. Fibroblasts from normal human foreskin (CRL 2091; American Type Culture Collection, Rockville, MD) were grown in monolayer for not more than 15 passages in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics (100 IU of penicillin + 100 µg of streptomycin/mL) in a humidified incubator (5% $CO_2$, 95% air) at 37°C. For experiments, confluent cell monolayers in 162 $cm^2$ stock flasks (Costar Corp., Cambridge, MA) were detached with 0.05% (vol/wt) trypsin and 0.02% (vol/wt) EDTA and cells were seeded into 10 cm Ø petri dishes (Costar Corp.) containing 9 mL of 10% FCS medium. In all experiments lasting more than 3 d the medium was replaced with fresh medium every 3 d. In some experiments the medium was changed at the indicated time points to medium with 10% LPDS with or without 25-hydroxycholesterol + cholesterol (1:20). In the experiments described in Figure 4, the cells were photographed using a 10× magnification lens with a phase contrast microscope (Carl Zeiss, Inc., Thornwood, NY) connected to a video printer (Mitsubishi Electric Chiyoda-ku, Tokyo, Japan).

*Determination of LDL receptor expression and activity*. Cellular degradation of $^{125}$I-LDL was used as a measure of LDL receptor activity. Monolayers were washed three times with 10 mL ice-cold phosphate-buffered solution (PBS; 140 mmol/L NaCl, 2.7 mmol/L KCl, 9.5 mmol/L $Na_2HPO_4$, and 9.5 mmol/L $KH_2PO_4$, pH 7.4) containing 1% LPDS before 8 mL 10% LPDS medium containing 10 µg/mL $^{125}$I-LDL was added. After 4 h, the medium was collected and trichloroacetic acid (TCA)–soluble radioactivity was determined in the medium, which represents degradation of products formed by the cells and released into the medium (1). Following removal of the incubation medium, the monolayers were washed with PBS, after which the cells were solubilized in 0.1 M NaOH for determination of cellular protein concentration.

*Determination of RNA levels*. The monolayers were washed three times with 10 mL ice-cold PBS; the cells were harvested with a cell scraper, and following centrifugation, the pellets were frozen in liquid nitrogen for later RNA analysis. An aliquot of the cells was also frozen for later HMG-CoA reductase activity analysis. LDL receptor and HMG-CoA reductase RNA levels in cells were determined by a quantitative RNA-RNA solution hybridization method (20). Briefly, total nucleic acids were prepared from 10–20 × $10^6$ cells that were homogenized in 4 mL of 1 × SET (1% sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.5, and 10 mM EDTA) with a Polytrone (Kinematica Type PT 16/35; Kriens, Luzern, Switzerland) for 10–15 s at setting 5–6 and DNA concentration was assayed by Hoechst fluorometry. Antisense and sense probes for LDL receptor RNA were prepared by *in vitro* transcription of a BamHI or HindIII cleaved plasmid, carrying a 265-bp fragment of cDNA encoding the human LDL receptor with T7 (in the presence of $^{35}$S-UTP and $^{35}$S-CTP) and SP6 (in the presence of unlabeled nucleotides only) RNA polymerases, respectively. HMG-CoA reductase RNA antisense and sense probes were prepared by *in vitro* transcription of a HindIII or EcoRI-cleaved plasmid, carrying a 50-bp oligonucleotide encoding human HMG-CoA reductase, with SP6 and T7 RNA polymerases, respectively. The nucleic acid extracts or unlabeled sense RNA were hybridized with the various antisense probes in solution. Following RNase treatment and precipitation with trichloroacetic acid, the RNase resistant precipitate was collected on a GF/C filter and the radioactivity was determined in a liquid scintillation counter (Packard Instrument Company, Downers Grove IL). RNA levels were quantified by comparing the hybridization signal with the linear part of the standard curve, which was generated by hybridizations with different concentrations of sense RNA. RNA values are presented as RNA copies per diploid genome (6 pg DNA).

*Other assays*. HMG-CoA reductase activity in cell-free extracts was determined from the rate of conversion of 3-hydroxy-3-methyl(3-$^{14}$C) glutaryl coenzyme A (25,000 dpm/nmol) to $^{14}$C-mevalonate in detergent-solubilized extracts as previously described (20,21). HMG-CoA reductase activity is expressed as picomoles of $^{14}$C-mevalonate formed per minute per milligram of detergent-solubilized protein. The protein concentration in extracts and whole cells was determined by the method of Lowry *et al.* (22) with human serum albumin as a standard. $^{3}$H-Thymidine incorporation was determined by the addition of 30 µCi $^{3}$H-thymidine (Amersham Pharmacia) to the cell monolayers. After 4 h of incubation at 37°C (simultaneously with the $^{125}$I-LDL degradation assay) the cells were washed with PBS three times and filtered through GF/C filters with ice-cold 10% TCA before radioactivity was determined (23).

## RESULTS

*Gene expressions in continuously growing fibroblasts.* Figures 1 and 2 demonstrate the profound effect of growth on the expression of both the HMG-CoA reductase and LDL receptor genes in cultured human fibroblasts. Although the gene expressions were severalfold higher in cells continuously growing in the absence of lipoproteins (Figs. 2A,B) compared with fetal calf serum, RNA levels and protein activities declined rapidly as the monolayers became confluent

*Sterol sensitivity in cells at different growth rates and cell densities*. We next studied whether LDL receptor sterol sensitivity varied with different growth rates. Two experimental setups were used. In the first experimental setup, concentration–effect curves regarding the inhibitory effect of sterols (25-hydroxycholesterol + cholesterol, 1:20) on LDL receptor

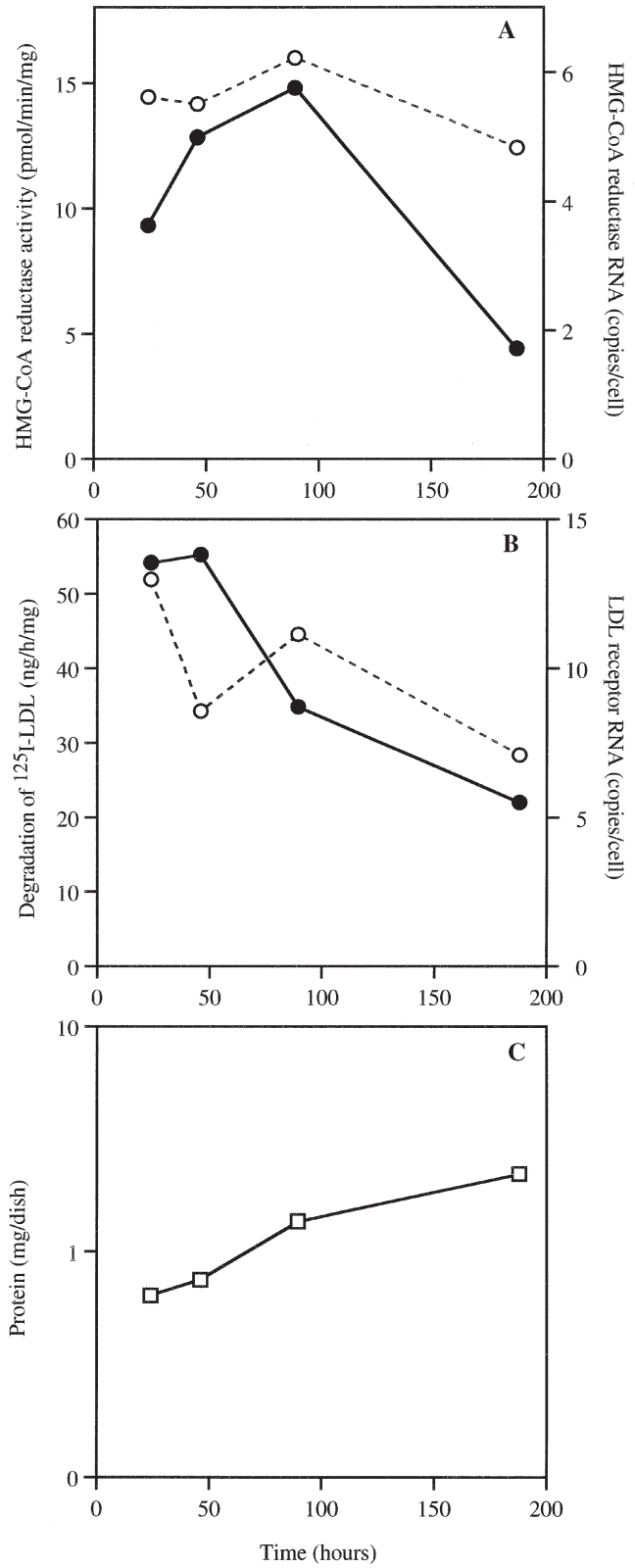



**FIG. 1.** (A) 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase RNA level (○) and protein activity (●), (B) low density lipoprotein (LDL) receptor RNA level (○) and protein activity (●), and (C) amount of cell protein/dish (log scale) in fibroblasts continuously growing in medium containing 10% fetal calf serum (FCS). Each RNA value represents the mean of three hybridizations and each degradation value represents the mean of duplicate incubations.

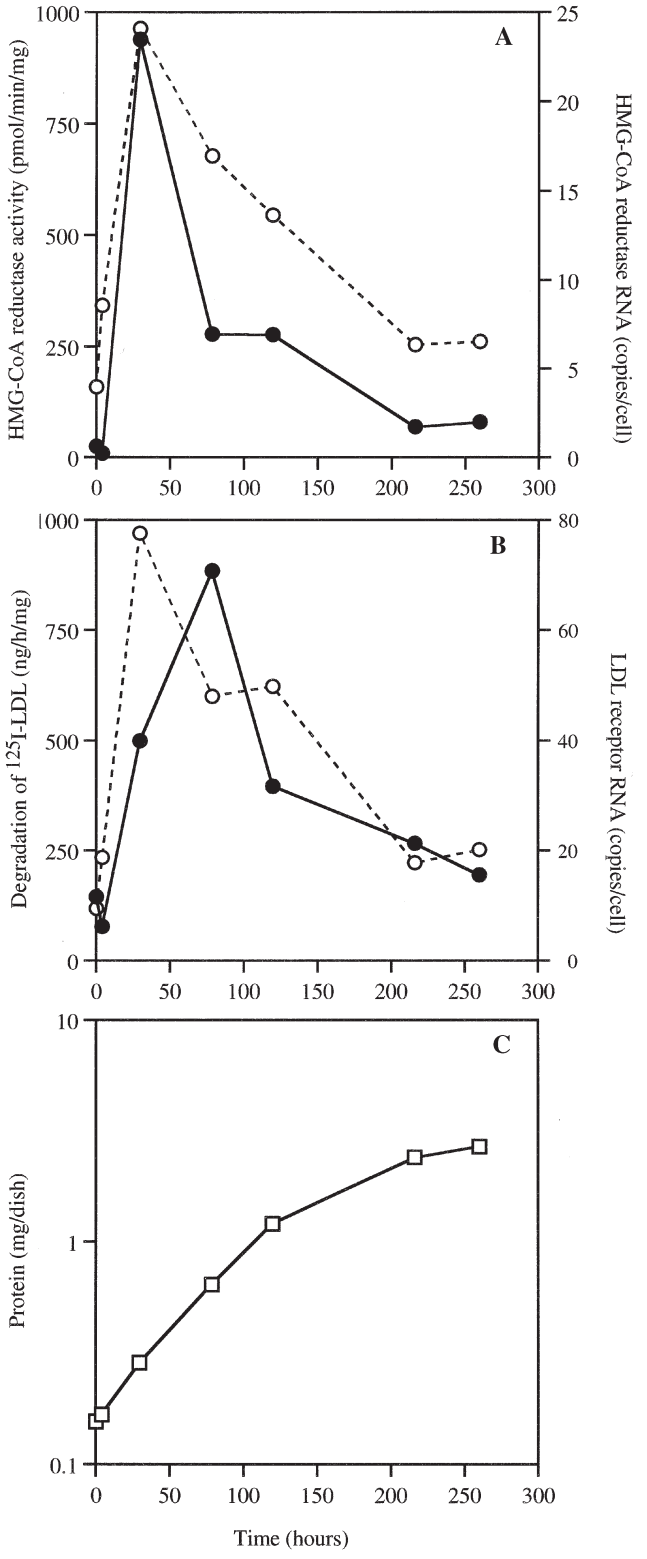
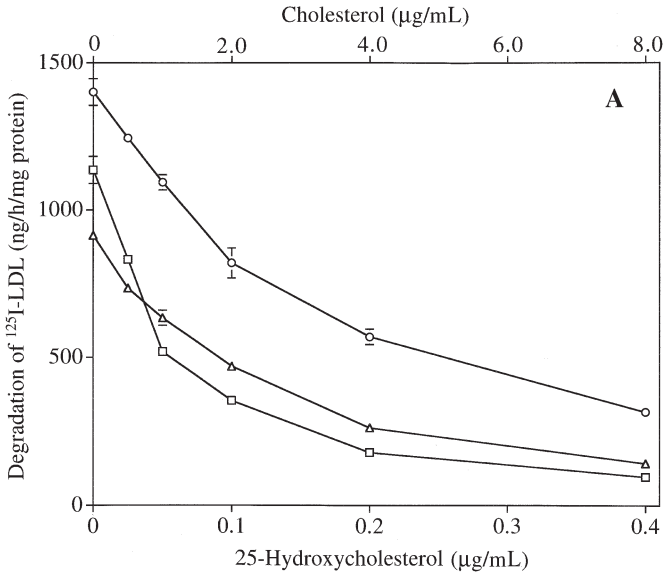
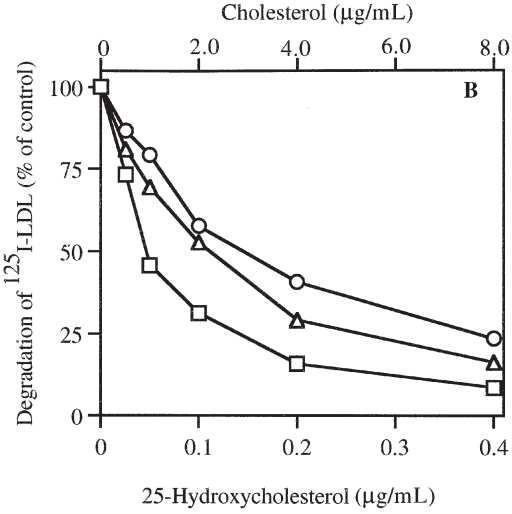



**FIG. 2.** (A) HMG-CoA reductase RNA level (○) and protein activity (●), (B) LDL receptor RNA level (○) and protein activity (●), and (C) amount of cell protein/dish (log scale) in fibroblasts continuously growing in medium containing 10% human lipoprotein-deficient serum (LPDS). The cells were seeded at day 0 in medium containing 10% FCS, and on day 1, when the monolayers still were sparse, the medium was changed to 10% LPDS medium. The number of hybridizations and incubations were as in Figure 1. See Figure 1 for other abbreviations.

activity were determined from cells plated at the same density but grown for different time periods (Fig. 3). Cells were seeded in 10% FCS medium and after 27, 44, and 99 h of growth, the medium was changed to 10% LPDS medium with or without sterols. The LDL receptor activity was determined after a further 16 h of incubation. The fractional growth (protein content after the 16 h incubation divided by protein content before) during the 16 h incubation in 10% LPDS was 1.8, 1.5, and 1.2 for the cells grown in 10% FCS for 27, 44, and 99 h, respectively. The lowest sensitivity to sterols and the highest LDL receptor activity was found in the cells grown for 44 h, which appeared subconfluent, whereas the more confluent cells grown for 99 h were more sensitive to sterols. The most sensitive cells were the sparse cells grown for 27 h. In a separate experiment we also determined the influence of sterols on HMG-CoA reductase activity in cells grown for 27 and 44 h. In contrast to the LDL receptor activity, the HMG-CoA reductase was equally sensitive to sterols at both cell densities (Fig. 4).

In the second experimental setup, sterol sensitivity was determined in cells seeded at a wide range of densities (from $3 \times 10^3$ to $24 \times 10^3$ cells/cm$^2$) and allowed to grow for the same time period. Thirty hours after seeding, the medium was changed to 10% LPDS medium with either a single concentration of sterols (25-hydroxycholesterol 0.1 µg/mL + cholesterol 2.0 µg/mL) or without sterols. Following a further 16 h of incubation the LDL receptor activity was determined (Fig. 5A). The fractional cell growth, during the 16 h of incubation in 10% LPDS, declined at cell protein contents above approximately 0.9 mg protein/dish (Fig. 5B). Also in this case there was a cell density-dependent decrease in sterol sensitivity up to approximately 0.8 mg protein/dish where the cells appeared subconfluent and LDL receptor activity reached its maximum.



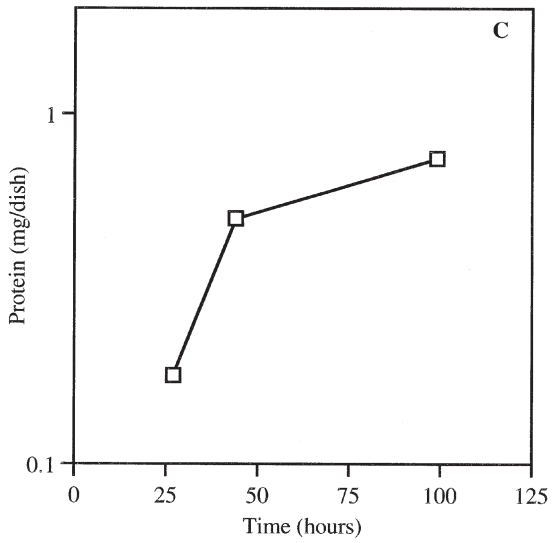




**FIG. 3.** Effect of sterols on $^{125}$I-LDL degradation rate in normal human fibroblasts grown for different time periods. (A) After 27 (□), 44 (○), and 99 (△) h of growth in medium containing 10% FCS, the medium was changed to medium containing 10% LPDS with the indicated concentrations of 25-hydroxycholesterol + cholesterol (1:20), and the cells were incubated for another 16 h before degradation of $^{125}$I-LDL was determined. (B) $^{125}$I-LDL degradation rate expressed as percentage of control cells incubated without sterols. Each degradation value represents the mean of triplicate incubations. (C) Amount of cell protein/dish (log scale) after growth for the indicated periods of time in medium with 10% FCS (27, 44, and 99 h). See Figures 1 and 2 for abbreviations.

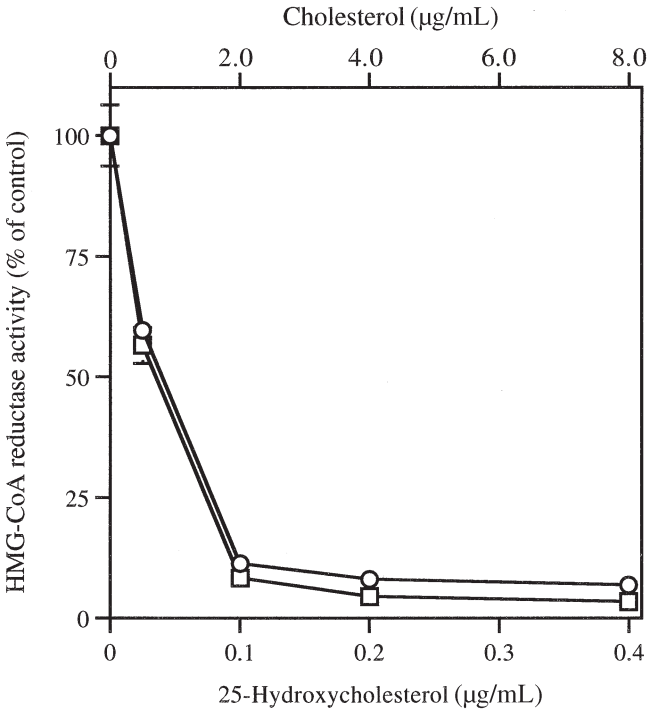



**FIG. 4.** Effect of sterols on HMG-CoA reductase activity in normal human fibroblasts grown for different time periods. After 27 (□) and 44 (○) h of growth in medium containing 10% FCS, the medium was changed to medium containing 10% LPDS with the indicated concentrations of 25-hydroxycholesterol + cholesterol (1:20), and the cells were incubated for another 16 h before HMG-CoA reductase activity was determined. Results are expressed as percentage of control cells incubated without sterols. Each value represents the mean of triplicate incubations. See Figures 1 and 2 for abbreviations.

At higher densities the sterol sensitivity again increased and LDL receptor activity decreased along with declining cell growth (Figs. 5A,B). The photographs in Figure 5C show representative illustrations of cells from the various cell densities.

In order to study in more detail growth modulation of LDL receptor and HMG-CoA reductase sterol sensitivity, we also investigated the RNA levels for both genes. $^3$H thymidine uptake was used as a measure of cell growth rate. This experiment was performed in a similar setup as that presented in Figure 5 but at two densities, sparse (seeded at $5 \times 10^3$ cells/cm$^2$) and medium (seeded at $10 \times 10^3$) density (Table 1). We found a significantly higher sensitivity to sterols at the LDL receptor RNA level in cells at low density (0.33 mg protein/dish) than at medium density (0.55 mg protein/dish), whereas the sterol sensitivity at the HMG-CoA reductase RNA level was similar at both cell densities. There was a trend toward higher $^3$H-thymidine incorporation in the low-density as compared to the medium-density cells ($P = 0.073$, Table 1).

## DISCUSSION

The objective of this study was to investigate if an intrinsic growth modulation of LDL receptor sterol sensitivity takes place in growing cells. The results demonstrate that growth modulation of LDL receptor sterol sensitivity takes place in normal human cells, both at RNA and protein levels. In contrast, HMG-CoA reductase activity was not modulated by cell growth. Essentially the same results were observed for the LDL receptor using two different experimental settings. Sterol sensitivity was determined at different cell densities obtained by either letting the cells grow for different periods of time (Figs. 3,4) or by seeding the cells at a wide range of densities (Fig. 5). Clearly, the highest LDL receptor activity and lowest sterol sensitivity were found in subconfluent cells at protein contents between 0.5 and 0.8 mg/dish (Figs. 5A,B). At cell densities above 0.8 mg protein/dish the LDL receptor activity declined and the sensitivity to sterols again increased.

Why then was LDL receptor activity lower and sterol sensitivity higher in sparse cells compared with subconfluent cells even though the growth rate was not lower in the sparse cells, as shown by data on $^3$H-thymidine uptake and fractional cell growth? One explanation could be that growing cells secrete factors that increase LDL receptor activity and lower the sensitivity to sterols. At low cell densities, the concentration of a putative factor that is secreted into the culture medium is low because of the low number of cells. The concentration of this factor, however, increases in the medium along with the increasing amount of cells resulting in increased LDL receptor activity and decreased sensitivity to sterols in the subconfluent cells.

We cannot rule out that cell to cell contact may be involved in the modulation of sterol responsiveness and LDL receptor activity. When cells come in close contact with each other, as, for example, in dishes with more than 0.8 mg protein, LDL receptor activity falls off and sterol responsiveness increases

**TABLE 1**
**High Affinity Degradation Rate of $^{125}$I-LDL, LDL Receptor RNA Levels, HMG-CoA Reductase RNA Levels, and $^3$H-Thymidine Uptake in Normal Human Fibroblasts at Low and Medium Density[a]**

| Cell density protein (mg/dish) | LDL-receptor RNA (copies/cell) | | | HMG-CoA reductase RNA (copies/cell) | | | $^3$H-Thymidine uptake |
|---|---|---|---|---|---|---|---|
| | Control | 25-OH-cholesterol | % of control | Control | 25-OH-cholesterol | % of control | (cpm/μg protein) |
| 0.333 (0.035) | 100.8 (17.2) | 48.7 (3.4) | 48.4 (3.3) | 31.8 (0.2) | 12.7 (2.3) | 40.06 (7.25) | 2381 (269) |
| 0.556 (0.058) | 70.7 (3.0) | 47.3 (3.3) | 66.9 (4.6) | 17.5 (0.6) | 8.6 (0.2) | 48.91 (1.37) | 1905 (209) |
| | | | $P = 0.005$[b] | | | $P = 0.106$[b] | $P = 0.073$[b] |

[a]Mean and standard deviation, $n = 3$.
[b]Student's *t*-test low density (0.333 mg/dish) vs. medium density (0.556 mg/dish). LDL, low density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A reductase.

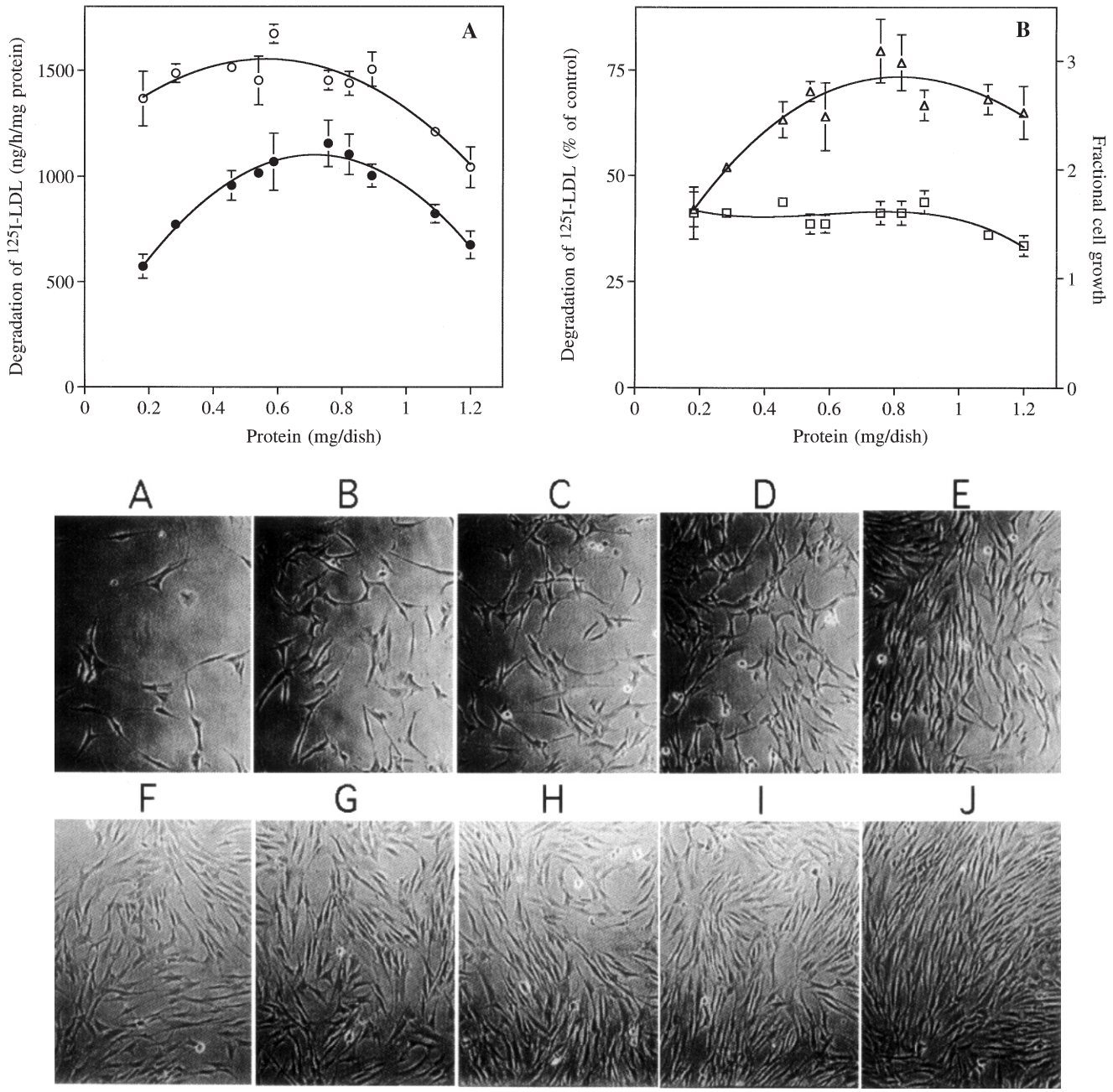



**FIG. 5.** Effect of sterols on $^{125}$I-LDL degradation rate by normal human fibroblasts at 10 different densities. Cells were seeded at 3000 to 24,000 cells/cm$^2$ in medium containing 10% FCS; after 30 h of growth, the medium was changed to medium containing 10% LPDS with (0.1 μg/mL 25-hydroxycholesterol + 2.0 μg/mL cholesterol) or without sterols. Following another 16 h of incubation, the $^{125}$I-LDL degradation rate was determined. Owing to the large number of dishes, the experiment was divided into two; the five lowest densities were seeded one week before the five highest densities. (A) Degradation of $^{125}$I-LDL in cells without (○) and with (●) sterols in the medium. (B) $^{125}$I-LDL degradation rate in the presence of sterols (△) expressed as percentage of control cells incubated without sterols and fractional cell growth (□) (calculated from the amount of protein after the 16 h incubation divided by the amount of protein before the 16 h incubation). Each value represents the mean of triplicate incubations. (C) The photographs show representative cell monolayers in the experiment in Figure 5; the mean protein contents (mg protein/dish) of the dishes are as follows: Picture A, 0.18; B, 0.28; C, 0.46; D, 0.54; E, 0.59; F, 0.76; G, 0.82; H, 0.89; I, 1.09; J, 1.20. For abbreviations see Figures 1 and 2.

rapidly. This explanation is also supported by the rapid decrease in RNA levels for both proteins when cell growth declines (Figs. 1,2). Alternatively, a high cell density may lead to a decreased available surface area of the cells that may add to the steep decrease in LDL receptor activity.

Several studies have shown that mitogenic stimulation by growth factors or phorbol esters activate LDL receptor gene expression (9,11,12,24). Activation with growth factors leads to an increased LDL receptor mRNA expression, and it has been shown that the sterol-responsive elements of the LDL

receptor are modulated by platelet-derived growth factor growth activation in human skin fibroblasts (25,26). Activation of the LDL receptor was also characterized by a decreased sensitivity to sterols, supporting our hypothesis that growth factors modulate LDL receptor sterol sensitivity and stimulate receptor activity in growing fibroblasts. Interestingly, Kenagy *et al*. (27) found that cell medium conditioned by cultured fibroblasts autostimulated LDL receptor activity, supporting the existence of such a factor. The data in the current study support that a growth-dependent autocrine modulation of sterol sensitivity takes place simply as a result of cell growth and that an exogenous addition of stimulants is not necessary in order to achieve a sterol-independent regulation of the LDL receptor.

Previous studies have shown that, in order to reach maximal LDL receptor expression, cells have to be actively growing and deprived of exogenous cholesterol (1,3,28,29). Because growing cells have higher cholesterol demands than nonproliferating cells, it is tempting to explain the strong suppression of LDL receptor and HMG-CoA reductase activities in confluent cells by a decreased cholesterol utilization for membrane synthesis leading to elevated intracellular cholesterol levels and decreased gene expression. However, results presented by Kruth *et al*. (5) show that cells at a higher density contained less cholesterol/mg protein compared to cells at a lower density both in the presence and absence of LDL cholesterol. This also supports the hypothesis that growth-dependent expression of the LDL receptor and HMG-CoA reductase is not mainly governed by the presence of cholesterol. We cannot, however, rule out that the concentration of cholesterol in a "regulatory compartment" differs from the whole cell level. That cholesterol is important for the expression of the LDL receptor and HMG-CoA reductase genes is obvious from the data in Figures 1 and 2, demonstrating that cells growing without an external source of cholesterol exhibited more than 10-fold higher expression levels than cells growing in 10% FCS.

It is possible that the growth-dependent modulation of LDL receptor sterol sensitivity, which in this study we have described in normal cells, could be abnormal in malignant cells and therefore be responsible for the elevated LDL receptor activity in AML cells. This is supported by the fact that previous studies have shown that the LDL receptor activity in leukemia cells from patients with AML has a decreased sensitivity to sterols (17). In addition to this, an *in vitro* autonomous growth that is related to an autocrine formation of growth factors (30,31) is frequently found in leukemia cells from AML patients. Modulation of sterol sensitivity may be an important mechanism to ensure an adequate cholesterol supply in growing cells.

## ACKNOWLEDGMENTS

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# Production of Docosahexaenoic Acid by *Crypthecodinium cohnii* Grown in a pH-Auxostat Culture with Acetic Acid as Principal Carbon Source

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**ABSTRACT:** *Crypthecodinium cohnii*, a marine alga used for the commercial production of docosahexaenoic acid (DHA), was cultivated in medium containing sodium acetate as principal carbon source; the pH was maintained at a constant value by addition of acetic acid, which also provided an additional carbon source in a controlled manner. The accumulation of lipid by *C. cohnii* in this pH-auxostat culture was significantly greater than previously reported for batch cultures using glucose as principal carbon source. Of six strains tested in pH-auxostat cultures, *C. cohnii* ATCC 30772 was the best, with the cells reaching 20 to 30 g dry weight per liter after 98 to 144 h and containing in excess of 40% (w/w) total lipid, with DHA representing approximately half of the total fatty acids in the triacylglycerol fraction. A productivity of 36 mg DHA $L^{-1}$ $h^{-1}$ was achieved during cultivation for 98 h using a 5% (vol/vol) inoculum, and DHA production was in excess of 3 g per liter of culture. Most of the DHA was present in neutral lipids.

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With the current increasing demand for oils rich in docosahexaenoic acid, DHA (22:6 n-3), not only for improved infant nutrition (1–3) but also as a dietary supplement for adults (4–7), attention has increasingly focused on the potential of microorganisms to meet these requirements (8,9). Nonmicrobial supplies of pure DHA are restricted to such sources as the eye-socket of the tuna or to expensive fractionation of fish oils (4), but in the latter case the DHA is nearly always accompanied by notable amounts of eicosapentaenoic acid (20:5n-3) (10), which may detract from the efficacy of DHA as a nutritional supplement (1–7). However, it has long been known that the marine dinoflagellate, *Crypthecodinium cohnii*, produces DHA as its sole polyunsaturated fatty acid (11). Development of this organism as a commercial source of oil rich in DHA has taken place over the past 6 to 8 yr, mainly through the efforts of Martek Co. Inc. (Columbia, MD) (12–14), and numerous patents have been taken out to protect the process and the product (e.g., Refs. 15–17).

In an attempt to improve upon the existing process, which uses glucose as the principal carbon source, we have discovered that the organism will grow to high cell densities, and also produce DHA at high concentrations within its intracellular oil, when it is grown on acetic acid. Preliminary work (de Swaaf, M.E., personal communication) indicated that *C. cohnii* could utilize sodium acetate, but the rise in pH that accompanied growth resulted in poor cell yield (<3 g/L). Also, published work (18) indicated that *C. cohnii*, as well as other microalgae, might be able to grow on acetate, but only if the concentration was kept low (1 g/L or less).

Growth of microorganisms on sodium acetate results in a rise in pH. This is because $Na^+$ remains in the medium and $CH_3COO^-$ is gradually replaced with $OH^-$ and other anions. Sodium hydroxide is a much stronger base than sodium acetate, and hence the pH rises. An increase in pH is characteristic of growth of microorganisms on salts of organic acids, or organic acids that have been neutralized by the addition of alkali. In the present work, we have used a pH-auxostat culture system in which a low initial concentration of sodium acetate is included in the initial growth medium and acetic acid is used to maintain a constant pH value and supply a further carbon source for growth. The concept of the pH-auxostat was first described in 1976 (19), and a pH-auxostat fed with acetic acid has been used to cultivate *Mucor circinelloides* for the production of biomass and an oil rich in γ-linolenic acid (20). In this paper we report the cultivation of *C. cohnii* in a pH-auxostat and DHA production in excess of 3 g per liter of culture.

## MATERIALS AND METHODS

*Growth of* C. cohnii. *Crypthecodinium cohnii* strain ATCC 30772 was used except where stated otherwise. Strains were grown at 27°C for 4–5 d in screw-capped tubes (25 mL) containing 5 mL of medium (ATCC Culture Medium 460 A2E6) and used to inoculate starter cultures. Starter cultures were grown at 27°C for 4 d in static flasks (250 mL) containing 100 mL medium containing (per liter): glucose (9 g), yeast extract (2 g), and sea salts (25 g). Shake-flask cultures were grown in flasks (250 mL) containing 100 mL medium composed of (per liter): glucose (27 g), yeast extract (3.8 g), and sea salts (25 g). These cultures were inoculated (10% vol/vol) with static culture and grown at 27°C for 3 d with shaking at 180 rpm.

Small pH-auxostat cultures were grown in fermenters (1 L) containing 800 mL of medium composed of (per liter): yeast extract (7.5 g), sea salts (25 g), and sodium acetate (1–16 g, as required). The pH of the medium was adjusted to

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Abbreviations: ATCC, American Type Culture Collection; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC, gas chromatography; TLC, thin-layer chromatography; vvm, volume of air/volume of medium/min.

approximately 6.5 with NaOH, prior to autoclaving for 60 min at 121°C. pH-auxostat cultures were inoculated (10% vol/vol) with culture grown in shake-flasks as described above. The pH was maintained at 6.5 by the automatic addition of acetic acid (50% vol/vol) and, if necessary, KOH (2 M). The temperature was maintained at 27°C. The cultures were stirred at 300–1000 rpm and aerated at 0.2 to 1.0 vol air/vol medium/min (vvm), increasing the stirring speed and aeration as required to maintain a dissolved oxygen concentration above 30% of air saturation. Foaming was controlled by addition of polypropylene glycol (2000 grade). For growth in a fermenter with glucose as principal carbon source, the shake-flask culture medium described above was used and inoculated (10% vol/vol) with culture grown in the same medium. The growth conditions were otherwise as described for pH-auxostat culture except that the pH was maintained at 6.5 by the automatic addition of KOH (2 M) and, if necessary, HCl (2 M).

Larger pH-auxostat cultures were grown in the same medium (3.5 L) in fermenters (5 L) and inoculated (5% vol/vol) with pH-auxostat culture grown for 3 d in a 1-L pH-auxostat culture. The culture was aerated at 1 vvm and the dissolved oxygen concentration was maintained above 30% of air saturation by increasing the stirrer speed as required, from an initial speed of 300 rpm to a maximum of 1000 rpm. The growth temperature was 27°C and the pH was maintained at 6.5 as described above. Foaming was controlled as before.

*Determination of dry weight.* Samples of culture (50 to 100 mL) were centrifuged (4000 × *g* at 5°C for 10 min) and washed twice with water. The resulting cells were freeze-dried until constant weight was achieved.

*Lipid extraction.* Lipid was extracted from freeze-dried cells (usually 250 to 500 mg) by standing overnight in approximately 100 mL chloroform/methanol (2:1, vol/vol) and then following established procedures (21), with the final lipid amount being determined gravimetrically.

*Lipid fractionation.* The total lipid extract (approximately 120 mg) was fractionated following the procedure of Christie (22) using a 70 mL/10 g SPE Isolute $NH_2$ column (International Sorbent Technology, Mid-Glamorgan, United Kingdom) which was washed initially with hexane, and then the lipid extract, in chloroform, was added. Fractions were eluted progressively with diethyl ether (to elute neutral triacylglycerols), acetone/pyridine (1:1, vol/vol) (to elute sterol esters, sterol glycosides, and any digalactosyldiacyl glycerols), methanol (to elute choline-containing lipids), and finally chloroform/methanol/28% (wt/vol) aqueous ammonia/0.05 M ammonium acetate (4:1:0.08:0.02, by vol), to remove polar phospholipids. The solvent from each lipid fraction was evaporated and the residue weighed. The identity of each lipid component was confirmed by standard thin-layer chromatography (TLC) analysis (23).

*Fatty acid analysis.* Fatty acyl groups of the total lipid or the lipid fractions were transesterified using trimethylsulfonium hydroxide (24) and analyzed by gas chromatography (25).

*Determination of polysaccharide.* Samples of culture (100 mL) were centrifuged (4000 × *g* at 5°C for 10 min). The supernatant (25 mL) was mixed with 2-propanol (75 mL) and allowed to stand at room temperature for 30 min. The precipitated material was transferred to a preweighed filter (Whatman GF/A) and dried to constant weight at 65°C.

## RESULTS

*Optimization of the initial nutrient concentrations for growth of* C. cohnii *in a pH-auxostat.* When *C. cohnii* ATCC 30772 was grown in a series of pH-auxostat cultures with the initial concentration of sodium acetate in the medium ranging from 1 to 16 g/L, the growth rate and cell density attained after growth for 140 h were greatest with sodium acetate at 8 g/L (Fig. 1A). This concentration also produced the highest lipid content in the cells (Fig. 1B). The concentration of sodium acetate in the medium had no significant effect on the proportion of DHA, which represented approximately 40 to 50% of the total fatty acids (Fig. 1C).

The effect of independently varying the concentrations of sea salts and yeast extract in the growth medium was tested. No significant improvement in lipid accumulation or DHA production was achieved by modest increases or decreases in concentration (data not shown).

*Performance of other strains in pH-auxostat culture.* The performance of six strains of *C. cohnii*, including ATCC 30772, all grown in pH-auxostat cultures with 8 g sodium acetate/L as the initial concentration, is summarized in Table 1. All the strains tested produced DHA, and the highest productivity was achieved using strain 30772. On the basis of the amount of acetic acid consumed, strain 30772 produced the lowest yield of cells and the highest yield of DHA.

All cultures of *C. cohnii* became viscous toward the end of the growth period, and material, presumably exopolysaccharide, could be precipitated from the supernatant fluid by treatment with 2-propanol (Table 1). The highest concentration of precipitable material was found for strain ATCC 30772.

*Effect of inoculum substrate on pH-auxostat growth.* It is well known that the utilization of acetate requires the induction of several enzymes that are not active in glucose-grown cells. The performance of *C. cohnii* in the pH-auxostat was, however, similar for cultures inoculated with glucose or acetate (pH-auxostat) cultures. No significant differences were seen with respect to growth performance (Fig. 2A), lipid production (Fig. 2B), or DHA production (Fig. 2C), and both lipid and DHA production were substantially greater than achieved during batch fermentation with glucose as carbon source (Figs. 2B, 2C). Presumably the inducible enzymes needed for the utilization of acetate were synthesized soon after exposure of the cells to acetate, and this occurred during the lag period, prior to rapid growth of the culture.

*Lipid fractionation.* The lipid produced by cells grown with glucose as principal carbon source was fractionated into its major components (Table 2). Comparable data for cells grown on acetate in a pH-auxostat are shown in the lipid from

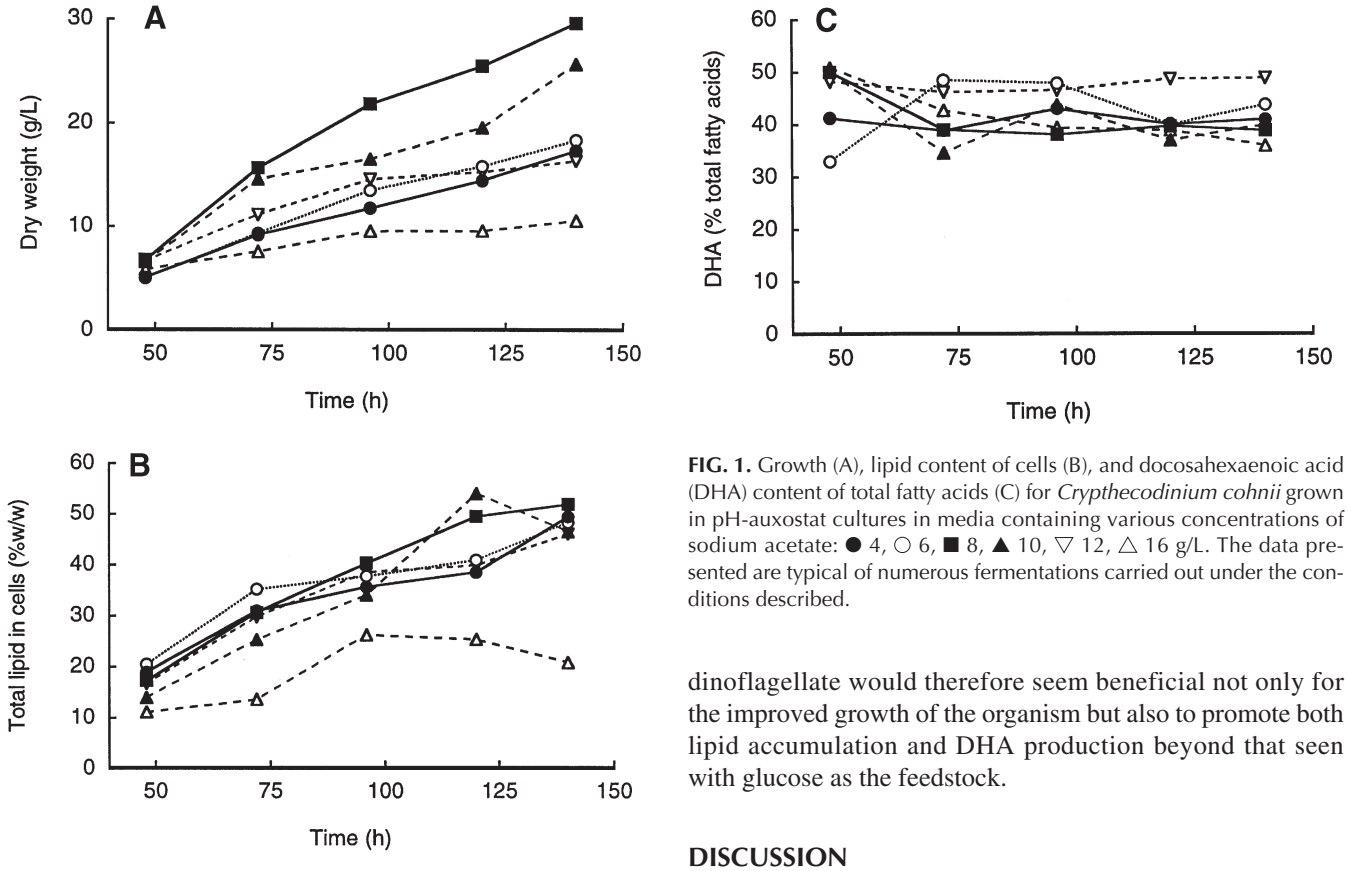


**FIG. 1.** Growth (A), lipid content of cells (B), and docosahexaenoic acid (DHA) content of total fatty acids (C) for *Crypthecodinium cohnii* grown in pH-auxostat cultures in media containing various concentrations of sodium acetate: ● 4, ○ 6, ■ 8, ▲ 10, ▽ 12, △ 16 g/L. The data presented are typical of numerous fermentations carried out under the conditions described.

Table 3. In both cases the major fraction, constituting 75% of the total lipids, was the neutral lipids. These were shown by TLC to be wholly triacylglycerols. The proportion of the other lipid components was also similar for cells produced in glucose and acetate pH-auxostat cultures. Of possible significance, though, was the increased content of DHA not only in the total extracted lipid but also in the triacylglycerol fraction obtained from the acetate-grown cells compared to the glucose-grown cells. The triacylglycerol fraction, which is the commercially most valuable part of the oil, contained over 50% more DHA when isolated from the acetate-grown cells than was in the same fraction obtained from the glucose-grown cells. Acetate as a feedstock for the cultivation of this dinoflagellate would therefore seem beneficial not only for the improved growth of the organism but also to promote both lipid accumulation and DHA production beyond that seen with glucose as the feedstock.

## DISCUSSION

The large-scale production of DHA-rich oil derived from *C. cohnii* has been carried out since the mid-1990s with the oil being offered as an over-the-counter dietary supplement for adults and as a beneficial and important polyunsaturated fatty acid for inclusion in infant formulae. Although technical aspects of the commercial process are obviously restricted, it is nevertheless understood that the organism is cultivated in large stirred fermenters with glucose as principal carbon source (12,13). Yields of biomass, lipid, and DHA are, of course, closely guarded company secrets.

Nevertheless the results being reported here would indicate that an alternative process based on acetic acid as the principal carbon feedstock might seek to rival the traditional glucose feedstock process. The performance of *C. cohnii*

**TABLE 1**
**Comparison of *Crypthecodinium cohnii* Strains After 140 h in pH-Auxostat Cultures**

| ATCC strain number | Dry weight ($g\ L^{-1}$) | Yield of cells[a] [mg (g acetic acid)$^{-1}$] | Lipid ($g\ L^{-1}$) | Lipid (% dry wt) | DHA (% total fatty acids) | DHA ($g\ L^{-1}$) | Yield of DHA[b] [mg (g acetic acid)$^{-1}$] | Precipitable material[c] ($g\ L^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| 30772 | 17.0 | 120 | 7.5 | 44 | 59 | 4.4 | 30 | 8.0 |
| 30541 | 45.5 | 200 | 8.3 | 24 | 33 | 2.7 | 12 | 2.8 |
| 50298 | 45.5 | 210 | 12.3 | 30 | 31 | 3.8 | 18 | 3.8 |
| 40750 | 17.0 | 220 | 3.3 | 22 | 46 | 1.5 | 26 | 3.3 |
| 30555 | 20.7 | 180 | 2.8 | 15 | 30 | 0.8 | 7 | 5.0 |
| 30557 | 25.6 | 160 | 4.7 | 18 | 40 | 1.9 | 10 | 3.9 |

[a,b]Yield calculated on basis of acetic acid utilized.
[c]Material precipitated by treatment of culture supernatant with 2-propanol and inferred to be polysaccharide (see Ref. 28).

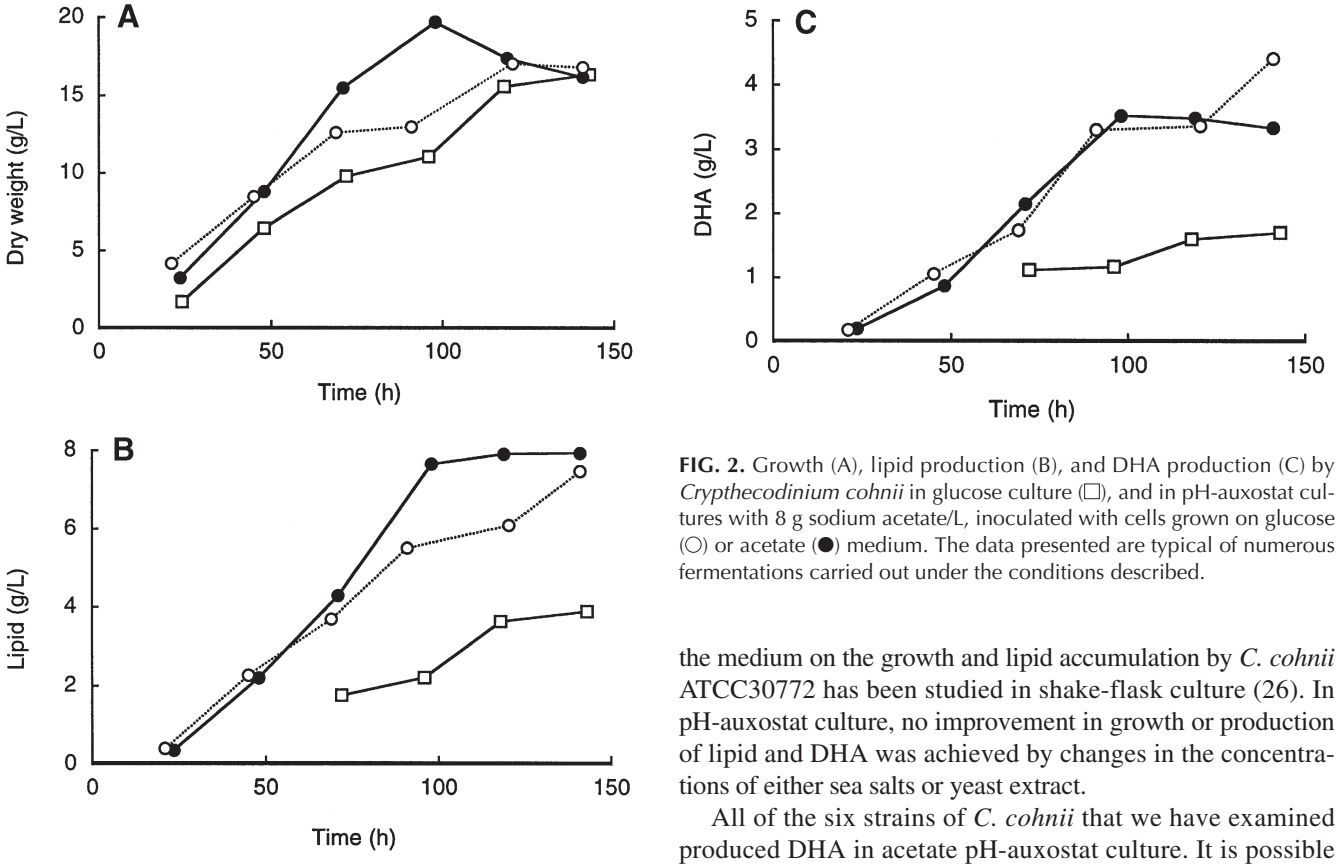


**FIG. 2.** Growth (A), lipid production (B), and DHA production (C) by *Crypthecodinium cohnii* in glucose culture (□), and in pH-auxostat cultures with 8 g sodium acetate/L, inoculated with cells grown on glucose (○) or acetate (●) medium. The data presented are typical of numerous fermentations carried out under the conditions described.

ATCC 30772 in acetate pH-auxostat culture is certainly superior to that achieved with this strain using medium containing glucose as principal carbon source (26). Overall productivities for total lipid and DHA were calculated as 78 and 36 mg $L^{-1}h^{-1}$, respectively, for 98-h cultivation in a pH-auxostat culture using a 5% inoculum taken from a similar culture (Figs. 2B, 2C). The influence of the concentrations of sea salts and yeast extract in the medium on the growth and lipid accumulation by *C. cohnii* ATCC30772 has been studied in shake-flask culture (26). In pH-auxostat culture, no improvement in growth or production of lipid and DHA was achieved by changes in the concentrations of either sea salts or yeast extract.

All of the six strains of *C. cohnii* that we have examined produced DHA in acetate pH-auxostat culture. It is possible that other strains of this organism (the American Type Culture currently lists some 45 strains) might have even better DHA productivity than strain ATCC 30772. The pH-auxostat is clearly a useful method of cultivation of *C. cohnii* on acetate, since the carbon source is delivered in a controlled manner so that its concentration never becomes inhibitory to growth. The pH-auxostat might also be of value for the cultivation of a wide range of oleaginous organisms on acetic acid, which is an inexpensive carbon source.

**TABLE 2**
**Fractionation, by Column Chromatography, of Lipid Extracted from *Crypthecodinium cohnii* ATCC 30772 Grown with Glucose as Principal Carbon Source**

| | Relative % (w/w) of fatty acyl groups | | | | |
|---|---|---|---|---|---|
| Fatty acid | Total lipid | Neutral lipids | Sterolglycosides and digalactosyldiacyl glycerols | Choline lipids | Acidic phospholipids |
| 10:0 | 7 | 7 | Trace[a] | Trace | Trace |
| 12:0 | 24 | 19 | 19 | 3 | 6 |
| 14:0 | 26 | 22 | 16 | 11 | 16 |
| 16:0 | 8 | 9 | 18 | 19 | 27 |
| 16:1 | 1 | 2 | 1 | 1 | 2 |
| 18:0 | 1 | 1 | 4 | 3 | 8 |
| 18:1 | 7 | 9 | 12 | 11 | 16 |
| 22:6 | 26 | 31 | 30 | 52 | 25 |
| Weight applied (mg) | 121 | — | — | — | — |
| Weight recovered (mg) | 111 | 82 | 9 | 18 | 1.3 |
| Recovery (% original lipid) | 92 | 68 | 7 | 15 | 1 |
| Recovery (% total recovered lipid) | (100) | 75 | 8 | 16 | 1 |

[a]Trace (<0.5%).

**TABLE 3**
**Fractionation, by Column Chromatography, of Lipid Extracted from *Crypthecodinium cohnii* ATCC 30772 Grown in pH-Auxostat Culture with Acetic Acid as Principal Carbon Source**

| | Relative % (w/w) of fatty acyl groups | | | | |
|---|---|---|---|---|---|
| Fatty acid | Total lipid | Neutral lipids | Sterolglycosides and digalactosyldiacyl glycerols | Choline lipids | Acidic phospholipids |
| 10:0 | Trace[a] | Trace | Trace | Trace | Trace |
| 12:0 | 7 | 8 | 13 | 5 | Trace |
| 14:0 | 21 | 16 | 18 | 13 | 16 |
| 16:0 | 18 | 16 | 19 | 15 | 28 |
| 16:1 | 2 | 2 | 2 | Trace | 2 |
| 18:0 | 2 | 1 | 3 | 4 | 5 |
| 18:1 | 11 | 10 | 11 | 11 | 14 |
| 22:6 | 39 | 47 | 34 | 52 | 35 |
| Weight applied (mg) | 132 | — | — | — | — |
| Weight recovered (mg) | 125 | 93 | 12 | 19 | 1.4 |
| Recovery (% original lipid) | 95 | 70 | 9 | 14 | 1 |
| Recovery (% total recovered lipid) | (100) | 74 | 10 | 15 | 1 |

[a]Trace (<0.5%).

Fractionation of the lipid produced from glucose (Table 2) and acetate (Table 3) cultures confirmed the observations (27) that DHA is present in both neutral and polar lipids of *C. cohnii* and that choline lipids contain the highest proportion of DHA. In the present study, however, most of the DHA is present in neutral lipids, simply because of the much greater lipid accumulation achieved.

The material that could be precipitated from supernatant fluid with 2-propanol has recently been identified as polysaccharide (28). The production of exopolysaccharide is likely to affect yield and is therefore significant for large-scale cultivation of *C. cohnii* for DHA production. Thus, carbon and energy source will be wasted in production of polysaccharide, and it becomes increasingly difficult to supply cultures with sufficient dissolved oxygen as their viscosity increases. The presence of polysaccharide may also interfere with the recovery of the DHA-containing oil.

A patent application (GB 00/02695) covering the main aspects of this work has been filed.

## ACKNOWLEDGMENT

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# Regiospecific Analysis of Conifer Seed Triacylglycerols by Gas–Liquid Chromatography with Particular Emphasis on Δ5-Olefinic Acids

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**ABSTRACT:** Dibutyroyl derivatives of monoacylglycerols (DBMAG) from conifer seed oil triacylglycerols (TAG) were prepared by partial deacylation of TAG with ethylmagnesium bromide followed by diesterification with *n*-butyryl chloride. The resulting mixtures were analyzed by gas–liquid chromatography (GLC) with a 65% phenylmethyl silicon open tubular fused-silica capillary column operated under optimal conditions and separated according to both their fatty acid structures and their regiospecific distribution. Seed oils of 18 species from 5 conifer families (Pinaceae, Taxaceae, Cupressaceae, Cephalotaxaceae, and Podocarpaceae) were analyzed. The chromatograms showed a satisfactory resolution of DBMAG containing palmitic (16:0), stearic (18:0), taxoleic (*cis*-5,*cis*-9 18:2), oleic (*cis*-9 18:1), *cis*-vaccenic (*cis*-11 18:1), pinolenic (*cis*-5,*cis*-9,*cis*-12 18:3), linoleic (*cis*-9,*cis*-12 18:2), α-linolenic (*cis*-9,*cis*-12,*cis*-15 18:3), and an almost baseline resolution of DBMAG containing gondoic (*cis*-11 20:1), *cis*-5,*cis*-11 20:2, sciadonic (*cis*-5,*cis*-11,*cis*-14 20:3), dihomolinoleic (*cis*-11,*cis*-14 20:2), juniperonic (*cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4), and dihomo-α-linolenic (*cis*-11,*cis*-14,*cis*-17 20:3) acids. We have observed that results for *Pinus pinaster* and *P. koraiensis* seed oils obtained with this new simple method compared favorably with literature data established with other usual regiospecific analytical techniques. Δ5-Olefinic acids are esterified mainly at the external positions of the glycerol backbone in all cases, in agreement with data obtained by other methodologies allowing validation of the GLC regiospecific method. To date, 45 gymnosperm species (mostly Coniferophytes) from 21 genera belonging to 9 families have been analyzed, all of them showing a definite enrichment of Δ5-olefinic acids in the external positions of TAG. These fatty acids (FA), with one exception only, represent between ~2 and 8% of FA esterified to the internal positions. For some species, i.e., *P. koraiensis* and *P. pinaster,* this asymmetrical distribution was established by at least three analytical procedures and confirmed by stereospecific analysis of their seed TAG.

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Abbreviations: DAG, diacylglycerol; DBMAG, dibutyroyl-monoacylglycerol; FA, fatty acid; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; MAG, monoacylglycerol; NMR, nuclear magnetic resonance; TAG, triacylglycerol; TLC, thin-layer chromatography; UPIFA, unsaturated polymethylene-interrupted fatty acid.

Although gymnosperm seed fatty acid (FA) compositions have been almost completely neglected for decades, ~200 species have been analyzed in our laboratory since 1995. This systematic study followed that of Takagi and Itabashi (1) published in 1982, who established the FA compositions of 20 species. In addition to the presence of Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA, or Δ5-olefinic acids), the seed triacylglycerols (TAG) show a particular regio- and stereospecific distribution of these FA. Both classical and recent procedures have shown that Δ5-UPIFA are esterified mainly at the external [*sn*-1(3)] positions and scarcely at the internal (*sn*-2) position. In the classical procedures, TAG are partially deacylated with a Grignard reagent (2) or pancreatic lipase (3), and FA resulting from the partially deacylated products [isolated by thin-layer chromatography (TLC)] are analyzed by gas–liquid chromatography (GLC). In the recent methods, $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy is used to assess Δ5-UPIFA at the external and internal positions (4–11). Both protocols indicate either a low content of Δ5-UPIFA, or very little Δ5-UPIFA, at the *sn*-2 position, respectively. However, each method has its limitations. With the lengthy classical methods, which include a TLC step, some intra-isomerization of monoacylglycerols (MAG) can occur, whereas $^{13}C$ NMR spectroscopy is unable to detect signals arising from the *sn*-2 position when Δ5-UPIFA are less than ~3% of the total FA at this position.

In more thorough experiments (12), the stereospecific distribution of Δ5-UPIFA was established in a few species. In these experiments, appropriate derivatives of *sn*-1,2- and *sn*-2,3-diacylglycerol (DAG) were separated by high-performance liquid chromatography (HPLC) on a chiral column. This clearly demonstrated that Δ5-UPIFA are essentially esterified at the *sn*-3 position of TAG (>80% of total Δ5-UPIFA). However, it is also possible in this procedure that Δ5-UPIFA may have migrated to some extent from one position to another during separation of DAG by TLC. It is noteworthy that TAG molecular species containing two Δ5-UPIFA were scarce, or even absent in two pine species (13).

To reduce any positional acyl migrations in MAG or DAG, we adopted a new regiospecific analytical procedure that considerably reduces the time of analysis (14,15; Destaillats, F., Angers, P., Wolff, R.L., and Arul, J., unpublished data). Briefly,

TAG are partially deacylated with ethylmagnesium bromide and immediately butylated with *n*-butyryl chloride. Dibutyroyl-MAG (DBMAG) are then directly analyzed by GLC under appropriate conditions (*sn*-1,2-DBMAG coelute with *sn*-2,3-DBMAG, but are separated from *sn*-1,3-DBMAG); thus, no TLC step is involved. The objectives of this study were to compare data obtained with the new method to earlier data obtained with classical procedures, and to determine, using this method, the regiospecific distribution of FA of conifer seed oils from species or genera that have not been analyzed to date.

## MATERIALS AND METHODS

*Samples and reagents.* The conifer species studied for their seed lipids are listed in Table 1. Details of their source, oil extraction, and the FA compositions of some species are found elsewhere (16,17). Phosphatidylcholine and standard TAG (tripalmitin, tristearin, triolein, tri-*cis*-vaccenin, trilinolein, tri-α-linolenin, trigondoin, trieicosadienoin, and trieicosatrienoin) were purchased from Sigma Chemical (St Louis, MO). Ethyl magnesium bromide, *n*-butyryl chloride, and triethylamine were obtained from Aldrich (Milwaukee, WI).

*DBMAG synthesis*. The method of Angers and Arul (14) was followed with only slight modifications. To a stirred solution of oil (10 mg, equivalent to a droplet) in anhydrous diethylether (500 μL), contained in a flame-dried flask under inert atmosphere ($N_2$), a solution of ethyl magnesium bromide in the same solvent (3.0 M, 40 μL) was added. After 30 s of stirring at room temperature, glacial acetic acid (10 μL) was added, followed by boric acid (10% aqueous solution, 300 μL). The mixture was quickly extracted with diethyl ether (3 × 500 μL) and the organic extracts were combined, washed sequentially with $Na_2CO_3$ (10% aqueous solution, 2 mL) and NaCl (saturated aqueous solution, 2 mL), dried over anhydrous $Na_2SO_4$, and concentrated under a stream of dry nitrogen. The residue was dissolved in dry chloroform (500 μL); triethylamine (100 μL) and *n*-butyryl chloride (50 μL) were added sequentially after homogenization, and the mixture was held at 40–60°C for 30 min in a closed vial with constant stirring. After the mixture was cooled to room temperature, distilled water (500 μL) was added, and after homogenization, the mixture was added to *n*-octane (1 mL) and sequentially washed with HCl (0.15 N, 2 mL), $Na_2CO_3$ (10%, 2 mL), and NaCl (saturated aqueous solution, 2 mL). The organic extract was dried over anhydrous sodium sulfate and filtered before GLC analysis.

**TABLE 1**
**List of Conifer Seed Oils Analyzed in the Present Study**

| Family | Sample no. | Genus and species |
|---|---|---|
| Pinaceae | 1 | *Abies nephrolepis* |
| | 2 | *Cedrus brevifolia* |
| | 3 | *Larix gmelinii* var. *olgensis* |
| | 4 | *Picea schrenkiana* |
| | 5 | *Pinus pinaster* |
| | 6 | *P. koraiensis* |
| | 7 | *P. sylvestris* (M)[a] |
| | 8 | *P. sylvestris* (U) |
| | 9 | *Hesperopeuce mertensiana*[b] |
| | 10 | *Pseudolarix amabilis* |
| | 11 | *Tsuga caroliniana* |
| Cephalotaxaceae | 12 | *Cephalotaxus drupaceae* |
| | 13 | *C. sinensis* |
| Podocarpaceae | 14 | *Nageia nagi* |
| Taxaceae | 15 | *Taxus chinensis* |
| | 16 | *Torreya californica* |
| Cupressaceae | 17 | *Austrocedrus chilensis* |
| | 18 | *Juniperus virginiana* |

[a]Geographical origin: M, Mongolia; U, Ukraine.
[b]Also known as *Tsuga mertensiana* (see Ref. 18).

*GLC analyses*. DBMAG were analyzed in a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a RTX-65TG (65% phenylmethyl silicon) open tubular fused-silica capillary column (30 m × 0.25 mm i.d., 0.10-μm film thickness; Restek, Brockville, CA). The injector (split mode) and the flame-ionization detector were maintained at 400°C. Quantitative data were calculated by a Hewlett-Packard GLC ChemStation.

Three different temperature programs were used to obtain an almost baseline separation of most of the DBMAG containing a Δ5-unsaturated FA. Identifications were performed by comparison with DBMAG prepared with authentic standards of usual TAG. The initial chromatographic runs were performed under conditions described below, which allowed the separation of DBMAG but not baseline resolutions of DBMAG containing a Δ5-unsaturation. The oven temperature was 270°C for 10 min and then increased to 360°C at 20°C/min. The inlet pressure of the carrier gas ($H_2$) was 120 kPa. Satisfactory resolutions of DBMAG of the $C_{18}$ group containing taxoleic (*cis*-5,*cis*-9 18:2), oleic (*cis*-9 18:1), *cis*-vaccenic (*cis*-11 18:1), pinolenic (*cis*-5,*cis*-9,*cis*-12 18:3), and linoleic acids were achieved with isothermal separation at 210°C for 50 min, then to 360°C at 20°C/min for 3 min. The inlet pressure of the carrier gas ($H_2$) was 140 kPa. Separations of DBMAG of the $C_{20}$ group containing 5,11-eicosadienoic (*cis*-5,*cis*-11 20:2), gondoic (*cis*-11 20:1), sciadonic (*cis*-5,*cis*-11,*cis*-14 20:3), dihomolinoleic (*cis*-11,*cis*-14 20:2), juniperonic (*cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4), and dihomo-α-linolenic (*cis*-11,*cis*-14,*cis*-17 20:3) acids were achieved with temperature programming. The oven was programmed from 100 to 300°C at a rate of 5°C/min, then to 360°C at 20°C/min. The inlet pressure of the carrier gas ($H_2$) was 84 kPa. Quantification of *sn*-2- and *sn*-1(3)-DBMAG was performed by normalizing each series to 100% and dividing each peak area by the respective sums. Results are expressed as area percentages, and no response factors were applied.

*GLC of FA methyl esters*. Methylation of FA was carried out in a sealed tube with 0.4 N sodium methoxide in methanol. Analyses of the methyl esters were performed with a Hewlett-Packard gas chromatograph model 5890 Series II, equipped with a flame-ionization detector and connected to a computer with a Hewlett-Packard ChemStation. Samples in hexane (1.0 μL) were injected on an open tubular DB-225

capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness; J&W). The injector and detector temperatures were maintained at 250°C, and the oven temperature was 190°C. Hydrogen was the carrier gas, with a head pressure of 140 kPa.

## RESULTS AND DISCUSSION

*Sampling.* Eighteen conifer seed oils were analyzed in this study. This sampling contained original species whose regiospecific distribution is not known and six species (*Taxus chinensis*, *Cephalotaxus drupaceae*, *Pinus koraiensis*, *P. sylvestris*, *P. pinaster*, and *Juniperus virginiana*) whose regiospecific profiles have already been determined by literature methods (1,4,6,8,12,19). The latter group was included for the purpose of comparison of the present method. Seed oils were extracted by the method of Folch *et al.* (20) and used without further purification.

*Experimental considerations.* The oil samples were crude and possibly contained components other than TAG, such as phospholipids, which can interfere in DBMAG synthesis. FA profiles of polar lipids and TAG fractions are known, and both fractions contain different proportions of Δ5-UPIFA (1). Egg yolk phosphatidylcholine was submitted to Grignard degradation and derivatization with *n*-butyryl chloride under conditions similar to seed oil as described above. GLC analysis of the resulting products did not exhibit any peaks attributable to DBMAG. Presumably *sn*-1-monoacyl-lysophospholipids and *sn*-2-monoacyl-lysophospholipids resulting from Grignard degradation are removed in the aqueous extract during work-up. Thus, phospholipids do not interfere with the regiospecific analysis of TAG by the present method, which can be performed directly on the crude lipid extracts without further purification of TAG by TLC or other methods.

*Optimization of analytical conditions.* Different GLC conditions were examined for the separation of DBMAG containing Δ5-UPIFA. A systematic search of optimal temperature and pressure conditions revealed that the retention of the $C_{18}$ and $C_{20}$ DBMAG on the stationary phase is different; consequently, they cannot be completely baseline-resolved under the same chromatographic conditions. The $C_{18}$ DBMAG group includes stearic, taxoleic, oleic, *cis*-vaccenic, pinolenic, linoleic, α-linolenic, and coniferonic acids; the $C_{20}$ group encompasses 5,11- 20:2, gondoic, sciadonic, dihomolinoleic, juniperonic, and dihomo-α-linolenic acids, at different regioisomeric positions.

For the $C_{18}$ regioisomers, isothermal conditions were used. The DBMAG elution order was established by comparison with stearic, oleic, *cis*-vaccenic, linoleic, and α-linolenic DBMAG in their *sn*-1(3) and *sn*-2 positions prepared from authentic TAG standards. Figure 1 shows the relative elution of these DBMAG at different temperatures when the gas

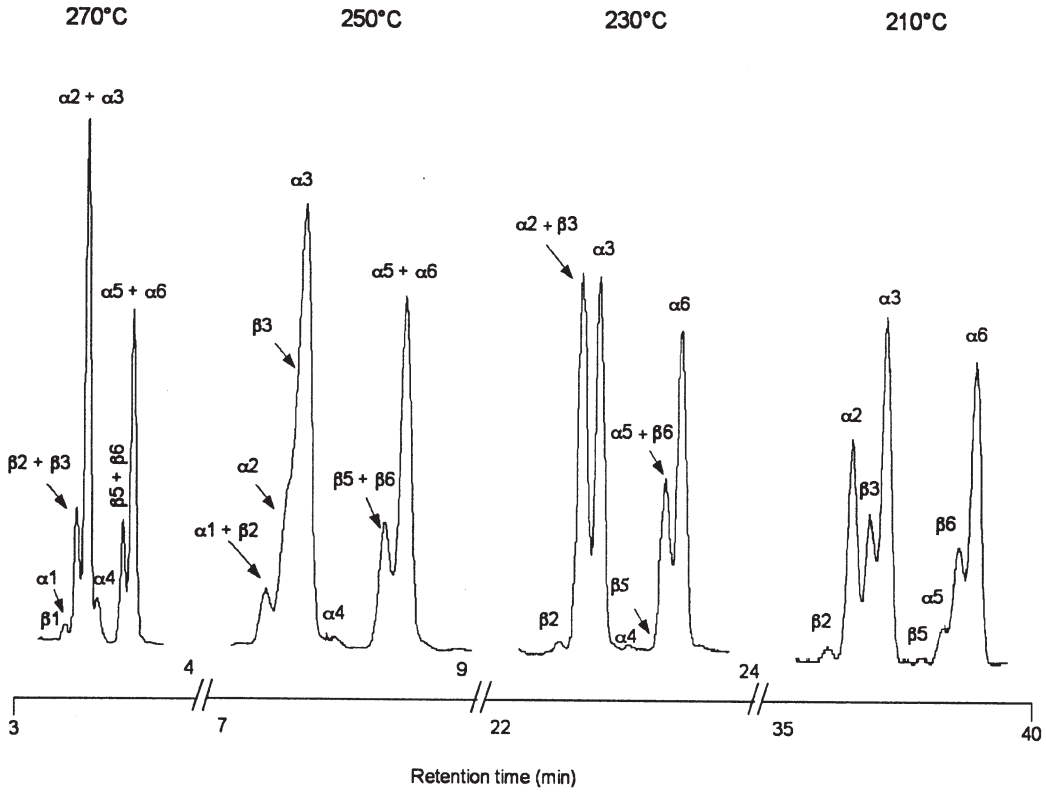


**FIG. 1.** Gas–liquid chromatography of $C_{18}$ dibutyroyl derivatives of monoacyglycerols prepared from *Taxus chinensis* on a 30-m RTX-65TG (Restek, Brockville, CA) column at different oven temperatures (see text for other operating conditions). Peak identification: α is for *sn*-1(3) and β for *sn*-2 positions; (1) stearic (18:0) acid; (2) taxoleic (*cis*-5,*cis*-9 18:2) acid; (3) oleic (*cis*-9 18:1) acid; (4) *cis*-vaccenic (*cis*-11 18:1) acid; (5) pinolenic (*cis*-5,*cis*-9,*cis*-12 18:3) acid; (6) linoleic (*cis*-9,*cis*-12 18:2) acid.

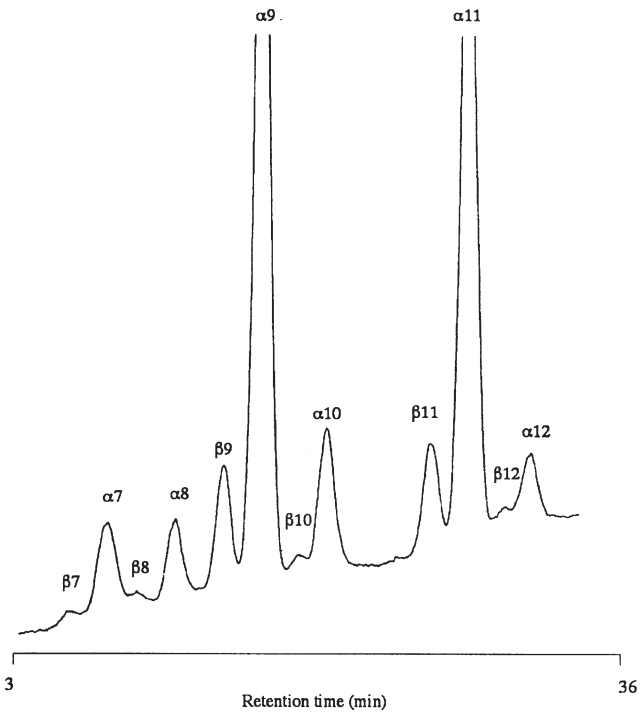


**FIG. 2.** Gas–liquid chromatography of $C_{20}$ dibutyroyl derivatives of monoacyglycerols prepared from *Taxus chinensis* on a 30-m RTX-65TG (Restek, Brockville, CA) column (see text for other operating conditions). Peak identification: α is for *sn*-1(3) and β for *sn*-2 positions; (7) *cis*-5,*cis*-11 20:2; (8) gondoic (*cis*-11 20:1) acid; (9) sciadonic (*cis*-5,*cis*-11,*cis*-14 20:3) acid; (10) dihomolinoleic (*cis*-11,*cis*-14 20:2) acid; (11) juniperonic (*cis*-5,*cis*-11,*cis*-14,*cis*-17 20:4) acid; (12) dihomo-α-linolenic (*cis*-11,*cis*-14,*cis*-17 20:3) acid.

velocity is maintained at 40 mL·cm$^{-1}$. Temperature had a significant effect on the resolution of Δ5-olefinic DBMAG. At 270°C, oleic and taxoleic acids at *sn*-1(3) elute together as well as linoleic and pinolenic acids. The same phenomenon also occurs for all *sn*-2-DBMAG. However, with a gradual temperature decrease, taxoleic acid esterified at the *sn*-1(3) and *sn*-2 positions undergoes a shift to the left-hand side of oleic acid (see Fig. 1, partial chromatograms at 250 and 230°C). Similarly, the elution order becomes *sn*-2 taxoleic, *sn*-1(3) taxoleic, *sn*-2 oleic, *sn*-1(3) oleic, *sn*-2 pinolenic, *sn*-1(3) pinolenic, *sn*-2 linoleic followed by linoleic acid at the *sn*-1(3) positions at 210°C (Fig. 1). This elution order is quite uncommon with medium polar capillary stationary phases because the octadecadienoate-DBMAG with a Δ5-ethylenic bond (*cis*-5,*cis*-9 18:2) elutes before DBMAG containing oleic acid; similarly, the octadecatrienate (*cis*-5,*cis*-9,*cis*-12 18:3) DBMAG elutes before DBMAG containing linoleic acid.

For the $C_{20}$-containing regioisomers in which the number of ethylenic bonds varies between 1 and 4, the number of corresponding DBMAG peaks may reach 12 for species such as *J. virginiana*, which contains not only sciadonic and juniperonic acids but also their precursors (*cis*-11,*cis*-14 20:2 and *cis*-11,*cis*-14,*cis*-17 20:3, respectively). Because isothermal chromatographic conditions did not permit baseline resolution of all regioisomers, we used temperature programming from 100 to 300°C at a linear rate of 5°C/min. Temperature programming coupled with a low carrier gas pressure of 84 kPa led to satisfactory separation of DBMAG containing $C_{20}$ regioisomers (Fig. 2). The order of elution for the $C_{20}$-DBMAG was the same as that for $C_{18}$-DBMAG (Fig. 2). The polymethylene-interrupted system with a double bond at position Δ5 close to the glyceryl bond is presumably hidden by the two $C_4$ chains of butyroyl groups, thereby leading to reduced solubility on the stationary phase and resulting in the observed elution order.

*Validation of the method.* Regiospecific distribution of Δ5-UPIFA is given in Table 2. Regiospecific distribution of Δ5-UPIFA for *Pinus pinaster* and *P. koraiensis* seed oils obtained with the present protocol is compared with the data published by Wolff *et al.* (12) and Blaise *et al.* (19). They contain four Δ5-UPIFA, two of which belong to the $C_{18}$ group (taxoleic and pinolenic acids) and two to the $C_{20}$ group (5,11-eicosadienoic and sciadonic acids). Blaise *et al.* (19) employed the method of Brockerhoff (2), which involved deacylation with Grignard reagent followed by TLC separation of DAG and FA analysis of their methyl esters by GLC. Wolff *et al.* (12) employed the same method except that DAG generated by partial deacylation were separated by TLC, and their

**TABLE 2**
**Regiospecific Distribution of Δ5-Olefinic Acids in *Pinus pinaster* and *P. koraiensis* Seed Triacylglycerols Obtained by Two Different Methods (Refs. 12 and 19) and Compared with the Methodology Used in This Study**

| | | *P. pinaster* | | | *P. koraiensis* | | |
|---|---|---|---|---|---|---|---|
| | | This study | (12) | (19) | This study | (12) | (19) |
| 5,9-18:2 | *sn*-1(3)[a] | 1.1 | 1.0 | 1.0 | 2.5 | 2.3 | 2.6 |
| | *sn*-2 | 0.2 | 0.0 | 0.0 | 0.3 | 0.3 | 0.3 |
| 5,9,12-18:3 | *sn*-1(3) | 9.8 | 8.9 | 7.8 | 19.7 | 15.0 | 19.8 |
| | *sn*-2 | 1.6 | 0.9 | 0.1 | 1.9 | 1.0 | 1.0 |
| 5,11-20:2 | *sn*-1(3) | 1.2 | 1.5 | 0.9 | 0.5 | — | — |
| | *sn*-2 | 0.3 | 0.2 | 0.2 | 0.1 | — | — |
| 5,11,14-20:3 | *sn*-1(3) | 6.4 | 6.5 | 7.2 | 0.9 | 1.0 | 0.9 |
| | *sn*-2 | 1.0 | 1.8 | 0.5 | 0.1 | 0.2 | 0.3 |

[a]Fatty acids esterified to the *sn*-1(3) and *sn*-2 positions of monoacylglycerol derivatives.

**TABLE 3**
**Regiospecific Distribution of Δ5-Unsaturated Polymethylene-Interrupted Fatty Acid (area %) in Triacylglycerols from 18 Conifer Seed Oils[a]**

| Fatty acid | | 1[b] | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C18:1 | Tot[c] | 6.7 | 7.7 | 2.4 | 4.2 | 1.0 | 2.1 | 2.8 | 2.9 | 2.3 | 8.1 | 1.9 | 0.0 | 0.0 | 0.0 | 16.6 | 0.0 | 0.0 | 0.0 |
| Δ5,9 | TAG[d] | 5.8 | 6.8 | 1.8 | 3.6 | 0.8 | 1.8 | 2.3 | 2.1 | 1.6 | 6.0 | 1.3 | 0.0 | 0.0 | 0.0 | 13.6 | 0.0 | 0.0 | 0.0 |
| | *sn*-1(3)[e] | 7.9 | 8.4 | 2.3 | 4.9 | 1.1 | 2.5 | 3.3 | 2.8 | 2.5 | 9.0 | 1.9 | 0.0 | 0.0 | 0.0 | 20.0 | 0.0 | 0.0 | 0.0 |
| | *sn*-2 | 1.8 | 3.8 | 0.9 | 0.8 | 0.2 | 0.3 | 0.3 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 |
| C18:3 | Tot | 14.4 | 11.2 | 28.6 | 20.4 | 7.9 | 14.6 | 21.8 | 21.5 | 20.0 | 7.7 | 19.7 | 0.1 | 0.0 | 0.0 | 3.3 | 0.2 | 0.2 | 0.1 |
| Δ5,9,12 | TAG | 12.3 | 9.3 | 24.3 | 17.6 | 7.1 | 13.8 | 18.6 | 17.9 | 18.7 | 7.0 | 15.3 | 0.0 | 0.0 | 0.0 | 3.1 | 0.0 | 0.0 | 0.0 |
| | *sn*-1(3) | 18.4 | 12.0 | 35.1 | 25.9 | 9.8 | 19.7 | 26.0 | 25.5 | 25.1 | 8.5 | 21.1 | 0.0 | 0.0 | 0.0 | 4.1 | 0.0 | 0.0 | 0.0 |
| | *sn*-2 | 0.0 | 4.0 | 2.9 | 1.0 | 1.6 | 1.9 | 3.8 | 2.8 | 5.9 | 3.8 | 3.7 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 |
| C20:2 | Tot | 0.3 | 0.0 | 0.2 | 0.1 | 0.9 | 0.1 | 0.6 | 0.6 | 0.1 | 1.2 | 0.7 | 0.9 | 0.9 | 0.2 | 0.2 | 1.3 | 0.9 | 0.8 |
| Δ5,11 | TAG | 0.6 | 0.0 | 0.2 | 0.6 | 0.9 | 0.4 | 0.8 | 0.7 | 0.6 | 4.7 | 0.9 | 1.4 | 1.2 | 0.2 | 0.9 | 1.8 | 1.1 | 0.8 |
| | *sn*-1(3) | 0.9 | 0.0 | 0.2 | 0.7 | 1.2 | 0.5 | 1.2 | 1.0 | 0.8 | 6.9 | 1.3 | 1.9 | 1.6 | 0.4 | 1.3 | 2.4 | 1.2 | 0.9 |
| | *sn*-2 | 0.0 | 0.0 | 0.3 | 0.3 | 0.3 | 0.1 | 0.0 | 0.1 | 0.1 | 0.4 | 0.3 | 0.3 | 0.5 | 0.0 | 0.3 | 0.7 | 0.9 | 0.6 |
| C20:3 | Tot | 1.9 | 0.5 | 0.6 | 0.9 | 7.5 | 1.0 | 5.2 | 5.2 | 1.4 | 3.7 | 2.7 | 9.9 | 9.9 | 29.6 | 2.1 | 5.0 | 3.2 | 10.0 |
| Δ5,11,14 | TAG | 1.7 | 0.0 | 0.4 | 0.9 | 4.6 | 0.6 | 4.1 | 4.6 | 1.5 | 5.0 | 4.4 | 7.8 | 6.0 | 24.0 | 2.6 | 5.8 | 2.7 | 8.2 |
| | *sn*-1(3) | 2.1 | 0.0 | 0.3 | 1.1 | 6.4 | 0.9 | 5.0 | 5.6 | 2.0 | 6.8 | 5.9 | 11.1 | 8.6 | 28.3 | 3.8 | 9.3 | 3.6 | 11.4 |
| | *sn*-2 | 0.7 | 0.0 | 0.4 | 0.6 | 1.0 | 0.1 | 2.2 | 2.4 | 0.7 | 1.2 | 1.3 | 1.2 | 0.9 | 15.5 | 0.3 | 0.9 | 1.0 | 1.8 |
| C20:4 | Tot | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.2 | 0.1 | 0.2 | 0.0 | 8.8 | 9.4 |
| Δ5,11, | TAG | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 7.0 | 6.9 |
| 14,17 | *sn*-1(3) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 9.7 | 9.3 |
| | *sn*-2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.7 | 2.0 |
| Σ Δ5- | Tot | 23.2 | 19.4 | 31.8 | 25.7 | 17.3 | 17.8 | 30.3 | 30.3 | 23.7 | 20.7 | 25.0 | 11.0 | 10.9 | 30.0 | 22.4 | 6.5 | 13.0 | 20.3 |
| UPIFA[f] | TAG | 20.4 | 16.1 | 26.8 | 22.6 | 13.3 | 16.5 | 25.7 | 25.2 | 22.5 | 22.6 | 21.9 | 9.1 | 7.2 | 24.3 | 20.2 | 7.7 | 10.9 | 15.8 |
| | *sn*-1(3) | 29.3 | 20.3 | 37.9 | 32.6 | 18.4 | 23.6 | 35.4 | 34.9 | 30.4 | 31.2 | 30.2 | 12.9 | 10.2 | 28.7 | 29.1 | 11.7 | 14.5 | 21.5 |
| | *sn*-2 | 2.5 | 7.8 | 4.5 | 2.7 | 3.1 | 2.4 | 6.3 | 5.8 | 6.7 | 5.5 | 5.3 | 1.5 | 1.3 | 15.5 | 2.3 | 1.6 | 3.5 | 4.4 |

[a]Values are means of duplicate analyses.
[b]The numbers refer to species listed in Table 1.
[c]Fatty acid composition of O-acylated species in the oil obtained by gas–liquid chromatography analysis of fatty acid methyl esters.
[d]TAG, fatty acid composition of triacylglycerols calculated from *sn*-1(3) and *sn*-2 positions.
[e]Fatty acid esterified to the *sn*-1(3) and *sn*-2 positions of monoacylglycerol derivatives.
[f]Sum of Δ5-UPIFA.

3,5-dinitrophenylurethane derivatives were separated by chiral HPLC. In *P. pinaster* seed oil, we detected 1.1% of taxoleic acid at the *sn*-1(3) and 0.2% at the *sn*-2 positions, whereas Wolff *et al.* (12) and Blaise *et al.* (19) found this FA only at the *sn*-1(3) positions at a level of 1.0%. In *P. koraiensis* seed oil, we detected the same FA at the level of 2.5% at the *sn*-1(3) and 0.3% at the *sn*-2 positions, whereas Wolff *et al.* (12) found 2.3% at the *sn*-1(3) and 0.3% at the *sn*-2 positions and Blaise *et al.* (19) found 2.6% at the *sn*-1(3) and 0.3% at the *sn*-2 positions. With respect to sciadonic acid, we found 6.4% at the *sn*-1(3) and 1.0% at the *sn*-2 positions in *P. pinaster* seed oil. Wolff *et al.* (12) determined a level of 6.5% at the *sn*-1(3) and 1.8% at the *sn*-2 positions, whereas Blaise *et al.* (19) reported 7.2% at the external positions of glycerol and 0.5% at the internal position. The regiospecific distribution of sciadonic acid in *P. koraiensis* seed oil also compares well with that of Wolff *et al.* (12) and that of Blaise *et al.* (19) (see Table 2). Regiospecific distributions of pinolenic and 5,11-eicosadienoic acids in *P. pinaster* and *P. koraiensis* seed oils are similar. Thus, the proposed GLC method compared well with the other two methods, with respect to its accuracy and reliability. The seed oils from these two pine species have also been analyzed by $^{13}$C NMR spectroscopy (4). Regiospecific analysis of Δ5-UPIFA on TAG was based on the chemical shifts of $C_1$ and $C_2$ carbonyl groups. For the $C_1$, two signals at 173.2 ppm in the external position of the glycerol backbone (α-chains) and 172.8 ppm in the internal position are detected. The occurrence of a Δ5-ethylenic bond in the aliphatic chain has the property to modify the $C_1$ and $C_2$ chemical shifts. The $C_1$ on external chains give values in the range of 173.01–173.07 ppm. No studies referred to a signal attributable to Δ5-UPIFA in the *sn*-2 position, which likely has a value slightly lower than 172.8 ppm. Blaise *et al.* (6) indicated that the minimum detection level of Δ5-UPIFA is 3% of fatty acids esterified at the *sn*-2 position.

*Regiospecific distribution of Δ5-UPIFA in conifer seed TAG.* Regiospecific distribution of Δ5-UPIFA is presented in Table 3. Δ5-UPIFA are preferentially located at the *sn*-1(3) positions compared with the *sn*-2 position, in agreement with previous studies. The distribution does not depend on the chain length of FA ($C_{18}$ and $C_{20}$) nor on the number of double bonds (2 to 4). However, certain discrepancies exist compared with the data obtained by methods based on $^{13}$C NMR spectroscopy, which is unable to detect Δ5-UPIFA in the *sn*-2 position of TAG. Unfortunately, all of the species analyzed here were not investigated by $^{13}$C NMR spectroscopy, and only an estimate of the lower value that can be registered by that technique can be made. More than 10 species analyzed here contain <4% Δ5-olefinic acids in their *sn*-2 position, which could not be detected by $^{13}$C NMR spectroscopy. This also holds for *Cedrus brevifolia,* a close relative of *C. atlantica* analyzed by $^{13}$C NMR spectroscopy, which contains

**TABLE 4**
**Regiospecific Distribution of Common Fatty Acids (area %) in Triacylglycerols from 18 Conifer Seed Oils[a]**

| Fatty acid | | 1[b] | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16:0 | Tot[c] | 3.1 | 4.5 | 2.8 | 3.3 | 4.2 | 5.0 | 3.2 | 3.3 | 3.7 | 4.2 | 4.9 | 6.2 | 6.0 | 3.7 | 3.1 | 7.3 | 5.4 | 5.1 |
| | TAG[d] | 3.3 | 3.9 | 3.2 | 3.5 | 3.4 | 4.4 | 3.8 | 3.3 | 5.3 | 4.6 | 7.1 | 6.8 | 7.6 | 4.6 | 4.6 | 11.1 | 5.7 | 6.1 |
| | *sn*-1(3)[e] | 4.7 | 5.7 | 4.4 | 5.0 | 5.0 | 6.4 | 5.5 | 4.5 | 7.9 | 6.8 | 10.5 | 9.6 | 11.0 | 6.3 | 6.6 | 16.5 | 8.2 | 9.0 |
| | *sn*-2 | 0.5 | 0.2 | 0.7 | 0.5 | 0.3 | 0.2 | 0.5 | 0.8 | 0.2 | 0.3 | 0.2 | 1.2 | 0.9 | 1.3 | 0.6 | 0.5 | 0.6 | 0.3 |
| 18:0 | Tot | 1.6 | 2.9 | 1.6 | 1.9 | 2.7 | 2.4 | 1.8 | 2.0 | 1.6 | 2.5 | 2.1 | 2.7 | 2.8 | 1.2 | 1.5 | 4.4 | 1.6 | 3.2 |
| | TAG | 2.0 | 3.6 | 2.5 | 2.3 | 2.8 | 2.5 | 2.4 | 2.3 | 1.9 | 2.0 | 2.3 | 3.0 | 3.6 | 1.5 | 1.0 | 3.8 | 1.8 | 4.3 |
| | *sn*-1(3) | 2.4 | 3.3 | 3.2 | 3.0 | 4.0 | 3.5 | 3.3 | 3.1 | 2.1 | 3.0 | 2.9 | 4.4 | 5.1 | 2.0 | 1.3 | 5.5 | 2.4 | 5.8 |
| | *sn*-2 | 1.2 | 4.4 | 1.2 | 0.9 | 0.6 | 0.3 | 0.7 | 0.9 | 1.5 | 0.0 | 1.0 | 0.4 | 0.6 | 0.6 | 0.3 | 0.4 | 0.7 | 1.3 |
| 9-18:1 | Tot | 24.7 | 45.1 | 17.9 | 20.8 | 19.2 | 27.3 | 15.1 | 14.6 | 24.0 | 27.6 | 14.3 | 44.6 | 45.0 | 12.1 | 34.7 | 36.1 | 16.0 | 15.0 |
| | TAG | 25.4 | 46.4 | 20.2 | 22.7 | 19.9 | 28.4 | 17.8 | 17.2 | 24.2 | 27.6 | 15.4 | 49.8 | 52.0 | 14.1 | 34.5 | 33.7 | 17.1 | 18.0 |
| | *sn*-1(3) | 24.4 | 43.9 | 17.4 | 19.5 | 20.8 | 28.3 | 16.8 | 16.4 | 22.5 | 23.9 | 14.6 | 38.6 | 42.8 | 13.8 | 27.3 | 27.8 | 12.7 | 12.7 |
| | *sn*-2 | 27.4 | 51.5 | 25.7 | 29.2 | 18.2 | 28.7 | 19.7 | 18.6 | 27.7 | 34.9 | 16.9 | 72.2 | 70.5 | 14.7 | 48.8 | 45.4 | 25.8 | 28.7 |
| 11-18:1 | Tot | 0.3 | 1.1 | 1.2 | 1.0 | 0.4 | 0.4 | 0.8 | 0.8 | 0.6 | 0.3 | 0.4 | 0.6 | 0.6 | 0.6 | 0.4 | 0.1 | 0.4 | 0.3 |
| | TAG | 0.4 | 0.9 | 1.3 | 1.2 | 0.9 | 0.5 | 1.2 | 0.7 | 0.5 | 1.7 | 1.5 | 0.6 | 0.6 | 0.6 | 0.6 | 0.0 | 0.6 | 0.5 |
| | *sn*-1(3) | 0.6 | 1.3 | 1.9 | 1.7 | 1.3 | 0.8 | 1.8 | 1.1 | 0.8 | 2.5 | 2.2 | 0.9 | 1.0 | 0.9 | 0.9 | 0.5 | 0.9 | 0.7 |
| | *sn*-2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 9,12-18:2 | Tot | 45.1 | 25.7 | 43.6 | 46.1 | 52.7 | 45.0 | 46.2 | 46.3 | 45.3 | 37.1 | 51.5 | 29.0 | 28.3 | 36.7 | 33.5 | 43.5 | 30.5 | 26.4 |
| | TAG | 46.2 | 27.2 | 44.5 | 46.2 | 55.4 | 46.2 | 46.4 | 49.0 | 43.9 | 33.7 | 49.2 | 25.3 | 23.2 | 41.2 | 33.3 | 40.6 | 32.3 | 27.9 |
| | *sn*-1(3) | 36.2 | 23.7 | 33.9 | 36.5 | 45.0 | 35.2 | 34.1 | 37.1 | 34.0 | 25.7 | 35.7 | 26.0 | 21.9 | 28.7 | 27.8 | 35.1 | 23.6 | 20.2 |
| | *sn*-2 | 66.1 | 34.1 | 65.8 | 65.6 | 76.4 | 68.1 | 71.0 | 72.7 | 63.6 | 49.8 | 76.1 | 24.0 | 26.0 | 66.3 | 44.2 | 51.7 | 49.7 | 43.5 |
| 9,12,15-18:3 | Tot | 0.4 | 0.1 | 0.4 | 0.4 | 1.3 | 0.2 | 0.4 | 0.4 | 0.0 | 5.4 | 0.5 | 0.4 | 0.4 | 0.2 | 2.1 | 0.3 | 29.4 | 26.5 |
| | TAG | 1.1 | 0.8 | 1.3 | 0.7 | 2.8 | 0.2 | 1.0 | 0.5 | 0.0 | 3.6 | 0.0 | 0.5 | 0.3 | 0.0 | 3.1 | 0.2 | 28.5 | 24.7 |
| | *sn*-1(3) | 0.5 | 0.6 | 1.0 | 0.6 | 3.7 | 0.2 | 0.9 | 0.4 | 0.0 | 0.8 | 0.0 | 0.5 | 0.3 | 0.0 | 2.8 | 0.3 | 33.1 | 26.3 |
| | *sn*-2 | 2.2 | 1.3 | 1.9 | 0.9 | 1.0 | 0.2 | 1.2 | 0.7 | 0.0 | 9.1 | 0.0 | 0.5 | 0.3 | 0.0 | 3.5 | 0.1 | 19.3 | 21.5 |
| 11-20:1 | Tot | 0.9 | 0.9 | 0.4 | 0.4 | 1.2 | 1.2 | 1.2 | 1.3 | 0.5 | 1.7 | 0.6 | 2.4 | 2.7 | 1.3 | 1.5 | 0.8 | 1.3 | 0.8 |
| | TAG | 0.8 | 0.6 | 0.1 | 0.2 | 0.8 | 1.0 | 0.9 | 0.8 | 0.9 | 3.1 | 1.1 | 2.1 | 2.7 | 0.9 | 1.9 | 1.3 | 1.2 | 0.7 |
| | *sn*-1(3) | 1.2 | 0.7 | 0.1 | 0.3 | 1.1 | 1.5 | 1.3 | 1.2 | 1.3 | 4.6 | 1.7 | 3.1 | 4.1 | 1.3 | 2.8 | 1.9 | 1.8 | 0.9 |
| | *sn*-2 | 0.0 | 0.4 | 0.1 | 0.1 | 0.1 | 0.0 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 | 0.1 | 0.3 |
| 11,14-20:2 | Tot | 0.6 | 0.4 | 0.4 | 0.5 | 1.0 | 0.6 | 1.0 | 1.1 | 0.6 | 0.5 | 0.7 | 3.0 | 3.3 | 14.1 | 0.7 | 1.0 | 1.5 | 1.6 |
| | TAG | 0.6 | 0.5 | 0.2 | 0.6 | 0.7 | 0.4 | 0.7 | 1.0 | 0.7 | 1.1 | 1.6 | 2.8 | 2.4 | 12.8 | 0.9 | 1.3 | 1.4 | 1.5 |
| | *sn*-1(3) | 0.8 | 0.5 | 0.2 | 0.8 | 0.8 | 0.5 | 0.8 | 1.2 | 1.0 | 1.4 | 2.2 | 4.1 | 3.5 | 18.3 | 1.2 | 1.8 | 2.0 | 2.2 |
| | *sn*-2 | 0.2 | 0.5 | 0.1 | 0.2 | 0.4 | 0.1 | 0.5 | 0.5 | 0.2 | 0.4 | 0.5 | 0.2 | 0.1 | 1.7 | 0.2 | 0.3 | 0.3 | 0.0 |
| 11,14,17-20:3 | Tot | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 | 0.8 | 0.8 |
| | TAG | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.6 | 0.5 |
| | *sn*-1(3) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.9 | 0.8 |
| | *sn*-2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

[a]Values are means of duplicate analyses.
[b]The numbers refer to species listed in Table 1.
[c]Fatty acid composition of O-acylated species in the oil obtained by fatty acid methyl esters analyses by gas–liquid chromatography.
[d]TAG, fatty acid composition of triacylglycerols calculated from *sn*-1(3) and *sn*-2 positions.
[e]Fatty acid esterified to the *sn*-1(3) and *sn*-2 positions of monoacylglycerol derivatives.

almost 8% of Δ5-UPIFA in the internal position. There is one species that appears exceptional in our study, *Nageia nagi*, a podocarp, which contains >15% of Δ5-UPIFA in the *sn*-2 position. This species was not examined by $^{13}$C NMR spectroscopy, but our data confirm the observations of Takagi and Itabashi (1) for that species (reported as *Podocarpus nagi*). A realistic limit of detection of Δ5-UPIFA in the internal position by $^{13}$C NMR would thus be 6–8%, rather than 3% as reported earlier for $^{13}$C NMR spectroscopy analyses (6).

*Regiospecific distribution of common FA.* Regiospecific distribution of FA is presented in Table 4. Palmitic and stearic acids are preferentially esterified at the *sn*-1(3) positions, whereas the distribution of monoenoic acids is variable. Oleic (*cis*-9 18:1) acid distribution is random. *Cis*-vaccenic (*cis*-11 18:1) acid appeared to be completely absent from the *sn*-2 position for all species. Except for *Cephalotaxus* spp., in which linoleic acid (*cis*-9,*cis*-12 18:2) was almost equally distributed between the internal and the external positions, this FA was more concentrated in the *sn*-2 position than in the *sn*-1(3) positions for all other species. α-Linolenic (*cis*-9,*cis*-12,*cis*-15 18:3) acid did not seem to follow any rule; however, it was most often equally esterified to both internal and external positions. An exception was *Pseudolarix amabilis*, in which α-linolenic acid was concentrated in the *sn*-2 position. In the few cases in which dihomo-α-linolenic (*cis*-11,*cis*-14,*cis*-17 20:3) acid occurs (Cupressaceae), it appeared to be excluded from the *sn*-2 position.

In conclusion, Table 5 summarizes Δ5-UPIFA regiospecific distribution in all species of conifer seeds analyzed to date, including families and species analyzed here. This table, including two *Ephedra* species (Cycadophytes), is based mainly on the recapitulation by Wolff *et al.* (12) and includes the species analyzed in the present study. Most Coniferophyte families have been screened, and with the exception of

**TABLE 5**
**Gymnosperm Orders, Families, and Species Showing a Preferential Location of Δ5-Unsaturated Polymethylene-Interrupted Fatty Acids at the α-Chains of Seed Triacylglycerols**

| Order | Family | Species | Method[a] | Reference[b] |
|---|---|---|---|---|
| Ginkgoatae | Ginkgoaceae | *Ginkgo biloba* | GR | 1 |
| Pinatae | Podocarpaceae | *Nageia nagi* | GR, GLC | 1, this study |
| | | *Prumnopitys andina* | NMR | 7 |
| | Taxaceae | *Torreya nucifera* | GR | 1 |
| | | *T. grandis* | NMR | 11 |
| | | *T. californica* | GLC | This study |
| | | *Taxus cuspidata* | GR | 1 |
| | | *T. baccata* | GR, NMR | 4,12,19 |
| | | *T. chinensis* | NMR, GLC | 11, this study |
| | Cephalotaxaceae | *Cephalotaxus sinensis* | GLC | This study |
| | | *C. drupaceae* | NMR, GLC | 7, this study |
| | Pinaceae | *Pinus armandi* | — | 21 |
| | | *P. koraiensis* | GR, NMR, GLC | 1,4,12,19, this study |
| | | *P. cembra* | — | 12 |
| | | *P. sylvestris* | NMR, GLC | 4,8, this study |
| | | *P. mughus* | NMR | 4 |
| | | *P. nigra* | NMR | 4 |
| | | *P. griffithii* | NMR | 4 |
| | | *P. pinaster* | GR, NMR, GLC | 4,12,19, this study |
| | | *P. pinea* | GR | 19 |
| | | *Larix decidua* | GR, NMR | 12,19 |
| | | *L. gmelinii var. olgensis* | GLC | This study |
| | | *L. leptolepis* | NMR | 5 |
| | | *L. sibirica* | NMR | U.R.[c] |
| | | *Picea jezoensis* | GR | 1 |
| | | *P. abies* | NMR | 5 |
| | | *P. sitchensis* | NMR | 5 |
| | | *P. schrenkiana* | GLC | This study |
| | | *Cedrus atlantica* | NMR | 5 |
| | | *C. brevifolia* | GLC | This study |
| | | *Abies concolor* | NMR | 5 |
| | | *A. alba* | NMR | 10 |
| | | *A. nephrolepis* | GLC | This study |
| | | *Pseudolarix amabilis* | GLC | This study |
| | | *Tsuga caroliniana* | GLC | This study |
| | | *Hesperopeuce mertensiana* | GLC | This study |
| | Sciadopityaceae | *Sciadopitys verticillata* | GR, NMR | 5,12,19 |
| | Taxodiaceae | *Cryptomeria japonica* | GR | 1 |
| | Cupressaceae | *Thuja occidentalis* | NMR | 5 |
| | | *Juniperus virginiana* | NMR, GLC | 5, this study |
| | | *J. communis* | GR | 19,21 |
| | | *Austrocedrus chilensis* | GLC | This study |
| | | *Biota orientalis* | PL, NMR | 22 |
| Gnetatae | Ephedraceae | *Ephedra nevadensis* | NMR | 9 |
| | | *E. viridis* | NMR | 9 |

[a]GR, Grignard reagent; NMR, $^{13}C$ nuclear magnetic resonance spectroscopy; PL, pancreatic lipase; GLC, gas–liquid chromatography.
[b]References 1,6,12,19, and 22 relate to purified triacylglycerols; data in References 4,5,7–9, and 11 were determined with total lipids, and those in Reference 10 were established with whole seeds. Reference 21, and experimental procedures therein, are in Japanese.
[c]Farines, M., and Wolff, R.L., unpublished results.

*N. nagi*, all of them show Δ5-UPIFA in the *sn*-2 position in the narrow range of 2.4–7.8% of fatty acid esterified at that position. Clearly, the present and earlier studies demonstrate that the distribution of Δ5-UPIFA in gymnosperm seed TAG is not random.

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# Sphingophosphonolipids, Phospholipids, and Fatty Acids from Aegean Jellyfish *Aurelia aurita*

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**ABSTRACT:** The goal of this study is to elucidate and identify several sphingophosphonolipids from *Aurelia aurita,* an abundant but harmless Aegean jellyfish, in which they have not previously been described. Total lipids of *A. aurita* were 0.031–0.036% of fresh tissue, and the lipid phosphorus content was 1.3–1.7% of total lipids. Phosphonolipids were 21.7% of phospholipids and consisted of a major ceramide aminoethylphosphonate (CAEP-I; 18.3%), as well as three minor CAEP (II, III, IV) methyl analogs at 1.3, 1.1, and 1.0%, respectively. The remaining phospholipid composition was: phosphatidylcholine, 44.5%, including 36.2% glycerylethers; phosphatidylethanolamine, 18.6%, including 4.5% glycerylethers; cardiolipin, 5.6%; phosphatidylinositol, 2.6%; and lysophosphatidylcholine, 5.0%. In CAEP-I, saturated fatty acids of 14–18 carbon chain length were 70.8% and were combined with 57.3% dihydroxy bases and 23.4% trihydroxy bases. The suite of the three minor CAEP methyl analogs were of the same lipid class based on the head group, but they separated into three different components because of their polarity as follows: CAEP-II and CAEP-III differentiation from the major CAEP-I was mainly due to the increased fatty acid unsaturation and not to a different long-chain base, but the CAEP-IV differentiation from CAEP-I, apart from fatty acid unsaturation, was due to the increased content of hydroxyl groups originated from both hydroxy fatty acids and trihydroxy long-chain bases. Saturated fatty acids were predominant in total (76.7%), polar (83.0%), and neutral lipids (67.6%) of *A. aurita*. The major phospholipid components of *A. aurita* were comparable to those previously found in a related organism *(Pelagia noctiluca),* which can injure humans.

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Phosphonolipids (PnL), in the broad sense of the word, are a class of lipids characterized by the presence of one or more carbon-to-phosphorus (C-P) bonds. The C-P bond(s) provides the molecule with a relative resistance to chemical hydrolysis and thermal decomposition compared with the more reactive N-P, S-P, and O-P linkages. Natural phosphonic compounds are found to exist as glycerol esters (glycerophosphonolipids, GPnL) or sphingosine esters (sphingophosphonolipids, SPnL, and sphingophosphonoglycolipids, SPnGL). The specific effects of the biological functions of PnL depend not only on the chemical stability of the C-P bond but also on the chemical inertness of PnL owing either to the existence of a glycerylether bond in GPnL or to the presence of hydroxy-fatty acids (hydroxy-FA) in SPnL (1–6). Because of the inert character of PnL, an important biological advantage might be conferred by the presence of these PnL in the biological membranes of those organisms that are able to synthesize such compounds. This reasoning may be applicable in the case of ciliated protozoa (*Tetrahymena pyriformis*), which are found in the rumen, an environment rich in hydrolytic enzymes, such as phosphatases (7). It is also generally agreed that PnL contribute to the protection of cellular membranes and survival of aquatic organisms such as cnidaria, mollusks, and sponges, since it has been proposed that PnL provide cationic buffering capacity or facilitate transport of essential ions. PnL were also conserved at the expense of phosphodiester bonds in starved organisms. Possibly, PnL are a storage mechanism for phosphorus in a phosphorus-deficient environment (1–6,8). Cold-temperature studies on the membrane lipid composition of *T. pyriformis* showed that PnL may contribute to its cold acclimation by increasing the level of unsaturated FA, e.g., 18:3n-3 (1,4). Another potential role for the unique structures of the aminophosphonates is in interspecies (or intraspecies) communication or recognition processes. An example of this type of communication is given in a report of a survival-enhancing relationship between the sea anemone *Anthopleura xanthogrammica* and the mussel *Mytilus californianus* (2).

The occurrence of SPnL in cnidaria was first reported in 1963 by Rouser *et al*. (9), with the discovery of ceramide 2-aminoethylphosphonate (CAEP) in the sea anemone *Anthopleura elegantissima* (class Anthozoa). CAEP together with its *N*-methyl analog ceramide 2-methylaminoethylphosphonic acid (CMAEP) is widely distributed in protozoa (10–13), mollusks (1,14–21), and cnidaria (22,23). A number of SPnGL have been identified in lipid extracts from various specific tissues (nervous and muscle tissue, nerve fibers, ganglia, etc.) of some marine mollusc species (24–26). PnL were also found in small amounts in human brain (6). In the milk and liver of cows and goats it has been hypothesized that PnL are exogenous in origin and not

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Abbreviations: AEP, 2-aminoethylphosphonic acid; CAEP, ceramide 2-aminoethylphosphonic acid; CCL, carbon chain length; CL, cardiolipin; CMAEP, ceramide 2-methylaminoethylphosphonic acid; ECL, equivalent chain length; FA, fatty acids; GC, gas chromatography; GPC, L-α-glycerylphosphorylcholine; GPE, L-α-glycerylphosphorylethanolamine; GPI, L-α-glycerylphosphorylinositol; GPnL, glycerophosphonolipid; HPTLC, high-performance thin-layer chromatography; LCB, long-chain base; L-PE, lyso-PE; L-PC, lyso-PC; MAEP, 2-methylaminoethylphosphonic acid; MUFA, monounsaturated fatty acids; NL, neutral lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PhL, phospholipid; PI, phosphatidylinositol; PL, polar lipids; PnL, phosphonolipid; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SPnGL, sphingophosphonoglycolipid; SPnL, sphingophosphonolipid; TDB, total dihydroxy bases; TL, total lipids; TLC, thin-layer chromatography; TMS, trimethylsilyl; TTB, total trihydroxy bases.

structural components of tissue (4,6). Moreover, PnL also were found in sea-stars, which feed on gastropod and bivalve mollusks, at up to 20% of the total phospholipids (PhL) (1,2,8).

Nakhel *et al*. (22) initiated extensive research into the composition and nature of PnL in jellyfish. They studied the occurrence of PnL in the Aegean jellyfish species *Pelagia noctiluca* (cnidaria), which is toxic to humans because, according to References 1 and 2, this phylum is very rich in PnL. The gastrointestinal tracts of cnidaria are known to contain many hydrolytic enzymes, and cell membranes may benefit from the presence of PnL. In the period 1982–1988, research on PnL species was stimulated by the infestation with swarms of *P. noctiluca*, along most Mediterranean coasts. These lipids were suspected of playing a vital role in the survival of the jellyfish in a very eutrophic environment where other organisms could not survive. The total lipids (TL) content of *P. noctiluca* was 0.19% of the whole body weight. PhL were found to represent 26.2% of TL and to contain 24.3% SPnL. The SPnL class consisted of a major CAEP species (21.0% of PhL) and 3.3% of two other minor SPnL species. However, the structural elucidation of the latter minor SPnL species was not achieved.

The purpose of this study was to elucidate and identify several SPnL species from the scyphozoan *A. aurita*, an abundant but harmless jellyfish of the Aegean Sea. The structural compositions of the PhL and the FA of neutral lipids (NL) and polar lipids (PL) were determined.

## MATERIALS AND METHODS

*Sample description and extraction of TL. Isolation of PL and NL classes.* Jellyfish *(A. aurita)* were collected from Eleusis Bay (Greece) by using a fishing net during August 1996 and May 1998. Four jellyfish (total weight 267 g, diameter 4–6 cm) were collected in 1996 and again in 1998 (total weight 830 g, diameter 10–15.5 cm). The organisms were placed in seawater and transferred to the laboratory alive for lipid extraction. Studies conducted by the Institute of Oceanographic and Fisheries Research in Greece (27) have shown that the salinity of Eleusis Bay is almost constant at 38.5‰ in the water column. The temperature is about 13°C during winter, while during summer, there is a temperature difference of about 10°C between surface and bottom samples (15–25°C). Eleusis Bay is one of the most eutrophic areas in Greece. It is small (67 $km^2$) and shallow (depth max. 33 m) and is subjected to urban and industrial pollution. Each individual jellyfish (1 vol) was drained as thoroughly as possible, transferred to a vessel containing 3 vol of chloroform/methanol (1:2, vol/vol) and immediately blended for 4 min at medium speed in a Sorvall Omni-Mixer. Extraction of lipids was performed according to the method of Bligh and Dyer (28), except that the liquid phase was separated by centrifugation (4°C, 10 min, 1465 × *g*), instead of filtration, and the residue was re-extracted with 1 vol chloroform/methanol (1:1, vol/vol) and centrifuged again. The two extracts were then combined. To the pooled extracts, 1 vol of chloroform and 1 vol of water was added. After phase equilibration, the lower chloroform layer of TL was evaporated to dryness, redissolved in chloroform/methanol (2:1, vol/vol), and stored at 0°C (23). The composite of *A. aurita* TL sample 1996 was recovered from the pooled lipid extracts of all four medusae to make one composite sample. The same procedure was followed for the sample of composite TL of 1998 *A. aurita* (four medusae).

The TL were fractionated into NL and PL classes by solid phase extraction as follows: A portion of TL extract (~10 mg solid) was dissolved in 0.5 mL chloroform/hexane (1:1, vol/vol) and applied to a prewashed 500-mg silica column. NL were eluted four times with 7-mL aliquots of chloroform, and PL were eluted three times with 6.5-mL aliquots of methanol. The apparatus used was a Waters SEP-PAK® Vacuum manifold, and the column used was a Waters SEP-PAK® VAR RC Silica cartridge (29).

*Thin-layer chromatography (TLC) of lipids*. Analysis of *A. aurita* PL by single-dimensional high-performance TLC (1D-HPTLC) was carried out on precoated silica gel 60 HPTLC plates (E. Merck, Darmstadt, Germany) with the following solvent systems: solvent **A**, chloroform/methanol/acetic acid/water (50:25:6:2, by vol); solvent **B**, chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol). The PL were further separated by two-dimensional HPTLC analysis (2D-HPTLC) carried out on precoated silica gel 60 plates (10 × 10 cm) with solvent system **C,** which consisted of the following: dimension 1, chloroform/methanol/acetic acid/water (50:25:6:2, by vol); dimension 2, chloroform/methanol/water (65:20:3, by vol). For separation of NL, system **D,** used for 1D-HPTLC was petroleum ether/ether/acetic acid (70:30:1, by vol). Solvent system **E,** used for sphingosine base analysis, was chloroform/methanol/water (65:25:4, by vol). Visualization of spots was effected by exposure to iodine vapor followed by spraying with ninhydrin reagent and/or the phosphomolybdenum blue reagent (30) for PhL, combined with the heating test of Stillway and Harmon (31) for PnL. Dragendorff reagent was used for detection of choline (32). Lipid spots were also visualized by using copper sulfate spray and heating at 180°C for 2–5 min (33,34). Testing for glyco- or sulfolipids was performed by using α-naphtholsulfuric acid reagent (35). The TLC of water-soluble products was carried out on precoated cellulose plates (0.1 mm thickness, Art. 5552, Merck) in the following solvent system: phenol/ethanol/acetic acid/water (80:12:10:20 wt/vol/vol/vol). Hanes and Isherwood spray (36) and ninhydrin spray were used for visualization of phospho- and amino-derivatives, respectively, and Dragendorff spray was used for staining of L-α-glycerylphosphorylcholine (GPC). Additionally, the following were used as lipid standards: synthetic CAEP and synthetic 2-methylaminoethylphosphonic acid (MAEP) were a gift from Prof. Akira Hayashi of Kinki University (Kowakae, Higashi-Osaka, Japan). 2-Aminoethylphosphonic acid (AEP) was purchased from Sigma, 2-dimethylaminoethylphosphonic acid and 2-trimethylaminoethylphosphonic acid were a kind gift from Prof. J. Donald Smith of University of Massachusetts (Dartmouth, MA). Cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol, lyso-PC (L-PC), lyso-PE (L-PE), L-α-glycerylphosphorylethanolamine (GPE),

L-α-glycerylphosphorylinositol (GPI), GPC, C18-sphingosine, C18-phytosphingosine, tripalmitin, oleic acid, palmitic acid methyl ester, cholesterol ester, and cholesterol were purchased from Sigma. C16-Sphingosine was purchased from Larodan.

*Fractionation of PL by preparative TLC (solvent B).* Five PhL bands were fractionated after separation of PL (500–800 μg phosphorus/sample) by solvent B on preparative TLC ($n$ = 2–5) carried out on chromatoplates of silica gel G, 0.5 mm thick (20 × 20 cm), activated at 120°C for 1 h, along with PhL standards (CL, PE, CAEP, PC, and L-PC). After visualization by exposure to iodine vapors, each PhL band was scraped off and eluted from silica gel with 31.6 mL chloroform/methanol/water (1:2:0.8, by vol) three times. Then 50 mL of chloroform/water (1:1, vol/vol) was added to the pooled solution, and after mixing, the chloroform extract of each band was taken to dryness and redissolved in chloroform/methanol (2:1, vol/vol).

*Fractionation of SPnL class by preparative TLC (solvent **A**).* The extracted lipid band of SPnL class from the preparative TLC, solvent **B**, was subjected to mild alkaline hydrolysis for removal of the glycerolipids components. The products (530 μg phosphorus) stable to mild alkali from SPnL class were fractionated on preparative TLC by solvent **A**. A major SPnL band and three minor SPnL bands were visualized, scraped off, and extracted as described previously.

*Analytical methods and quantitation of lipid components.* Total phosphorus and phosphonate phosphorus were determined by the methods of Long and Staples (37) and Kapoulas *et al.* (38), respectively. Sugars were determined according to the method of Dubois *et al*. (39). Esters were determined by the method of Snyder and Stephens (40), long-chain bases by the method of Lauter and Trams (41), glycerylethers by the method of Hanahan and Watts (42), and nitrogen according to the method of Hashmi *et al*. (43). Mild alkaline hydrolysis (deacylation) was performed with NaOH (1.2 N) as described previously (21). Each class of NL and PL, in bulk, was quantitated by weight after elution from solid-phase extraction columns.

Individual PhL components were quantitated after separation of PL on 2D-TLC (20 × 20 cm, 0.25 mm thick), scraping each PhL spot directly into digestion tubes, with determination of total phosphorus as described previously (21).

*Preparation of SPnL derivatives.* Acid methanolysis of each isolated and purified SPnL species was performed by a modification of the Vance and Sweely method (44) as follows: 500 μL of 3 N methanolic HCl (10% wt/vol; Supelco Inc.) reagent was added to each SPnL sample (25–70 μg phosphorus), methylation was allowed to proceed (100°C, 5–6 h), and molecular ratios for structural data were determined in each methanolysate aliquot. The SPnL FA methyl esters were extracted from methanolysate with petroleum ether and analyzed by gas chromatography (GC). To the remaining solution of methanolysate, suitable volumes of chloroform, methanol, and water were added to give a ratio of chloroform/methanol/water (8:4:3, by vol) (Folch partition). For the isolation of water-soluble products (aminoalkylphosphonic acids), the upper aqueous layer was evaporated to dryness on a rotary evaporator, and the last traces of HCl were removed under nitrogen by repeated evaporations with water (45). For the isolation of long-chain bases (LCB), the lower chloroform layer was evaporated to dryness in a stream of nitrogen and the residue was dissolved in a small amount of chloroform/methanol (95:5 vol/vol). The LCB were analyzed by GC after silylation.

*GC analysis. (i) FA methyl esters.* To each 1-mg lipid sample (TL, NL, and PL in bulk), 500 μL of 3 N methanolic HCl (10% wt/vol; Supelco Inc.) reagent was added and methylation was allowed to proceed at 100°C for 5–6 h. FA methyl esters were extracted four times with 0.8-mL aliquots of petroleum ether (29), and after drying were redissolved in hexane for GC analysis. The same transesterification procedure was performed for analyzing the FA moieties of individual isolated and purified SPnL species. FA methyl esters were separated using a Hewlett-Packard 6890 gas chromatograph equipped with a flame-ionization detector. The carrier gas and the make-up gas were He. A capillary column SGE BPX-70 (70% cyanopropyl liquid phase; 25 m × 0.32 mm, 0.25 mm film thickness) was used. The oven temperature was programmed from 100 to 200°C at a rate of 4°C/min, with initial and final holding times of 0 and 3 min, respectively. The splitless injector temperature was 250°C, and the detector temperature was 300°C. The FA methyl esters were identified by comparison with three standard mixtures of methyl esters: bacterial FA methyl esters (CP™ Mix, Catalog No. 114; Matreya Inc., Pleasant Gap, PA); mixture special preparation no. 461, Nu-Chek-Prep Inc. (Elysian, MN); and mixture 68 A, Nu-Chek-Prep Inc. Mass spectrometry was not used to confirm any of the FA structures. The FA analysis of each transesterified lipid class was repeated three times (three injections on the gas chromatograph), and the standard deviations of the percentages of FA were between ±0.1 and ±0.5.

*(ii) LCB silylation.* Trimethylsilyl (TMS) derivatives of LCB moieties from each isolated and purified SPnL species were prepared according to the method of Sweeley *et al*. (46) and separated on the same Hewlett-Packard chromatograph using a capillary column HP-5 (5% diphenyl dimethylsiloxane; 25 m × 0.32 mm, 0.25 mm film thickness). The oven temperature was 200°C, the splitless injector temperature was 250°C, and the detector temperature was 260°C. The silylated LCB were identified by their equivalent chain length (ECL), determined relative to C18-sphingosine (47–49).

## RESULTS

*Lipid fractionation and class composition.* By using the TL sample from composite *A. aurita* 1996 we had the opportunity to design initial large-scale experiments in order to provide a deeper insight into the composition and nature of individual SPnL species as well as the rest of the lipid classes present. The above 1996 experiments indicated that *A. aurita* contains a major and several minor SPnL species as well as glycerolipids. In the final large-scale experiment, we used the composite 1998 TL in order to isolate and identify all the individual glycerophospholipids and SPnL classes, together

with the FA composition of PL and NL classes. The extracted TL amounted to 0.031 and 0.036% (w/w) of fresh tissue, and the lipid phosphorus content of the TL accounted for 1.7 and 1.3%, respectively, for 1996 and 1998 composite samples; the coefficients of variation of lipid phosphorus content ($n$ = 4) were 23.5 and 7.6%, respectively. On the basis of phosphonate-phosphorus determinations, the PnL were found to represent 22.3 ± 0.6 and 21.7 ± 0.3% (mol/mol) of total PhL ($n$ = 4), respectively, for both composites. TL samples were separated by solid-phase extraction into PL and NL lipid classes. The PL in bulk recovered from silicic columns were 37.5 and 35.8% of TL, respectively, for 1996 and 1998 composites.

Glycerylether determinations were performed on TL, PL, and NL samples from composite 1998, and the data showed 8.3 ± 0.4, 16.3 ± 0.3, and 3.7 ± 0.2% glycerylethers ($n$ = 4), respectively; furthermore, the carbohydrate content of TL was negligible.

NL were separated by analytical 1D-TLC using solvent system **D**. Based on their TLC behavior along with standards, the NL major component was found to consist mainly of free FA. The NL were also found to contain substantial proportions of cholesterol esters, cholesterol, triglycerides, and trace amounts of glycerylethers and fatty alcohols.

PL were separated into nine subclasses by using analytical 1D-HPTLC with solvent systems **A** and **B**. In system **B** (Fig. 1), all the components of SPnL class, a major and three minor species, had similar $R_f$ values, but they were situated far from PC; therefore, the isolation and extraction of the SPnL class of lipids was more effective on preparative TLC by system **B**. The difference between solvent systems **A** and **B** was that the minor SPnL species were separated into three narrow bands with solvent system **A**, and each one migrated far from the major one, but the slowest minor SPnL spot was at nearly the same $R_f$ as that of PC. By using solvent system **A** on analytical 1D-HPTLC, we observed that the PL fraction contained nine lipid components including eight phosphocompounds. The main phosphorus-positive components were those that co-chromatographed with standards of PC, PE, and CAEP. Below the CAEP position, a group of three minor phosphocomponents, positive to ninhydrin and to the Stillway test, had migrated and were also designated as SPnL species (CAEP II, III, and IV). Two more phosphocompounds co-chromatographed with CL and L-PC standards. Another ninhydrin-positive component free of phosphorus migrated near the origin and was designated *z*. As determined by 2D-HPTLC in system **C**, the PL fraction revealed 11 components, which were designated as CL, $PE_1$, $PE_2$, CAEP-I, PI, CAEP-II, CAEP-III, CAEP-IV, PC, L-PC, and *z* (Fig. 2), namely, two more phosphocomponents than the foregoing 1D-HPTLC fractionation. The spots designated as $PE_1$ and $PE_2$ had the same $R_f$ values as the PE standard by 1D-HPTLC but could be resolved (by 2D-HPTLC) into two phosphorus and ninhydrin-positive spots in the second run. The spots, designated as PI and CAEP-I, had similar $R_f$ values by 1D-HPTLC with the CAEP standard. Component PI was ninhydrin- and Stillway test-negative but was slightly positive

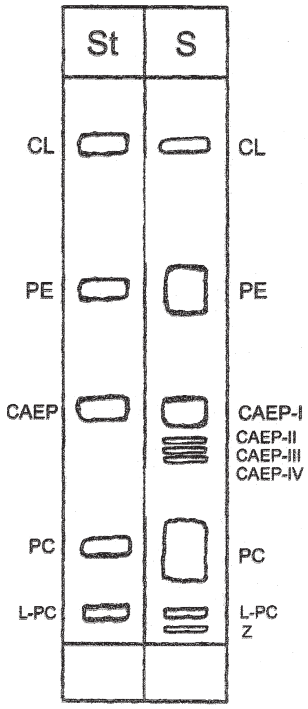


**FIG. 1.** Preparative thin-layer chromatography (TLC) fractionation of *Aurelia aurita* polar lipids on a TLC silica gel 60 plate. The diagram (St) on the left shows these standards: CL, cardiolipin; PE, phosphatidylethanolamine; CAEP, ceramide 2-aminoethylphosphonic acid; PC, phosphatidylcholine; and L-PC, lysophosphatidylcholine. Plates were developed with solvent **B**: chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol). Visualization: exposure to iodine vapors. S, sample; St, standard. For explanation of CAEP-I, -II, -III, -IV see text; *z*, unidentified ninhydrin-positive component free of phosphorus.

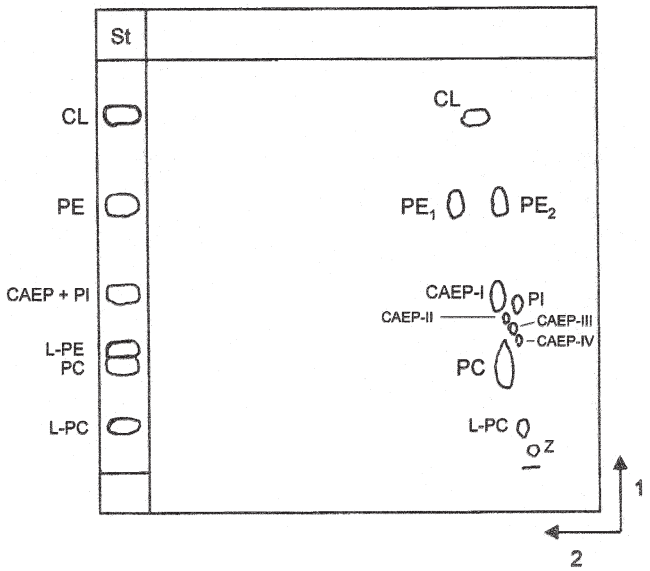


**FIG. 2.** Two-dimensional TLC separation of *A. aurita* polar lipids on high-performance TLC silica gel 60. Plates were developed with solvent **C**: direction 1, chloroform/methanol/acetic acid/water (50:25:6:2, by vol); direction 2, chloroform/methanol/water (65:20:3, by vol). The diagram (St) contains these standards (St): CL, PE, CAEP, PI (phosphatidylinositol), L-PE (lysophosphatidylethanolamine), PC, and L-PC. The plate was stained by copper sulfate/$H_3PO_4$ and charring. Samples contained: $PE_1$ and $PE_2$, two different molecular species of PE; CAEP-I, major sphingophosphonolipid species; CAEP II, III, IV, minor sphingophosphonolipid species of *Aurelia*. The letter *z* denotes an aminolipid component of *Aurelia* as described in the text. For other abbreviations see Figure 1.

**TABLE 1**
**Separation and Composition (% of lipid P) of *Aurelia aurita* Polar Lipids from Composites 1996 and 1998**

| | 1996 | 1998 | | 1998 |
|---|---|---|---|---|
| Lipid bands | Separation by preparative TLC[a,b] | | Lipid spots | Separation by 2D-TLC[c,d] |
| Cardiolipin | 6.0 ± 0.3 | 5.2 ± 0.6 | CL | 5.6 ± 0.4 |
| Phosphatidylethanolamine | 18.7 ± 0.5 | 19.1 ± 0.8 | $PE_1$ | 8.9 ± 0.2 |
| | | | $PE_2$ | 9.7 ± 0.3 |
| SPnL class | 23.8 ± 0.6 | 23.5 ± 0.5 | Major CAEP (CAEP-I) | 18.3 ± 0.6 |
| and phosphatidylinositol | | | Minor CAEP (II, III, IV) | 3.4 ± 0.4 |
| | | | PI | 2.6 ± 0.2 |
| Phosphatidylcholine | 44.9 ± 0.9 | 43.1 ± 1.1 | PC | 44.5 ± 0.6 |
| Lysophosphatidylcholine | 4.8 ± 0.3 | 4.6 ± 0.3 | L-PC | 5.0 ± 0.3 |
| Recovery | 98.2 | 95.5 | Recovery | 98.0 |

[a]Lipid sample (1.0 mg phosphorus/composite 1996; 3.0 mg phosphorus/composite 1998) was fractionated on 20 × 20 cm silica gel plate (solvent **B**).
[b]Values are means ± standard deviations of three replicate phosphorus determinations performed on each lipid band.
[c]Lipid sample (18 μg phosphorus) was separated on 20 × 20 cm two-dimensional thin-layer chromatography (2D-TLC) (solvent **C**).
[d]Values are means ± standard deviations obtained from phosphorus determinations performed on each 2D-TLC plate ($n$ = 3). Abbreviations: CL, cardiolipin; PE, phosphatidylethanolamine; CAEP, ceramide 2-aminoethylphosphonic acid; PI, phosphatidylinositol; SPnL, sphingophosphonolipid; PC, phosphatidylcholine; L-PC, lyso-PC.

to an α-naphthol reagent test, suggesting the presence of PI in this spot. The major SPnL species CAEP-I, as well as the three minor SPnL species groups CAEP II, III, and IV, were, as mentioned above in the 1D-TLC, positive to ninhydrin and the Stillway test (Fig. 2).

Samples of PL originating from composites of either 1996 or 1998 were separated into five PhL bands (CL, PE, SPnL class, PC, L-PC) by preparative TLC with solvent system **B** (Fig. 1). Table 1 shows the lipid-phosphorus distribution of each of the five extracted lipid bands. Alternatively, similar analytical data were obtained by quantitation of total phosphorus using a PL sample (from composite 1998) after separation by 2D-TLC with solvent system **C** into eight PhL components comprising CL, $PE_1$, $PE_2$, the major SPnL (CAEP-I) component, the sum of three minor SPnL spots, which were obtained as one spot, and PI, PC, and L-PC as described in the Materials and Methods section and shown in Table 1.

*FA*. The FA composition of TL, as well as NL and PL FA, was determined using GC as described in the Materials and Methods section. The data on FA and the proportion of the saturated FA (SFA), monounsaturated FA (MUFA), polyunsaturated FA (PUFA), and hydroxy FA are given in Table 2. SFA were predominant in all lipid fractions of *A. aurita*. The *cis*-14:1n-5 fatty acid, included as "other" in Table 2, was not detected in TL and NL. Also, the 17:0 cyclo, 20:0, and *cis*-20:2n-6 fatty acids, included as "other" in Table 2, were not found in the NL and PL.

*Isolation of SPnL class (a major and three minor species)*. After fractionation of PL by preparative TLC (solvent **B**), the lipid band of the SPnL class (including, apart from CAEP-I, three minor SPnL species, CAEP II, III, and IV, and the contaminant glycerolipid of PI) was recovered, and the lipids were extracted (Table 1). A part of the extracted band of the SPnL class (705 μg phosphorus) was subjected to phosphonate-

**TABLE 2**
**Fatty Acid Composition (wt%) of *Aurelia aurita* Lipid Classes from Composite 1998**

| Fatty acids[a] | TL[b] | NL[b] | PL[b] |
|---|---|---|---|
| 12:0 | 1.2 | — | — |
| 14:0 | 4.1 | 4.1 | 2.4 |
| a-15:0[c] | 0.5 | 0.6 | 0.4 |
| 15:0 | 2.8 | 1.7 | 4.7 |
| 2OH-12:0 | 0.5 | 0.4 | 0.6 |
| 16:0 | 42.3 | 32.8 | 55.1 |
| 16:1n-7 *trans* | — | 1.0 | 0.1 |
| 16:1n-7 *cis* | 2.6 | 3.0 | 0.6 |
| 3OH-12:0 | 2.6 | 1.5 | 4.1 |
| a-17:0 | — | 0.8 | 1.1 |
| 17:0 | 2.9 | 2.5 | 3.8 |
| 17:1n-7 *cis* | — | — | 0.5 |
| 18:0 | 22.2 | 24.3 | 15.0 |
| 18:1n-9 *trans* | 0.5 | — | — |
| 18:1n-9 *cis* | 8.3 | 10.8 | 1.2 |
| 3OH-14:0 | 3.1 | 3.2 | 1.3 |
| 18:2n-6 *cis* | 1.8 | 2.7 | 0.6 |
| 19:0 | 0.7 | 0.8 | 0.5 |
| 20:4n-6 *cis* | 1.4 | 2.9 | 3.8 |
| 20:3n-3 *cis* | — | 1.2 | 0.4 |
| 20:5n-3 *cis* | 0.7 | 1.9 | 2.6 |
| 22:4n-6 *cis* | — | 0.5 | — |
| 22:5n-3 *cis* | — | 1.9 | — |
| 22:6n-3 *cis* | — | 0.6 | 0.5 |
| Σ Saturated | 76.7 | 67.6 | 83.0 |
| Σ Unsaturated | 15.3 | 26.5 | 10.3 |
| Σ Hydroxy | 6.2 | 5.1 | 6.0 |
| Σ Monounsaturated | 11.4 | 14.8 | 2.4 |
| Σ Polyunsaturated | 3.9 | 11.7 | 7.9 |
| Other | 1.8 | 0.8 | 0.7 |

[a]Mass spectrometry was not used to confirm fatty acid structures.
[b]Abbreviations: TL, total lipids; NL, neutral lipids; PL, polar lipids.
[c]i, iso; a, anteiso; iso and anteiso are isomers of branched saturated fatty acids. "Other" includes all fatty acids present at <0.5 wt%, i.e., 14:1n-5 *cis*, i-15:0, 17:0 cyclo, 20:0, 20:2n-6 *cis*.

phosphorus determination and amounted to 89.3 ± 1.2% of the total phosphorus content. Another part of the extracted lipid band of the SPnL class (650 µg phosphorus) was subjected to mild alkaline hydrolysis in order to remove any glycerolipids (e.g., PI), and the alkali-stable products (576 µg phosphorus) were tested by 2D-HPTLC for purity confirmation. The results confirmed that one major SPnL, CAEP-I, and three minor SPnL species, CAEP II, III, and IV (positive to ninhydrin, to molybdenum blue, and to the Stillway test), were present and their phosphonate-phosphorus analysis showed that 96.1 ± 0.8% of lipid-phosphorus was phosphonate-phosphorus. Individual SPnL species, both the major one and each of the three minor species, were isolated from the alkaline-stable products (530 µg phosphorus) and separated by preparative TLC (system **A**) into CAEP-I (18.3% of PhL) and three CAEP (II, III, IV in the amounts of 1.3, 1.1, and 1.0% of PhL, respectively).

*Identification of major SPnL species, CAEP-I.* After testing the purity of the major SPnL by 2D-HPTLC analysis, a sample of purified major SPnL species (70 µg phosphorus) was subjected to dry-acid methanolysis (see Materials and Methods section). The hydrolysis products contained total nitrogen, LCB, esters, and total phosphorus in molar ratios of 2.1:1.1:1.1:1.0, respectively, and 95.5% of its lipid-phosphorus was phosphonate-phosphorus. FA methyl esters were extracted from methanolysate with petroleum ether and were analyzed by GC (Table 3). After Folch partition of the remaining methanolysate, the water-soluble products were analyzed by TLC on cellulose and found to contain only one component, which co-migrated with the AEP standard and was ninhydrin- and phosphorus-positive (Fig. 3). Therefore, the structure of ceramide aminoethylphosphonic acid was confirmed for the major SPnL species of *A. aurita*, CAEP-I. The chloroform soluble products of Folch partition (LCB) were converted to their TMS derivatives and analyzed by GC. The results are listed in Table 4.

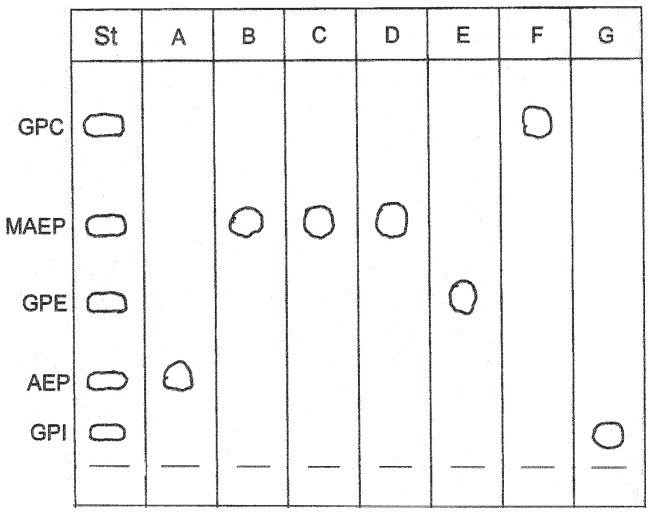


**FIG. 3.** TLC analysis on cellulose TLC of water-soluble components derived from hydrolyses of CAEP-I (A), CAEP-II (B), CAEP-III (C), CAEP-IV (D), PE (E), PC (F), and PI (G) polar lipids of *A. aurita*. The diagram (St) on the left shows these standards: GPC, L-α-glycerylphosphorylcholine; MAEP, 2-methylaminoethylphosphonic acid; GPE, L-α-glycerylphosphorylethanolamine; AEP, 2-aminoethylphosphonic acid; and GPI, L-α-glycerylphosphorylinositol. Developing solvent system: phenol/ethanol/acetic acid/water (80:12:10:20 wt/vol/vol/vol). Visualization: Hanes-Isherwood reagent. For other abbreviations see Figures 1 and 2.

**TABLE 3**
**Fatty Acid Composition (wt%) of *Aurelia aurita* SPnL from Composite 1998**

| | Major SPnL | Minor SPnL | | |
|---|---|---|---|---|
| Fatty acids[a] | CAEP-I | CAEP-II | CAEP-III | CAEP-IV |
| 12:0 | 0.8 | 1.0 | 1.0 | — |
| 13:0 | — | 0.6 | — | — |
| 14:0 | 9.1 | 17.4 | 27.8 | 26.1 |
| a-15:0 | 0.1 | 1.1 | 1.3 | — |
| 15:0 | 5.1 | 4.0 | 3.6 | — |
| 2OH-12:0 | 0.8 | — | — | — |
| 16:0 | 59.5 | 26.8 | 16.0 | 18.0 |
| 16:1n-7 *trans* | 1.5 | 7.7 | 10.6 | 9.4 |
| 16:1n-7 *cis* | 0.6 | 2.3 | 2.8 | 3.3 |
| 3OH-12:0 | 6.4 | 1.9 | 0.4 | 0.4 |
| a-17:0 | 0.9 | 0.6 | — | — |
| 17:0 | 2.1 | — | 1.3 | — |
| 17:0 cyclo | — | 1.5 | — | — |
| 17:1n-7 *cis* | 0.5 | 1.4 | — | — |
| 18:0 | 2.2 | 8.4 | 8.5 | 7.3 |
| 18:1n-9 *trans* | 0.3 | 1.3 | 2.1 | — |
| 18:1n-9 *cis* | 2.4 | 8.6 | 11.5 | 9.3 |
| 3OH-14:0 | — | 1.8 | 3.7 | 3.8 |
| 18:2n-6 *cis* | 1.9 | 1.8 | 2.8 | 5.3 |
| 19:0 cyclo | 1.6 | 5.9 | — | — |
| 2OH-16:0 | — | 1.3 | 0.7 | 10.9 |
| 20:0 | 0.2 | 0.6 | — | — |
| 20:2n-6 *cis* | — | — | 0.6 | — |
| 22:0 | 0.1 | 0.5 | — | — |
| 22:1n-9 *cis* | 1.3 | 2.8 | 5.0 | 6.2 |
| 24:0 | 0.5 | — | — | — |
| 24:1n-9 *cis* | 0.6 | — | — | — |
| 22:6n-3 *cis* | 0.7 | — | — | — |
| Σ Saturated | 82.2 | 68.4 | 59.5 | 51.4 |
| Σ Unsaturated | 9.8 | 25.9 | 35.4 | 33.3 |
| Σ Hydroxy | 7.2 | 5.0 | 4.8 | 15.1 |
| Σ Monounsaturated | 7.2 | 24.1 | 32.0 | 28.2 |
| Σ Polyunsaturated | 2.6 | 1.8 | 3.4 | 5.3 |
| Other | 0.8 | 0.7 | 0.3 | — |

[a]Mass spectrometry was not used to confirm fatty acid structures. "Other" includes all fatty acids present at <0.5%, i.e., i-15:0, 18:3n-3 *cis*, 22:5n-3 *cis*. For abbreviations see Tables 1 and 2.

CAEP-I ceramides were composed mainly of 70.7 % total FA (Table 3), ranging from 14- to 16-carbon chain length (CCL). In contrast, total FA with 18 CCL amounted only to 6.8%. Total LCB of CAEP-I ceramides contained a large amount, 91.9%, of bases with 16–18 CCL, which were distributed primarily in total dihydroxy bases (TDB) (57.3% with a preponderance of C18-sphingosine, 35.6%) and in total trihydroxy bases (TTB) (23.4% with a preponderance of C16-phytosphingosine, 17.6%) (Table 4).

*Identification of three minor species, CAEP II, III, IV.* Each of the three isolated and purified minor SPnL species, CAEP II, III, and IV, was subjected to the same procedure, dry-acid methanolysis, for identification as described above for the major component. The respective molar ratios for

**TABLE 4**
**Long-Chain Base Composition (wt%) of *Aurelia aurita* SPnL from Composite 1998**

| | | Major SPnL | Minor SPnL | | |
|---|---|---|---|---|---|
| Long-chain bases | ECL[a] | CAEP-I | CAEP-II | CAEP-III | CAEP-IV |
| $C_{14}$-S[a] | 14.00 | — | 10.2 | — | — |
| $C_{13}$-P[a] | 14.82 | — | — | 20.0 | — |
| $C_{15}$-S | 15.00 | — | 9.1 | — | 1.1 |
| $C_{15}$-$SH_2$[a] | 15.32 | 1.3 | — | — | 2.3 |
| $C_{14}$-P | 15.81 | — | — | 2.5 | — |
| $C_{16}$-$SH_2$ | 16.32 | 4.0 | 4.7 | — | 4.0 |
| $C_{17}$-S | 17.00 | 8.1 | 7.1 | 3.5 | — |
| $C_{17}$-$SH_2$ | 17.32 | 2.6 | 6.1 | — | 5.0 |
| 3-O-Me-$C_{18}$-S[a] | 17.42 | 11.3 | — | — | — |
| $C_{16}$-P | 17.80 | 17.6 | — | — | — |
| $C_{18}$-S | 18.00 | 35.6 | 17.9 | 8.9 | 20.6 |
| $C_{18}$-$SH_2$ | 18.32 | 7.0 | — | 1.6 | — |
| 3-O-Me-$C_{19}$-S | 18.42 | — | 15.3 | — | 7.3 |
| $C_{19}$br-S[a] | 18.64 | — | — | 39.5 | — |
| $C_{17}$-P | 18.82 | 2.2 | 9.0 | 3.1 | 5.5 |
| $C_{17}$br-P | 19.49 | 3.6 | 7.4 | — | 5.6 |
| $C_{18}$-P | 19.82 | — | 13.2 | 20.9 | 47.3 |
| $C_{19}$-dehydro-P | 20.56 | — | — | — | 1.3 |
| $C_{21}$br-S | 20.64 | 6.7 | — | — | — |
| ∑ Dihydroxy | | 65.3 | 55.1 | 53.5 | 33.0 |
| ∑ Trihydroxy | | 23.4 | 29.6 | 46.5 | 59.7 |

[a]Abbreviations: ECL, equivalent chain length; br, branched; Me, methyl; S, sphingosine; P, phytosphingosine; $SH_2$, dihydrosphingosine; for other abbreviations see Table 1.

total nitrogen, LCB, esters, and total phosphorus were: 2.1:1.2:1.3:1.0 for CAEP-II, 2.0:1.1:1.3:1.0 for CAEP-III, and 2.2:1.2:1.1:1.0 for CAEP-IV. Furthermore, over 96% of lipid-phosphorus of each minor species was found to be phosphonate-phosphorus. Therefore, the structure of ceramide aminoethylphosphonic acid was confirmed for each of the minor SPnL species. FA of each of the minor SPnL species are shown in Table 3. The water-soluble products from each of the minor SPnL species were found to contain only MAEP, which was ninhydrin- and phosphorus-positive (Fig. 3). These data suggest the molecular structure of CMAEP for each. Their LCB compositions are listed in Table 4.

CAEP-II ceramides were composed mainly of 74.3% total FA (Table 3) ranging from 14 to 18 CCL with 52.6% SFA and 21.7% unsaturated FA. Total LCB of CAEP-II ceramides (Table 4) contained a large amount (84.7%) of bases with 14–18 CCL. Their TDB content was 55.1%, and their TTB amounted to 29.6%, mainly C18-phytosphingosine (13.2%).

CAEP-III ceramides were composed mainly of 82.1% total FA (Table 3) ranging from 14 to 18 CCL with 52.3% SFA and 29.8% unsaturated FA plus 4.8% total hydroxy FA. Total LCB of CAEP-III ceramides had a CCL range from 13 to 19, and their TDB was 53.5% with a preponderance of branched C19-sphingosine (39.5%), and their TTB content was 46.5%. Among these, phytosphingosine with 13 and 18 CCL (20.0 and 20.9%, respectively) were predominant (Table 4).

CAEP-IV ceramides were composed mainly of 78.7% FA (Table 3) ranging from 14 to 18 CCL with 51.4% SFA and 27.3% unsaturated FA as well as 15.1% total hydroxy FA. Total LCB of CAEP-IV ceramides (Table 4) contained a large amount (88.0%) of bases with 16–18 CCL. Their TDB content was 29.6% with a preponderance of C18-sphingosine (20.6%), while their TTB amounted to 58.4%, principally C18-phytosphingosine (47.3%).

The i-15:0 fatty acid, included as "other" in Table 3, was not detected in CAEP-IV. Also, *cis*-18:3n-3 was not detected in the three minor SPnL species. Finally, *cis*-22:5n-3, which is also included with "other" in Table 3, was not found in CAEP-III and CAEP-IV.

*Glycerol lipids*. After extraction of the lipid bands that co-chromatographed with PC, CL, and PE standards on preparative TLC (Table 1) of PL (system **B**), each one was repurified by one more preparative TLC step. Upon testing the purity of the PE lipid band by 2D-HPTLC, two species ($PE_1$, $PE_2$) were found. A sample of each PC, CL, and PE lipid band (19.3, 3.0, and 11.2 μmol phosphorus, respectively) was subjected to mild alkaline hydrolysis. The bulk of the lipid phosphorus of the CL band was distributed in the water-soluble products; therefore, this finding suggests a basic molecular structure of CL. The alkali-stable products of PC and PE lipid bands were found to contain 7.0 and 6.5 μmol lipid-phosphorus, respectively, which were co-chromatographed on 2D-HPTLC with L-PC and L-PE standards, respectively. The saponifiable products of the PC and PE lipid bands were analyzed by TLC on cellulose, and only one component was found to co-chromatographed with both GPC and GPE standards (Fig. 3). Therefore, by combining the data, the basic molecular structure of PC and PE was confirmed for PC and PE lipid bands of *A. aurita*. Glyceryl ether determination of PC and PE lipid classes indicated that PC contained 36.2 ± 0.9% glycerylethers (16.1 ± 0.6% of PL), while PE contained glycerylether analogs in a proportion of 4.5 ± 0.2% (0.8 ± 0.1% of PL).

The identification of PI was based on the analysis of water-soluble products derived from mild alkaline hydrolysis of extracted lipid band of SPnL class from the preparative TLC (system B) of PL. The 11.4% of lipid-phosphorus (2.6% of PhL) was distributed in the water-soluble products corresponding to the PI component. This finding is in agreement with the result of quantitative analysis of this lipid spot after separation of PL on 2D-TLC (Fig. 2, Table 1). Furthermore, the water-soluble products were found to contain only one component that co-chromatographed with the GPI standard and also was Hanes-Isherwood positive (Fig. 3). Therefore a molecular structure of PI could be suggested for the PI component of *A. aurita*.

## DISCUSSION

*Lipid content*. The TL contents found in extractions of *A. aurita* composites 1996 and 1998 were almost constant, 0.031 and 0.036% (w/w) of the fresh tissue (0.72% of dry mass, assuming 95% water). The above results are comparable to that reported for *A. aurita* from Chesapeake Bay, 0.027% of fresh tissue or 0.54% of dry mass (5), while in *P. noctiluca* from the Aegean Sea (22) and *Chrysaora quinquecirrha* from Chesapeake Bay (5) they were found to be higher, 0.19 and 0.2% of

fresh tissue, respectively, or 3.8 and 4.0% of dry mass. According to an earlier report of Larson and Harbison (50) the average TL content of Antarctic gelatinous zooplankton is approximately 3%, ranging from 0.4 to 6% of dry mass, whereas Arctic gelatinous zooplankton have an average 8% lipid of dry mass, range 1.5–22%. Recently, Nelson *et al*. (51) reported that in several species of Antarctic gelatinous zooplankton, including cnidaria (*Calycopsis borchgrevinki*, *Diphyes antarctica*, *Stygiomedusa gigantea*, *Atolla wyvillei,* and *Dimophyes arctica*) and ctenophora (*Beroe cucumis*, *B. forskalii*, *Pleurobrachia pileus*, *Bolinopsis infundibulum*), the TL content ranged from 0.01 to 0.5% of fresh tissue (0.3–10.2% of dry mass). It is noteworthy that the TL contents of jellyfish from the Aegean Sea such as *P. noctiluca* (22) and *A. aurita* and those from Chesapeake Bay such as *A. aurita* and C. *quinquecirrha* (5) are similar to those Antarctic jellyfish (51), but they are lower than TL contents of Arctic jellyfish.

The PL percentage of Aegean *Aurelia* in each composite sample 1996 and 1998 was nearly the same (37.5 and 35.8% of the TL), and similar results were obtained from Aegean *P. noctiluca* (e.g., 26.2% PL and 73.8% NL) (22). Currently, Nelson *et al*. (51) reported that in Antarctic gelatinous zooplankton, PL are the major lipid class, 59–96% of TL. It is noteworthy that the PL contents of Aegean jelly fish *P. noctiluca* (22) and *A. aurita* are significantly lower than those of Antarctic jellyfish (51). The similarly low relative percentages of Aegean jellyfish PL (*A. aurita* 1996, 1998 and *P. noctiluca* 1988) derived under the same lipid extraction conditions do not permit us to say that these data reflect greater feeding activity. It is notable that the NL of *A. aurita* 1998 composite contained 3.7 ± 0.2% glycerylethers.

*FA*. The FA composition of *A. aurita* TL from the 1998 composite sample is characterized by high percentages of SFA (76.7%), which included three major FA, 16:0 (42.3%), 18:0 (22.2%), and 14:0 (4.1%) (Table 2). The FA profile of the NL and PL fractions was similar to that of TL, containing also high percentages of SFA, 67.6 and 83.0%, respectively. The very high percentage of SFA, 83.0% (Table 2) in PL of *A. aurita* is in good agreement with that of FA from CAEP-I, 82.2% (Table 3). MUFA levels of *A. aurita* TL were 11.4% while PUFA levels were 3.9%. The principal MUFA of *A. aurita* TL was *cis*-18:1n-9 (8.3%).

The FA profile of TL from *A. aurita* of the Atlantic Ocean (which live in the cold waters off North America, particularly Canada) was appreciably different from that of Aegean *A. aurita*, containing a high percentage (62.7%) of unsaturated FA (which included 20:1, 13.7%, and 18:1, 11.8% (5). The TL, PL, and NL of Aegean *A. aurita* exhibited a low 16:1/16:0 ratio, which is characteristic of many shellfish, and is consistent with a phytoplankton diet. This ratio decreases in temperate and tropical waters (5). Nelson *et al*. (51) reported that the high 16:1/16:0 ratio is a characteristic feature of diatom FA profiles, particularly in combination with elevated proportions of the FA 20:5n-3. The differences in the FA composition of medusae could be attributed to the fact that the content in some FA is influenced by the environmental temperature and the diet of jellyfish, i.e., is dependent on whether the diet is predominantly phytoplankton, zooplankton, or small fishes (5).

*Glycerophospholipids*. The PL of *A. aurita* are characterized by the absence of some lipid classes that are widespread in other animals, such as phosphatidylserine, sphingomyelin, and glycolipids. As shown in Table 1, there is no variation in the relative composition of individual phospholipid bands (CL, PE, SPnL class + PI, PC, L-PC) between 1996 and 1998 composite samples ($P = 0.05$, *t*-test). These data are in agreement with those reported for *P. noctiluca* (22). The percentage of glycerylether-type total PhL of *A. aurita* from the 1998 composite was 16.3 ± 0.3%. These glycerylethers were concentrated in the molecular species of the PE class and also in PC (4.5 ± 0.2 and 36.2 ± 0.9%, respectively). The glycerylether-type PhL found in the jellyfish *P. noctiluca* represent 36.1% of total PhL and were distributed in both PE and PC at 55.0 and 62.0%, respectively (22). The low percentage of glycerylether (4.5%) found in PE of *Aurelia* in relation to the high percentage of alkali-stable products (57.9%) after mild alkaline hydrolysis of intact PE ($PE_1 + PE_2$) could suggest the possible presence of alkenylether analogs (plasmalogens) of PE in a proportion of 53.4%. Finally, it is interesting that PI (2.6% of PhL) was found in *A. aurita* PL (Table 1).

*SPnL*. According to the present structural studies, the PL of *A. aurita* are characterized by the presence of a significant amount of PnL (21.7% of PhLs) (Table 1), which belong to the ceramide aminophosphonic acid type. Their chromatographic resolution on TLC showed a faster-moving major CAEP-I (18.3% of PhL) and several minor SPnL species, which consisted of three chromatographically distinguisable *N*-methyl analogs, CAEP II, III, and IV, in percentages of 1.3, 1.1, and 1.0% of PhL, respectively.

CAEP-I ceramides were mainly characterized by a high content of total SFA acids, 70.8%, in the range of 14–18 CCL and a very low content of total unsaturated FA, 6.7% (Table 3). Total LCB of CAEP-I ceramides have a ratio of TDB to TTB, both having primarily 16–18 CCL, equal to 2.45 (Table 4). It is noteworthy that in the CAEP-I molecule, the principal SFA (70.8%) with 14–18 CCL, combine with 57.3% dihydroxy LCB with 16–18 CCL and 23.4% trihydroxy LCB. Theses results are not comparable to those obtained previously from the *P. noctiluca* major CAEP species (21.0% of PhL), which consisted mainly of SFA (about 96%) with 14–16 carbon atoms, and C18-sphingosine, with over 85% of total LCB (22). Only a few reports concerning the occurrence of CAEP species in cnidaria other than in *P. noctiluca* have been published. A major ceramide aminoethylphosphonic acid has been isolated from the lipids of the sea anemone *Metridium senile*. The main FA was found to be palmitic (52%), but 35% of the total FA were branched-chain acids. Moreover, its LCB were found to be D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4,*trans*-8-octadecadiene (95%) and sphingosine (5%) (23).

In comparison to CAEP-I, the CAEP-II methyl analog had reduced mobility on TLC owing to an increase of total unsaturated FA with 16–18 carbons (Table 3) and because the ratio

of TDB/TTB$_{CAEP-I}$ = 2.45 is decreased in it to 1.86 (value of TDB/TTB$_{CAEP-II}$) (Table 4).

CAEP-III methyl analog in comparison to the CAEP-II methyl analog had reduced mobility on TLC mainly because the value 1.86 for TDB/TTB$_{CAEP-II}$ decreased to 1.15 (value of TDB/TTB$_{CAEP-III}$) (Table 4), and because of an increase of + 8.1% total unsaturated FA content, ranging from 16 to 18 carbons (Table 3).

CAEP-IV methyl analog in comparison to CAEP-III also had reduced mobility mainly owing to an increase of hydroxy FA of 12–16 carbons (Table 3), and because the ratio of TDB/TTB$_{CAEPIII}$ = 1.15 decreased to 0.50 (value of TDB/TTB$_{CAEP-IV}$) (Table 4). The CAEP-IV molecule had 15.1% hydroxy FA with 12–16 carbons, together with 51.4% SFA (with 14–18 carbon atoms) and 27.3% unsaturated FA (with 16–18 carbon atoms), which combine with 58.4% trihydroxy LCB (two more hydroxyl groups) and 29.6% dihydroxy LCB (one more hydroxyl group), with 16–18 carbons.

If we compare each one of the three minor CAEP (II, III, IV) methyl analogs with the major CAEP-I, it is obvious that the three minor analogs have the same head group, but they differentiate from the major CAEP-I mainly because of the increase of their FA unsaturation and not because of a different LCB. Indeed, we see that the ratio SFA/unsaturated FA$_{CAEP-I}$ = 8.4 decreases to 2.6 (value of SFA/unsaturated FA$_{CAEP-II}$), 1.7 (value of SFA/unsaturated FA$_{CAEP-III}$), and 1.5 (value of SFA/unsaturated FA$_{CAEP-IV}$); apparently, the polarities of CAEP-III and CAEP-IV in comparison with CAEP-I polarity have no significant differentiation based on the FA unsaturation (ratios 1.7 and 1.5). We see also that the ratio SFA/hydroxy FA$_{CAEP-I}$ = 11.4 is increased 13.7 (value of SFA/hydroxy FA$_{CAEP-II}$) and 12.4 (value of SFA/hydroxy FA$_{CAEP-III}$). In contrast, for CAEP-IV this ratio is decreased 3.4; thus, CAEP-II and CAEP-III polarities in comparison with CAEP-I polarity have no significant differentiation based on the FA hydroxyl groups (ratios 13.7 and 12.4). In relation to the LCB moieties, we see that the ratio TDB/TTB$_{CAEP-I}$ = 2.8 is decreased to 1.9 (value of TDB/TTB$_{CAEP-II}$), 1.2 (value of TDB/TTB$_{CAEP-III}$), and to 0.6 for CAEP-IV; thus, CAEP-IV polarity in comparison with CAEP-I polarity is higher owing to the hydroxyl groups derived from the increased TTB content. Consequently, the three minor phosphonolipids could not be combined as one component in terms of their FA and LCB composition.

In the present study, a diversity of SPnL species was identified from the whole body of Aegean jellyfish *A. aurita,* including a major ceramidephosphonolipid CAEP, as well as three minor methyl CAEP analogs. This is the first report of the occurrence of methyl CAEP analogs in jellyfish. It is known that in marine invertebrates *Aplysia kurodai* and *Turbo cornutus*, CAEP species and its methyl analog are distributed in various tissue types such as the nervous tissue, ganglion, and fibers of *Aplysia* (54), and also in other tissues, such as viscera (52,53) and muscle tissue (16,19) of *Turbo*. Methyl analogs of SPnGL have also been found in skin of *Aplysia* (55). The biological significance of these distributions is not clear, but the differences in the FA and LCB composition may suggest differences in the metabolism of these molecules among the tissues (54). It would be of further interest to determine if the presence of each of the above major CAEP or minor methyl CAEP analogs is localized in specific tissues of *A. aurita*.

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# Separation and Identification of Phospholipid Peroxidation Products

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**ABSTRACT:** The molecular species in mixtures of phospholipid hydroperoxides are difficult to separate and identify by typical chromatographic and mass spectrometric techniques. As reported by Havrilla and coworkers, silver ion coordination ion-spray mass spectrometry (CIS–MS) has proven to be a powerful technique for the identification of mixtures of hydroperoxides. This ionization technique, which involves the formation of $Ag^+$ adducts of the hydroperoxides, provides valuable, unambiguous structural information about the hydroperoxides. Herein, we report a method for the analysis and identification of phospholipid hydroperoxides using CIS–MS. We also report an improved method for the separation of phospholipid hydroperoxides by reversed-phase high-performance liquid chromatography (RP–HPLC), which, for the first time, separates some of the hydroperoxide isomers. CIS–MS can be coupled with this RP–HPLC method by the addition of $AgBF_4$ to the mobile phase or to the HPLC effluent postcolumn, thus allowing powerful HPLC–MS techniques to be used to identify complex mixtures of phospholipid hydroperoxides.

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Lipid peroxidation has been widely studied because of its implicit role in the pathogenesis of a number of human diseases including cancer (1), neurodegenerative diseases (2,3), and atherosclerosis (4). Considerable evidence supports the hypothesis that oxidative modification of the lipids in low density lipoproteins (LDL) may play an important role in the onset of atherosclerosis (5). Phospholipids, as the major lipid components of the surface layer of LDL, are a primary target for oxidation (6). Linoleic acid and arachidonic acid are the most abundant unsaturated fatty acids esterified to phospholipids in LDL. Lipids containing these fatty acids are especially susceptible to free radical chain oxidation (7–9).

Some two decades ago, we reported that neat films of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) and 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine (SAPC) react readily with oxygen at room temperature to give a mixture of phospholipid hydroperoxides (10). These oxidized products could be separated from the unoxidized phospholipid using reversed-phase high-pressure liquid chromatography (RP–HPLC). However, separation of the molecular species of the oxidized phospholipid could not be achieved with the chromatographic options available at that time. Since then, improvements have been made in the HPLC separation of intact phospholipid molecular species. The addition of ion-pairing agents, such as ammonium acetate (11) and choline chloride (12), to HPLC mobile phases leads to improved separation and peak shape.

These improvements in chromatography along with advances in mass spectrometry (MS) have helped make the analysis of intact phospholipid hydroperoxides possible. In 1994, Zhang *et al.* (13) reported the first method to analyze intact hydroxyeicosatetraenoyl-*sn*-glycero-3-phosphatidylcholine (HETE PC) species by fast atom bombardment–tandem mass spectrometry (FAB–MS/MS). Subsequently, an HPLC–MS technique (thermospray MS) used to analyze phospholipid hydroperoxides has been reported (14), and electrospray MS (ESI–MS) has been applied to phospholipid hydroperoxide analysis (15–17).

Analyzing phospholipid hydroperoxides by positive ion ESI–MS allows detection of the parent ion, but MS/MS experiments do not give structural information about the position of oxidation on the side chain. Only the mass-to-charge ratio (*m/z*) of the phosphocholine head group can be detected because it is the only part of the molecule that is charged. The negative ion ESI–MS/MS experiments reported by Hall and Murphy (16) do give structural information about the position of oxidation on the side chains when a high orifice potential is applied during the ionization process. The high potential causes in-source fragmentation of the phospholipid species so that the parent ion, as well as two daughter ions that correspond to the *m/z* for the carboxylate anions of the fatty acid side chains, is observed. Collision-induced dissociation (CID) experiments where the ion with the *m/z* for the oxidized fatty acid of interest is activated give fragment ion peaks that are indicative of the position of oxidation on the side chain. These studies only report separation of the

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Abbreviations: BHT, butylated hydroxytoluene; CD, circular dichroism; CE, Cotton effect; CID, collision-induced dissociation; CIS–MS, coordination ion-spray mass spectrometry; DLI, direct liquid infusion; ESI–MS, electrospray MS; FAB–MS, fast atom bombardment MS; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high-pressure liquid chromatography; HPODE, hydroperoxyoctadecadienoic acid; LC, liquid chromatography; LDL, low density lipoproteins; MS, mass spectrometry; methyl-9-HETE, methyl 9-hydroxy-5(*Z*),8(*E*),11(*Z*),14(*Z*)-eicosatetraenoate; MS/MS, tandem MS; NMR, nuclear magnetic resonance; ODS, octadecylsilane; PC, phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidycholine; PLPC-OH, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidycholine alcohol; PLPC-OOH, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidycholine hydroperoxide; PMC, pentamethylchromanol; $PPh_3$, triphenylphosphine; RP, reversed phase; SAPC, 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine; SRM, selected reaction monitoring; TLC, thin-layer chromatography; UV, ultraviolet.

phospholipid species by class according to their head group and not by the position of oxidation on the side chains.

As an alternative to protonation, organic molecules containing hard or soft Lewis basic sites can be ionized by cations such as $Li^+$, $Na^+$, or $Ag^+$ (18–20). This ionization technique involves the formation of charged analyte complexes formed by the addition of a suitable coordination ion to the analyte. Combined with ESI–MS, coordination ion-spray mass spectrometry (CIS–MS) (21) has proved useful for analyzing complex mixtures of cholesteryl ester peroxides (22). Herein we report a method for the analysis and identification of phospholipid hydroperoxides using CIS–MS that eliminates some of the problems associated with ESI–MS. We also report an improved HPLC method that separates some of the isomers of linoleate hydroperoxides derived from PLPC and all of the arachidonate hydroperoxide isomers derived from SAPC.

## MATERIALS AND METHODS

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) or from Sigma Chemical Company (St. Louis, MO) and used without further purification. PLPC was purchased as a powder and SAPC was purchased as a chloroform solution. Solvents, such as methanol, water, and 2-propanol, were HPLC quality and purchased from either Fisher Chemical (Phillipsburg, NJ) or EM Science (Gibbstown, NJ). Hexanes were purchased from Burdick & Jackson (Muskegon, MI). All other reagents were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification.

Reactions involving hydroperoxides were monitored by thin-layer chromatography (TLC) using a stain of 1.5 g of *N,N′*-dimethyl-*p*-phenylenediamine dihydrochloride/25 mL of $H_2O$/125 mL of MeOH/1 mL acetic acid. Hydroperoxides yield an immediate pink color. TLC was carried out using 0.2-mm layer thickness, silica-coated aluminum columns (EM Science) that were visualized at 254 nm, phosphomolybdic acid char, or the peroxide stain. In general, hydroperoxides were stored as dilute solutions with 1 mol% butylated hydroxytoluene (BHT) in benzene at −78°C and never exposed to temperatures >40°C.

*Instruments.* Analytical HPLC was conducted on a Waters model 600 HPLC instrument, with a Waters model 486 tunable absorbance detector operating at 234 nm and with output to a Hewlett-Packard 3396 Series III integrator. Semipreparative HPLC was conducted on a Waters model 600E HPLC instrument with a Waters model 481 variable wavelength detector operating at 234 nm and with output to a Fisher Record-All Series 5000 strip chart recorder. Chiral HPLC was conducted on a Waters model 590 instrument with a Hewlett-Packard 1040A photodiode array detector. Semipreparative chiral HPLC was conducted on a Waters model 600 instrument with an LDC/Milton Roy Spectromonitor 3000 variable wavelength detector operating at 234 nm and with output to a Hitachi D-2500 Chromato-Integrator.

$^1$H Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker DRX-400 (400 MHz) spectrometer in $CDCl_3$. Immediately prior to sample preparation, $CDCl_3$ was passed through a plug of basic alumina to remove any HCl.

*Mass spectrometry.* CIS–MS was performed using a Finnigan Thermoquest TSQ-7000 (San Jose, CA) triple quadrupole mass spectrometer equipped with a standard API-1 electrospray ionization source outfitted with a 100-µm deactivated fused-Si capillary. Data acquisition and spectral analysis were conducted using ICIS software, version 8.3.2, running on a Digital Equipment Alpha Station 200 4/166. Nitrogen gas served both as the sheath and auxiliary gas; argon served as the collision gas. The electrospray needle was maintained at 4.6 kV, and the heated capillary temperature was 250°C. The tube lens and capillary voltages were optimized to maximize ion current for electrospray; the optimal values were determined to be 90 and 10 V, respectively, for phospholipid analysis. For MS/MS experiments, the collision gas pressure was typically 2.6–2.9 mTorr. To obtain fragmentation information on the $Ag^+$-phospholipid adducts, the dependence of collision energy on relative abundance was studied. Positive ions were detected scanning from 100 to 1000 amu with a total scan duration of 1 s. Profile data were recorded for 1 min (~60 scans) and averaged from analysis.

Samples were introduced either by direct liquid infusion (DLI) or HPLC. For DLI experiments, samples were introduced with a Harvard Apparatus (Cambridge, MA) syringe pump at a flow rate of 10 µL/min. For HPLC sample introduction, a Waters model 2690 Separation Model instrument was used. The HPLC was equipped with a Discovery octadecylsilane (ODS) column (4.6 × 250 mm, 5 µm; Supelco, Bellefonte, PA) and operated with a mobile phase of methanol/water (95:5, vol/vol) at a flow rate of 1 mL/min. A splitting tee after the column permitted 240 µL/min to be passed through an Applied Biosystems 785A programmable absorbance ultraviolet (UV) detector operating at 234 nm before entering the mass spectrometer. The remainder of the effluent was collected as waste.

*Liquid chromatography (LC)–CIS–MS of phospholipid hydroperoxides.* For these experiments, the hydroperoxides were isolated from the unoxidized phospholipid using analytical HPLC (methanol/water, 95:5, vol/vol, 1 mL/min). The oxidized fraction for PLPC (13.5–15.5 min) or SAPC (20–28 min) was collected, concentrated, and analyzed by LC–CIS–MS. A stock solution of the oxidized lipid was prepared (1–1.25 mg/mL) and 25–50 µL of the solution was injected per analysis. Offset voltages for selected reaction-monitoring (SRM) experiments, 28–33 eV, were determined by optimization in DLI experiments.

*Studies on PLPC, autoxidation.* To a solution of PLPC (25 mg, 0.033 mmol) in 2.5 mL of $CH_2Cl_2$ was added 10 mol% 2,2,5,7,8-pentamethyl-6-chromanol (PMC; 73 µL of a 10 mg/mL stock solution). The solution was evaporated to dryness under vacuum so that the mixture formed a thin layer on the inside of a 10-mL round-bottomed flask. The flask was then heated to 37°C and exposed to an atmosphere of dry air. After 24 h, the mixture was dissolved in benzene and BHT (~1–2 mg) was added to stop the reaction. TLC of the product mixture indicated the formation of hydroperoxides. The hydroperoxide products were then analyzed by HPLC.

*HPLC analysis of the PLPC hydroperoxides (PLPC-OOH).* In order to analyze the peroxidation products of PLPC, it was first necessary to optimize HPLC conditions for their separation. During the optimization process, two different C-18 analytical columns were tried; one was a Supelcosil column (4.6 × 250 mm, 5 μm; Supelco) while the other was a Discovery column (4.6 × 250 mm, 5 μm; Supelco). Mobile phases used in the optimization were mixtures of varying concentrations of methanol, hexanes, and water containing varying concentrations (0–25 mM) of ammonium acetate or choline chloride. The optimal conditions, which gave the best separation and peak shape, were obtained using the Discovery C-18 analytical column with a mobile phase of simply methanol/water (95:5, vol/vol) at a flow rate of 1 mL/min. Analysis of the PLPC-OOH using these HPLC conditions showed the formation of two major fractions at $t_R$ = 14.04 and 14.97 min. Each fraction was collected, concentrated, and rechromatographed to ensure its purity. The fractions were then converted to the corresponding methyl hydroxyoctadecadienoates (HODE), which were previously characterized (23), for identification.

*Conversion of PLPC-OOH to methyl HODE.* Methyl hydroperoxyoctadecadienoates (HPODE) were prepared as described previously (23). This mixture of hydroperoxides and the isolated fractions of PLPC-OOH were treated with an excess of triphenylphosphine ($PPh_3$) to generate the corresponding alcohols (PLPC-OH). To convert the alcohols to the methyl esters, each isolated fraction was dissolved in benzene (0.5 mL) and treated with an excess of NaOMe (1 mL of a 0.5 M solution in methanol) for 2 h. The reactions were worked up by the addition of deionized water (5 mL) and acetic acid (100 μL). The aqueous layer was extracted with hexanes (2 × 5 mL). The combined organic layers were dried, concentrated, and analyzed by HPLC. To analyze the methyl HODE, the HPLC was equipped with two tandem Ultrasphere silica columns (4.6 × 250 mm, 5 μm; Beckman, Fullerton, CA). The compounds were eluted with 0.6% 2-propanol in hexanes containing 0.1% acetic acid at a flow rate of 3 mL/min. The elution order of the methyl HODE from the standard mixture corresponds to that previously reported (24). Injection of the transesterified fractions showed that phospholipid hydroperoxides **1**, **2**, and **3** elute from the HPLC in the first fraction, $t_R$ = 14.04 min. Injection of the transesterified fractions showed that the second fraction, $t_R$ = 14.97 min, contains phospholipid hydroperoxide **4**. $^1$H NMR of each phospholipid fraction and LC–CIS–MS experiments support this assignment.

*$^1$H NMR of the PLPC-OOH.* $^1$H NMR spectra were taken on each of the isolated PLPC-OOH fractions. The vinyl region of the spectrum taken on the first fraction consists of signals at δ 5.45 (*m*, 1H), 5.62 (*m*, 1H), 5.99 (*m*, 1H), and 6.51 (*m*, 1H). These signals are consistent with those signals previously reported for the *cis,trans* isomers of oxidized cholesteryl linoleate (25). The spectrum of this fraction also contains small signals that correspond to the signals due to the *trans,trans* isomer. These signals at δ 5.55 (*m*, 1H), 5.72 (*m*, 1H), 6.03 (*m*, 1H), and 6.21 (*m*, 1H) are the major vinyl signals observed in the second fraction and are consistent with those signals reported for the *trans,trans* isomers of oxidized cholesteryl linoleate. The data support the assignment of compounds **1**, **2**, and **3** in the first fraction, and the assignment of compound **4** to the second.

*Studies on SAPC, autoxidation.* To a solution of SAPC (25 mg, 0.029 mmol) in 2.5 mL of $CH_2Cl_2$ was added 10 mol% PMC (64 μL of a 10 mg/mL stock solution). The solution was evaporated to dryness under vacuum so that the mixture formed a thin layer on the inside of a 10-mL round-bottomed flask. The flask was then heated to 37°C and exposed to an atmosphere of dry air. After 24 h, the mixture was dissolved in benzene and BHT (~1–2 mg) was added to stop the reaction. Analytical HPLC (Discovery line ODS column, methanol/water, 95:5, vol/vol, 1 mL/min, λ = 234 nm) indicated the formation of six major fractions. These fractions were then identified by LC–CIS–MS: **I**, **11**, $t_R$ = 20.93 min; **II**, **9**, $t_R$ = 22.03 min; **III**, **10**, $t_R$ = 22.78 min; **IV**, **7**, $t_R$ = 24.21 min; $\mathbf{V_a}$, **(*R,R*)-8**, $t_R$ = 24.89 min; $\mathbf{V_b}$, **(*S,R*)-8**, $t_R$ = 25.28 min; and **VI**, **6**, $t_R$ = 27.33 min.

*Studies on fractions $V_a$ and $V_b$.* Fractions $\mathbf{V_a}$ and $\mathbf{V_b}$ were isolated from each other using analytical HPLC (Discovery ODS column, methanol/water, 93:7, vol/vol., 1 mL/min, λ = 234 nm). The fractions were concentrated and rechromatographed to ensure their purity. The compounds were then converted to the corresponding methyl HETE in an analogous procedure to that described above for the PLPC-OOH. The transesterified products were purified using the same analytical HPLC system described for the methyl HODE. The collected products were concentrated for analysis by chiral HPLC equipped with a Chiralpak AD column (4.6 × 250 mm, 10-mm; Chiral Technologies, Exton, PA) using a mobile phase of hexanes/methanol (100:2, vol/vol) at a flow rate of 1 mL/min (26). Coinjection of each transesterified fraction with a racemic mixture of methyl 9-HETE confirmed the elution order. The transesterified product from fraction $\mathbf{V_a}$, $t_R$ = 8.65 min, was found to correspond to the *R* enantiomer of methyl 9-HETE; the transesterified product from fraction $\mathbf{V_b}$, $t_R$ = 9.08 min, was found to correspond to the *S* enantiomer of methyl 9-HETE.

*Determination of the elution order of methyl (9*R*)- and (9*S*)-hydroxy-5(*Z*),8(*E*),11(*Z*),14(*Z*)-eicosatetraenoate in chiral HPLC analyses, autoxidation of eicosatetraenoic acid (arachidonic acid).* In a round-bottomed flask, arachidonic acid (500 mg, 1.64 mmol) was dissolved in 1,4-cyclohexadiene (3.2 mL, 34 mmol) and benzene (5 mL) to give a solution 0.20 M in lipid and 4.2 M in 1,4-cyclohexadiene. Di-*tert*-butylhyponitrite (~3–5 mg) was added to the reaction, and the flask contents were allowed to stir at 37°C for 24 h. At this time, TLC indicated the formation of hydroperoxide products. BHT (~2 mg) was added to stop the reaction. Benzene and 1,4-cyclohexadiene were removed by vacuum, and the mixture was rediluted in hexanes for separation by semipreparative HPLC [Dynamax-60 A silica 83-121-C column (Rainin Instrument Co. Inc., Woburn, MA), 21.4 × 250 mm, 8 μm, 2% 2-propanol in hexanes with 1% acetic acid, 10 mL/min]. The elution order for the HPETE is known (27). 9-HPETE was collected, $t_R$ = 32 min, into a round-bottomed flask containing BHT (~2 mg). The solvent was removed *in vacuo,* and the racemic mixture of 9-HPETE was converted to the corresponding alcohols by treatment with excess $PPh_3$ in $Et_2O$.

*Synthesis of methyl 9-hydroxy-5(Z),8(E),11(Z),14(Z)-eicosatetraenoate (methyl 9-HETE).* The crude alcohol, 9-HETE, was dissolved in $Et_2O$ (1 mL) and treated with an excess of diazomethane generated in a Micro Diazomethane Generator (Ace Glass, Inc.) with 1-methyl-3-nitro-1-nitrosoguandine as the precursor. The reaction was stirred at 0°C for 2 h. The reaction mixture was allowed to sit open to the air in a hood for 30 min to allow evaporation of any excess diazomethane. The ether was removed *in vacuo,* and the methyl ester was purified by analytical HPLC (two tandem Beckman silica columns, 1.6% 2-propanol in hexanes, 1 mL/min, $\lambda = 234$ nm).

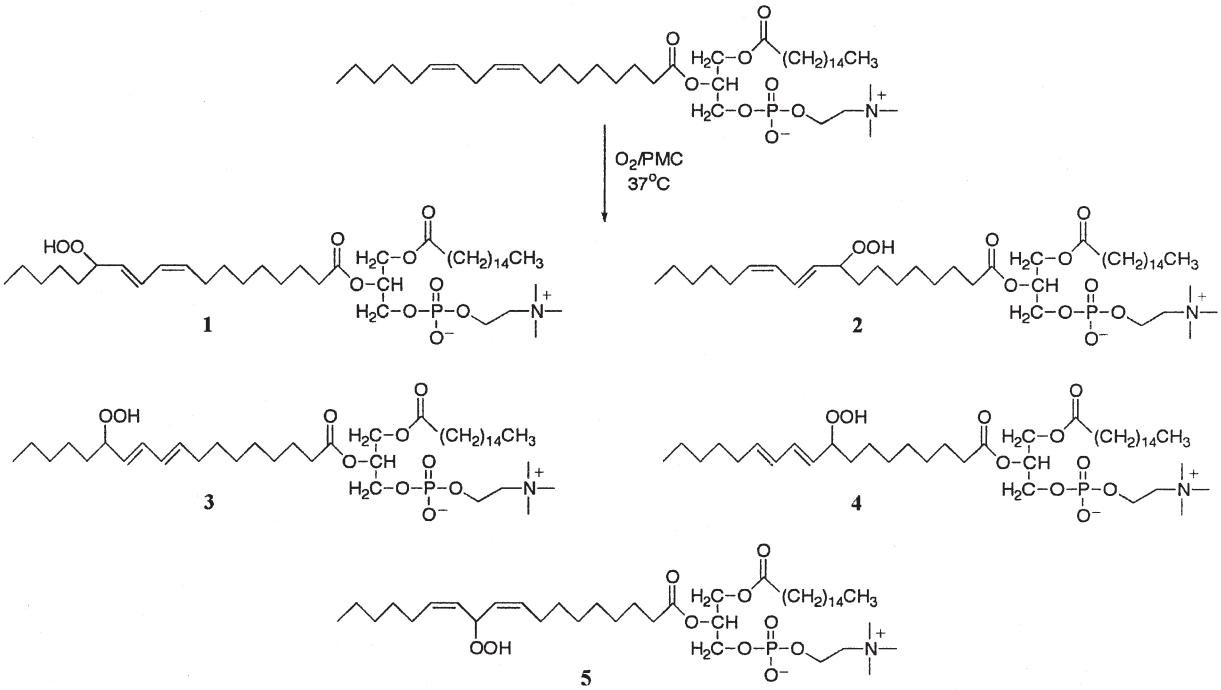
*Separation of racemic methyl 9-HETE by chiral HPLC (26).* The racemic methyl 9-HETE was separated using semi-preparative chiral HPLC. The HPLC was equipped with a Chiralpak AD column (10 × 250 mm, 10-mm; Chiral Technologies) using a mobile phase of hexanes/methanol (100:2, vol/vol) at a flow rate of 3 mL/min. Each enantiomer was collected, concentrated, and reinjected to ensure its purity: $\mathbf{V_a}$, $t_R$ = 13.82 min; and $\mathbf{V_b}$, $t_R$ = 14.47 min.

*Circular dichroism (CD) spectroscopy (28).* Each purified enantiomer was dissolved in 50 µL of dry acetonitrile. One microliter of 1,8-diazabicyclo[5.4.0]undec-1-ene and a few grains of 1-(2-naphthoyl)imidazole were added to the solution. The reaction was kept at room temperature overnight. The solvent was evaporated, and the residue was redissolved in $CH_2Cl_2$ (2 mL). The solution was washed twice with water (1 mL), and the $CH_2Cl_2$ was removed. The naphthoate derivatives were purified by analytical HPLC (two tandem Beckman silica columns, 1% 2-propanol in hexanes, 1 mL/min, $\lambda = 239$ nm). For CD spectroscopy, the purified naphthoate derivatives were dissolved in 400 µL of dry acetonitrile. CD was measured with a Jasco J-720 spectropolarimeter (cell volume 400 µL; cell path length 1 mm). The configuration of the collected enantiomer $\mathbf{V_a}$ was determined to be *R*, whereas the configuration of the collected enantiomer $\mathbf{V_b}$ was determined to be *S*.

## RESULTS AND DISCUSSION

As previously reported (10), the peroxidation products, **1–4**, that form when a neat film of PLPC is incubated in an atmosphere of air at 37°C are from linoleoyl side-chain oxidation (Scheme 1). The mechanism by which the four peroxidic products of linoleate form is well understood (7–9). Initial hydrogen atom extraction at the C-11 position on the linoleoyl side chain yields a pentadienyl radical. A molecule of oxygen then adds to this stable radical at either the C-9 or the C-13 positions to give *cis,trans* peroxyl radicals that, in the presence of a good hydrogen atom donor, will be trapped as the *cis,trans* hydroperoxides **2** and **1**, respectively (in the presence of large amounts of a good hydrogen atom donor, such as α-tocopherol, the 11-hydroperoxide of linoleate is formed; 29). In the absence of hydrogen atom donors, the *trans,trans* hydroperoxides **3** and **4** form. The ratio of *cis,trans* to *trans,trans* products formed in a reaction mixture is a good measure of the competition between hydrogen atom abstraction from hydrogen donor molecules and β-fragmentation.

In our previous study, **1–4** were separated from unoxidized PLPC by HPLC, but all four phospholipid hydroperoxides eluted as one broad, tailing peak. There was no separation of the various hydroperoxide isomers (10). Our first goal in this study was to improve chromatography for the separation of phospholipid hydroperoxides by methods that were compatible with CIS–MS techniques. Therond *et al.* (24) achieved




**SCHEME 1**

good separation of the molecular species of soybean PC and their corresponding hydroperoxides, formed from incubation with soybean lipoxygenase, using an analytical Spherisorb C-18 column and a mobile phase of methanol/10 mM ammonium acetate (95:5, vol/vol) at 1 mL/min. Chromatography of a mixture of **1–4** on a Supelcosil C-18 column with a mobile phase of methanol/hexanes/water (100:5:5, by vol) containing 10 mM ammonium acetate gave sufficient separation of the PLPC-OOH from unoxidized PLPC, but there was little separation of the different isomers **1–4**. Better separation was achieved with a mobile phase of methanol/hexanes/water (100:1:5, by vol) containing 10 mM choline chloride. Using choline chloride as an ion-pairing agent resolved the tailing peak observed when ammonium acetate was used. The best separation of the phospholipid hydroperoxides was achieved using a newly marketed HPLC column, the Discovery C-18 column from Supelco, with a mobile phase of methanol/water (95:5, vol/vol) containing no ion-pairing agent.

With the optimal chromatographic conditions, two resolved hydroperoxide peaks with retention times ($t_R$) of 14.04 and 14.97 min were observed by UV detection at $\lambda$ = 234 nm. To determine the elution order of **1–4**, compounds eluting in these two peaks were isolated. The collected phospholipid hydroperoxides in each peak were converted to the corresponding HODE methyl esters by reduction ($PPh_3$) and transesterification ($NaOCH_3$, $CH_3OH$). The HODE methyl esters are well characterized, and the chromatography of these compounds by normal-phase HPLC has been reported (23). This analysis showed that the first-eluting peak, $t_R$ = 14.04 min, contained both of the 13-substituted hydroperoxides, **1** and **3**, and the 9-*cis,trans* hydroperoxide of PLPC, **2**. The second peak, $t_R$ = 14.97 min, contained only the 9-*trans,trans* hydroperoxide, **4**.

It is possible to calculate the *cis,trans* to *trans,trans* product ratio based on the UV absorbance of each eluting peak, assuming that **3** is formed to the same extent as **4**, and that **1** is formed to the same extent as **2.** (This assumption is made based on the calculated extinction coefficients for the *trans,cis* and *trans,trans* hydroperoxides of methyl linoleate; 30.) By using a mixture of hydroperoxides **1–4** that were generated from a standard oxidation of PLPC without a hydrogen atom donor present, the *cis,trans* to *trans,trans* ratio was calculated to be 0.43 by HPLC under the optimal conditions described above. A portion of this oxidation mixture was converted to the corresponding HODE methyl esters, and the *cis,trans* to *trans,trans* ratio was determined by standard normal-phase HPLC–UV analysis. This number was also found to be 0.43, comparable with that calculated using the intact phospholipid hydroperoxides. Being able to determine the product ratio from the intact phospholipid hydroperoxides eliminates the need for converting **1–4** to the methyl esters and may be beneficial in future oxidation studies of complex phospholipid mixtures.

Having developed a chromatographic method that resolved some of the PLPC-OOH isomers, we explored analytical methods that would permit identification of oxidized phospholipid molecular species without conversion or derivatization. CIS–MS has proved to be useful for obtaining structural information for several classes of cholesteryl ester peroxides and hydroperoxides (22), and we therefore turned to CIS–MS for the analysis of intact phospholipid hydroperoxides. In the first experiments, **1–4** were isolated from unoxidized PLPC by HPLC. The collected hydroperoxides were introduced into the mass spectrometer *via* DLI as a mixture in methanol with 4 equivalents of $AgBF_4$. The resulting CIS–MS spectrum is shown in Figure 1A. The dominant ions observed are the $[M + Ag]^+$ adducts at *m/z* = 896 and 898, formed from $^{107}Ag$ and $^{109}Ag$ isotopes that are present in a ratio of ~1:1.

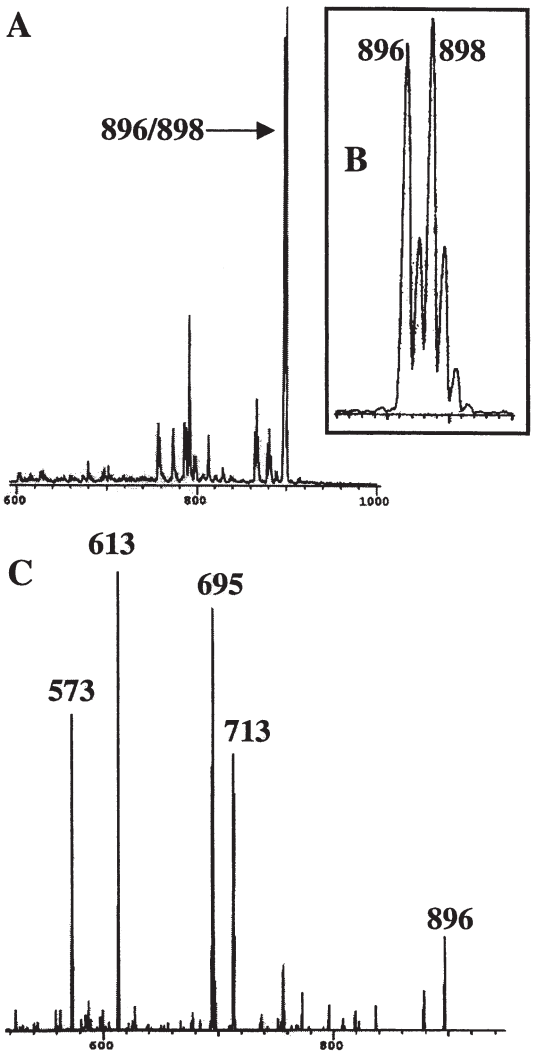
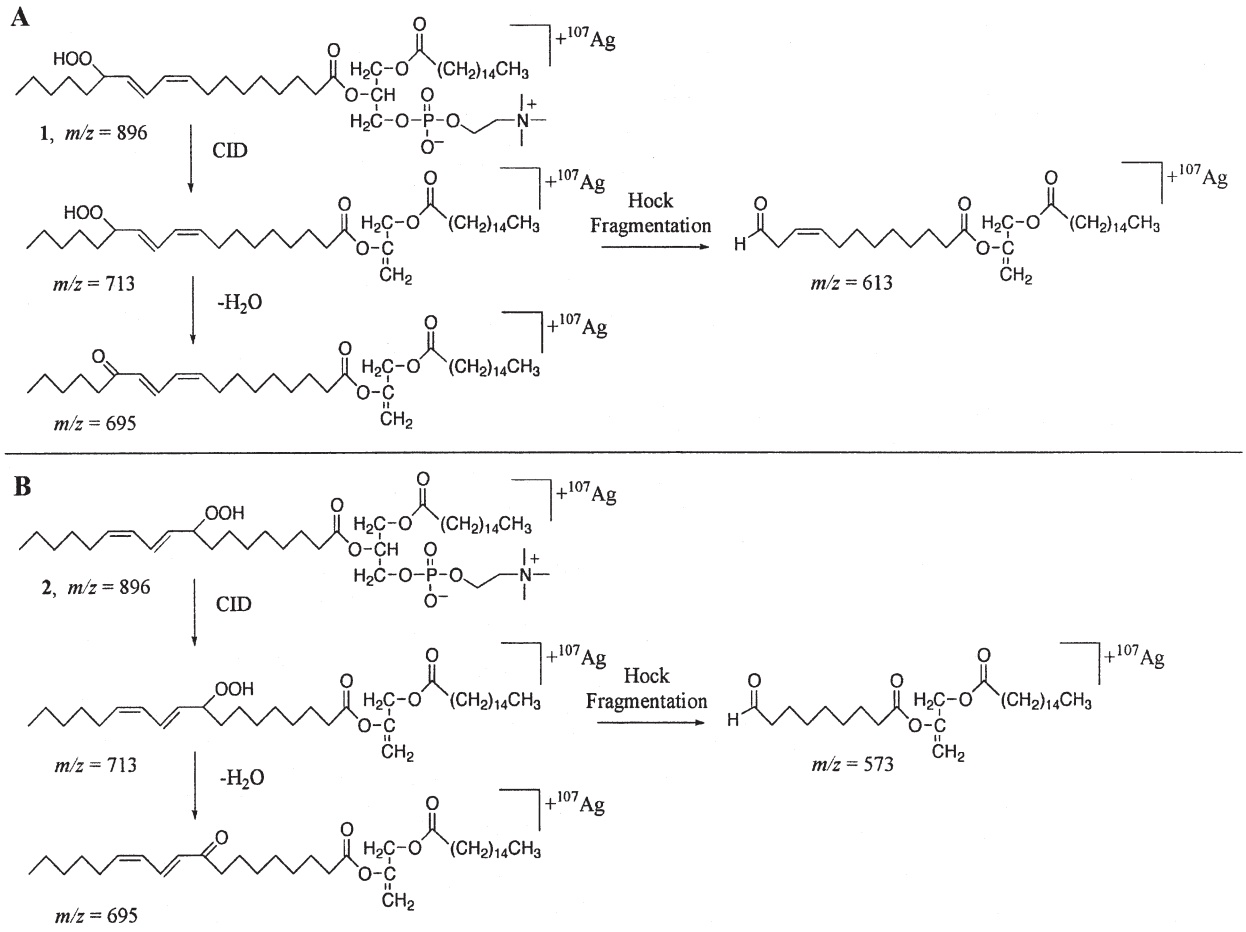


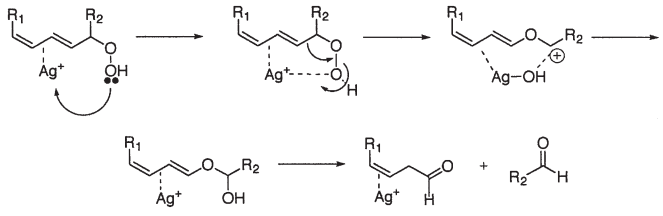

**FIG. 1.** Silver ion coordination ion-spray mass spectrum of a mixture of **1–4** obtained in direct liquid infusion experiments: (A) full scan, (B) blow-up of the $[M + Ag]^+$ region, and (C) collision-induced dissociation spectrum of *m/z* = 896.

CID experiments on the complex of **1–4** with $^{107}Ag^+$ at *m/z* = 896 gave fragment ions at *m/z* = 713, 695, 613, and 573 (Fig. 1C). The proposed structures for each of the fragment ions are shown in Scheme 2. Each fragment showed loss of the phosphocholine head group as a neutral loss of 183. The fragment at *m/z* = 713 $[M + Ag - 183]^+$ exhibits only loss of the head group, whereas the fragment at *m/z* = 695 $[M + Ag - 183 - H_2O]^+$, in




**SCHEME 2**

addition to head group loss, shows dehydration of the hydroperoxide on the side chain. We suggest that the fragments observed at *m/z* = 613 and 573 are from Hock fragmentation of the silver ion hydroperoxide complex. This fragmentation was the primary mechanism of cholesteryl ester hydroperoxide fragmentation in CIS–MS studies (22). This commonly observed fragmentation (or rearrangement) of lipid hydroperoxides is promoted by protic or Lewis acids in solution at moderate temperatures (31–34). In the collision cell of the mass spectrometer, this fragmentation is most likely catalyzed by the silver ion, a Lewis acid (Scheme 3). The Hock fragments provide valuable information about the position of the hydroperoxide on the linoleoyl side chain. The fragment ion observed at *m/z* = 613 results from collisional activation of the 13-substituted hydroperoxides **1** and **3**, while the fragment at *m/z* = 573 results from activation of the 9-substituted hydroperoxides **2** and **4**.



**SCHEME 3**

Coupling HPLC and LC–CIS–MS was achieved by either postcolumn mixing of 0.50 mM $AgBF_4$ in methanol with the HPLC effluent or by addition of $AgBF_4$ to the HPLC mobile phase to yield a 0.15 mM solution. Chromatography was carried out using the new Supelco Discovery C-18 analytical column with a mobile phase of methanol/water (95:5, vol/vol) at a flow rate of 1 mL/min. A flow splitter was inserted online so that 240 μL/min was directed through a UV detector and into the mass spectrometer while 760 μL/min was collected as waste. Typical chromatograms from the mass spectrometer for a mixture of PLPC-OOH are shown in Figure 2. Panel A shows the total ion current chromatogram for the silver ion adducts. Panel B shows the chromatogram of the 13-substituted hydroperoxides **1** and **3** that co-elute. Panel C shows the chromatogram of the 9-substituted hydroperoxides **2** and **4** that separate under these chromatographic conditions.

These chromatograms result from the mass spectrometer being operated in the SRM mode. In this mode, a specific precursor-to-product mass conversion produced at a characteristic energy in the collision cell is monitored. SRM separates the differently substituted hydroperoxides based on the difference in their Hock fragments. The elution pattern of the hydroperoxides in these SRM experiments is consistent with the elution order determined by separation of the hydroper-

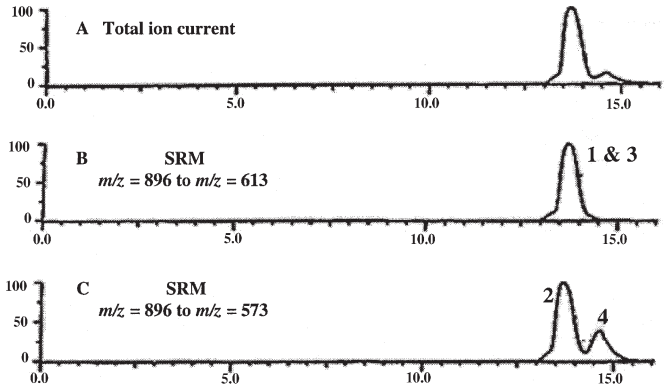



**FIG. 2.** Chromatograms of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine oxidation mixture formed when oxidized in the presence of 0.1 equivalents of pentamethylchromanol: (A) high-pressure liquid chromatography–coordination ion-spray–mass spectrometry (HPLC–CIS–MS) total ion current is a sum of (B) and (C); (B) HPLC–CIS–MS in selected reaction monitoring (SRM) mode for $m/z = 896 \rightarrow 613$; (C) HPLC–CIS–MS in selected reaction monitoring mode for $m/z = 896 \rightarrow 573$.

oxides by HPLC and subsequent conversion to the corresponding HODE methyl esters.

LC–CIS–MS experiments were also used to identify the 11-substituted hydroperoxide of PLPC, **5** (Fig. 3). The 11-substituted hydroperoxides of methyl linoleate (29) and cholesteryl linoleate (35) were only observed when the oxidation was performed in the presence of large amounts of α-tocopherol. Thus, to identify **5**, PLPC was oxidized as a thin film with 0.75 equivalents of α-tocopherol at 37°C for 24 h. By operating the mass spectrometer in SRM mode and monitoring for the conversion of **5** to its two possible Hock fragments at $m/z = 599$ and 587, the 11-substituted hydroperoxide was identified as the peak eluting at 13.77 min in Figure 3.

With the chromatographic and mass spectrometric techniques developed for analyzing the phospholipid hydroperoxides from PLPC, the hydroperoxides from another phospholipid, SAPC, were studied. The oxidation mixture from SAPC is more complex than that from PLPC because there are more sites of unsaturation on the arachidonoyl side chain. The six hydroperoxides, **6–11**, obtained from oxidizing a neat film of SAPC in the presence of a good hydrogen atom donor, PMC, are shown in Scheme 4. These products, which we previously reported (10), are formed in an analogous way to the hydroperoxides derived from PLPC.

A typical UV chromatogram obtained for **6–11** at λ = 234 nm is shown in Figure 4A, and the corresponding alcohols gave the chromatogram shown in Figure 5. The phospholipid hydroperoxides were then analyzed using LC–CIS–MS with the mass spectrometer being operated in both full-scan and SRM modes. It is of importance to note here that the starting phospholipid used in these studies was purchased from the Sigma Chemical Company. In our initial experiments, SAPC

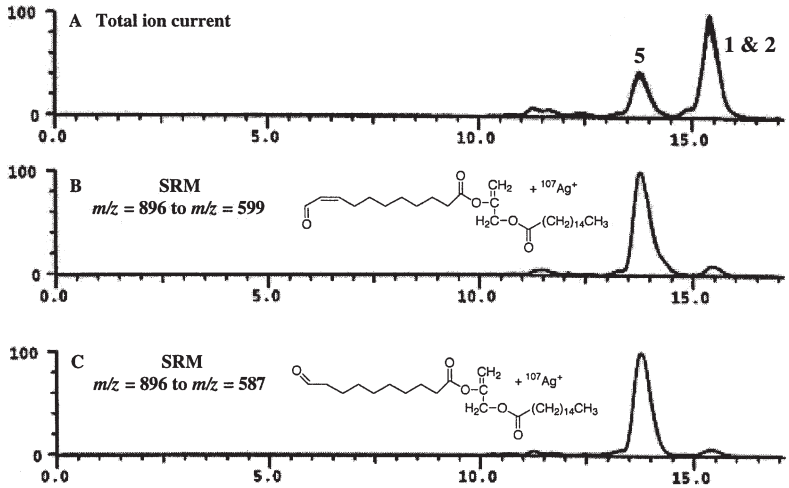
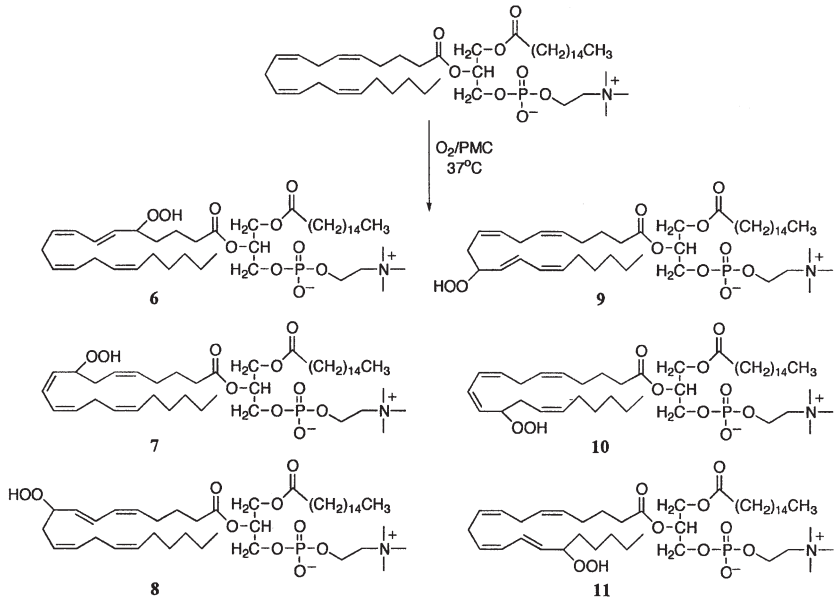



**FIG. 3.** Chromatograms of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine oxidation mixture formed when oxidized in the presence of 0.75 equivalents of α-tocopherol: (A) HPLC–CIS–MS total ion current is a sum of (B), (C), and the SRM experiments monitoring for the 9- and 13-hydroperoxides (as in Fig. 2); (B) HPLC–CIS–MS in SRM mode for $m/z = 896 \rightarrow 599$; (C) HPLC–CIS–MS in SRM mode for $m/z = 896 \rightarrow 587$. For abbreviations see Figure 2.




**SCHEME 4**

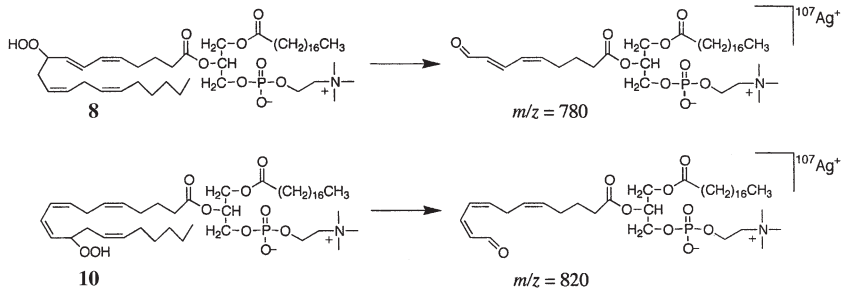
purchased from Avanti Polar Lipids was used, and a much more complex chromatogram was obtained. We speculate that this complexity was due to oxidation of 1-arachidonyl-2-stearoyl-*sn*-glycero-3-phosphatidylcholine, a positional isomer of SAPC present in the Avanti phospholipid. Avanti reports that the positional purity of their phospholipids can be as low as 80%, but Sigma reports a positional purity of 97%.

In scanning from *m/z* = 600 through 1000, the dominant ions in the LC–CIS–MS spectra for the phospholipid hydroperoxides **6–11** were *m/z* = 948 and 950. The elution order of the phospholipid hydroperoxides from the HPLC was determined using LC–CIS–MS with the mass spectrometer being operated in SRM mode (Fig. 4B–G).

Hydroperoxides **6** and **11** were easily identified as peaks **VI** and **I**, respectively, in Figure 4A because their Hock fragments are unique. However, hydroperoxides **7** and **8**, identified by SRM as peaks **IV** and **V**, have identical Hock fragments, as do hydroperoxides **9** and **10**, identified as peaks **II** and **III**. These hydroperoxides were distinguished from each other by monitoring unique, less abundant fragments from **8** and **10** in SRM mode. These fragments at *m/z* = 780 and 820, shown in Scheme 5, result from cleavage of the C9-C10 bond α to the hydroperoxide in **8** and the C12-C13 bond α to the hydroperoxide in **10**, respectively. This fragmentation pattern, analogous to that observed in the analysis of HETE and HETE PC by negative-ion FAB–MS and ESI–MS (13,16,36,37), helped identify **8** as peak **V** and **10** as peak **III**.

In the UV chromatogram (Fig. 4A), peak **V** actually appears to be two closely eluting peaks. This same pattern for peak **V** is observed in the SRM experiments monitoring for **8** [9-hydroperoxyeicosatetraenoyl-*sn*-glycero-3-phosphatidylcholine (9-HPETE PC)]. This suggested that both of these peaks, $\mathbf{V_a}$ and $\mathbf{V_b}$, are 9-HPETE PC and not a co-eluting contaminant. Unoxidized phospholipids are chiral molecules with a stereogenic center at $C_2$ of the glycerol backbone; the configuration of this center is *R*. When the phospholipid is oxidized to the hydroperoxide, diastereomers are formed because oxygen addition can occur to either face of the pentadienyl radical, and we suspected that these diastereomers of 9-HPETE PC were being separated as peaks $\mathbf{V_a}$ and $\mathbf{V_b}$.

To test the hypothesis that we were observing separation of diastereomers, compounds eluting in peaks $\mathbf{V_a}$ and $\mathbf{V_b}$ were isolated by HPLC and converted to the corresponding methyl HETE. Methyl HETE contain only one stereogenic center at the position of oxygen addition and exist as enantiomers that can be separated using chiral HPLC as reported




**SCHEME 5**

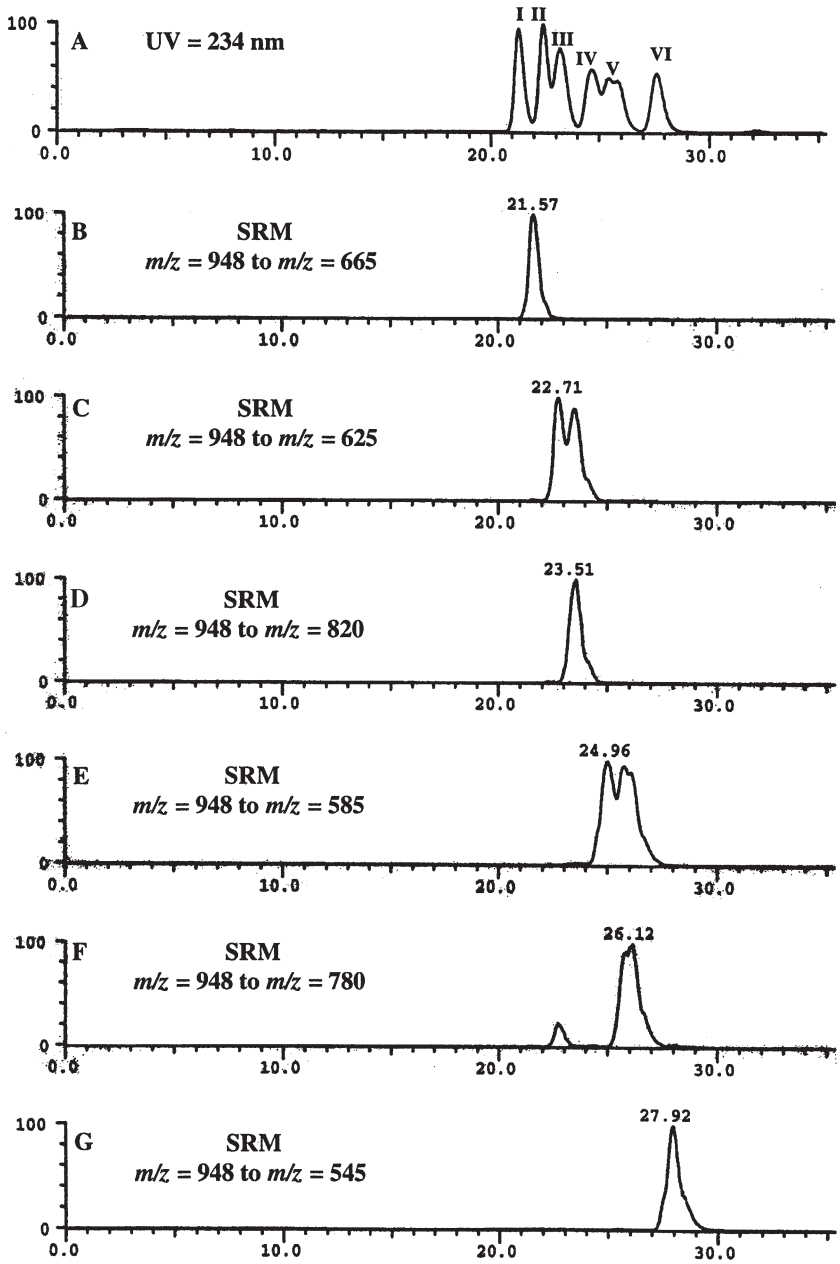



**FIG. 4.** Chromatograms of the product mixture obtained from a 24-h oxidation of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine containing 0.1 equivalents of pentamethylchromanol (mobile phase, methanol/water, 95:5, vol/vol; Discovery C-18 analytical column, Supelco, Bellefonte, PA): (A) ultraviolet detection at λ = 234 nm: **I** = 15-HPETE PC, **II** = 11-HPETE PC, **III** = 12-HPETE PC, **IV** = 8-HPETE PC, **V** = 9-HPETE PC, **VI** = 5-HPETE PC; (B–G) HPLC-CIS-MS in SRM mode. HPETE, hydroperoxyeicosatetraenoic acid; PC, phosphatidylcholine; for other abbreviations see Figure 2.

by Schneider *et al.* The methyl esters derived from collected peaks $\mathbf{V_a}$ and $\mathbf{V_b}$ were analyzed using an analytical Chiralpak AD column with a mobile phase of hexanes/methanol (100:2, vol/vol). Comparison with a standard mixture of racemic methyl 9-HETE supported the notion that the methyl ester derived from peak $\mathbf{V_a}$ was one enantiomer of methyl 9-HETE, and the methyl ester derived from peak $\mathbf{V_b}$ was the other enantiomer (Fig. 6). While it is of importance to report that the diastereomers of 9-HPETE PC are separable by RP-HPLC, we have no explanation for this fact of nature observed for the 9-HPETE PC and not the other HPETE PC.

To determine the elution order of the methyl 9-HETE enantiomers so that configurations could be assigned to the diastereomers of **8** eluting as peaks $\mathbf{V_a}$ and $\mathbf{V_b}$, it was necessary to synthesize derivatives of the compounds and determine their absolute configuration using circular dichroism (CD) spectroscopy. CD spectroscopy uses the interaction of two chromophores at the chiral center in order to define absolute config-

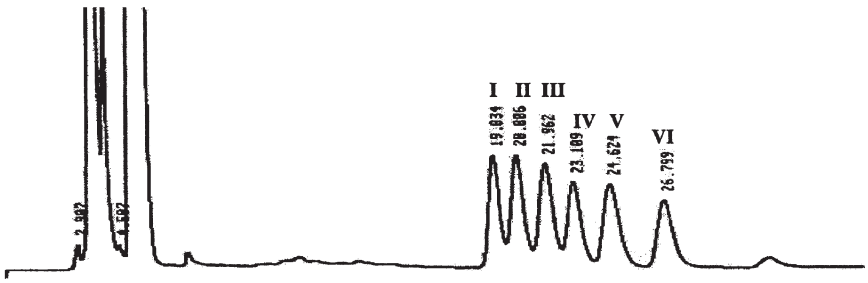



**FIG 5.** HPLC chromatogram of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine alcohol (SAPC-OH) formed by reduction of the SAPC-OOH (Scheme 4, Fig. 4) with triphenylphosphine. The elution order of the alcohols is identical to that of the hydroperoxides. For abbreviation see Figure 2.

uration. Conversion of the isolated 9-HETE methyl esters to their 2-naphthyl esters gave derivatives that provide information about the absolute configurations of the compounds (28).

The derivative of the first-eluting 9-HETE methyl ester enantiomer showed a negative first Cotton effect (CE) at $\lambda$ = 243 nm ($\Delta\varepsilon$ −50.8) and a positive second CE at $\lambda$ = 226 nm ($\Delta\varepsilon$ +41.6) (By definition, if the chirality of the electronic transition moments of the chromophore of highest wavelength to the chromophore of the lowest wavelength is counterclockwise when the molecule is represented in the Newman projection, the CD shows a negative first and a positive second CE and vice versa). Thus, this enantiomer was assigned an absolute configuration of *R*. The derivative of the later-eluting enantiomer showed a positive first CE at $\lambda$ = 243 nm ($\Delta\varepsilon$ +19.6) and a negative second CE at $\lambda$ = 226 nm ($\Delta\varepsilon$ −16.1) and was assigned an absolute configuration of *S*. Using this information, the first-eluting diasteromer of **8** ($\mathbf{V_a}$) has the *R,R* configuration, and the second-eluting diastereomer ($\mathbf{V_b}$) is *S,R*.

CIS–MS is a powerful technique for the identification of phospholipid hydroperoxides. Also, by using the new Supelco Discovery C-18 columns and a mobile phase of only methanol and water, separation of phospholipid hydroperoxide isomers has been achieved for the first time. Currently, we are using these analytical techniques to identify phospholipid hydroperoxides formed in oxidations of low-density lipoproteins.

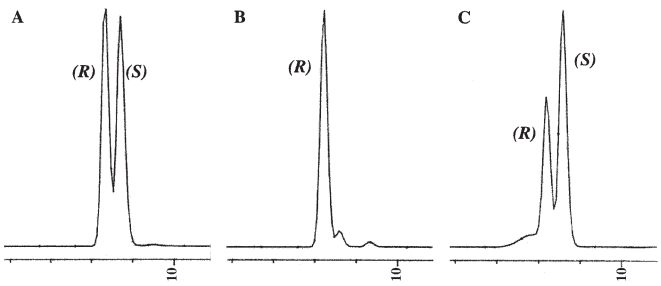



**FIG 6.** Chiral HPLC chromatograms of the 9-HETE methyl ester enantiomers (mobile phase, hexanes/methanol, 100:2, vol/vol; Chiralpak AD analytical column, Chiral Technologies, Exton, PA; UV detection at $\lambda$ = 234 nm): (A) racemic methyl 9-HETE; (B) methyl 9-HETE derived from fraction $\mathbf{V_a}$; (C) methyl 9-HETE derived from fraction $\mathbf{V_b}$. HETE, hydroxyeicosatetraenoic acid; for other abbreviation see Figure 2.

## ACKNOWLEDGEMENTS

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# Quantitative Determination of $^{13}C$-Labeled and Endogenous β-Carotene, Lutein, and Vitamin A in Human Plasma

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**ABSTRACT:** Quantitative procedures employing liquid-chromatography/particle beam–mass spectrometry (LC/PB–MS) and gas chromatography–mass spectrometry (GC–MS) were applied to the determination of the endogenous and $^{13}C$-labeled β-carotene, lutein, and retinol in plasma of a subject who consumed kale (*Brassica oleracea*) that had been grown in a $^{13}CO_2$-enriched atmosphere. All compounds were analyzed in the negative chemical ionization (NCI) mode using methane as the moderating reagent gas. β-Carotene and lutein were analyzed using LC/PB–MS applying reversed-phase high-performance liquid chromatography (HPLC) separation procedures to resolve the analytes. The concentrations of the β-carotene isotopomers in the plasma over a several-week period were determined using $^2H_8$-β-carotene as an internal standard. The total plasma concentrations of all *trans*-lutein were quantified by HPLC analysis with a photodiode array detector using β-apo-8′-carotenal as an internal standard, and the ratio of the $^{13}C$:$^{12}C$ isotopomers of lutein was determined by PB–MS. The retinol isotopomers were collected from individual HPLC fractions of the plasma extract and then analyzed as the trimethylsilyl ethers by GC–MS in the NCI mode. The $^{13}C$- and $^{12}C$-retinol isotopomers were quantified using $^2H_4$-retinol as an internal standard. These methods demonstrate the application of highly sensitive procedures employing NCI MS for the quantitative determination of carotenoids and vitamin A for the purpose of conducting metabolism studies of phytonutrients.

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The nutritional importance of β-carotene and vitamin A has been studied extensively and reviewed recently (1). In addition to the nutritional value of β-carotene as a provitamin A compound, carotenoids and related xanthophylls may possess other beneficial nutritional effects, such as those related to their antioxidant properties. Epidemiologic studies indicate that consumption of foods rich in these compounds may lower the risk of certain types of cancer (2), perhaps through the ability of some carotenoids to form stable radicals by scavenging phenoxyl radicals (3). It was shown recently in an *in vitro* study that lutein and zeaxanthin in the membranes of the retina may protect photoreceptor cells against ultraviolet (UV) light damage and chemically induced lipid oxidation (4). In this regard it has also been proposed that the consumption of foods enriched with these compounds may prevent the onset of age-related macular degeneration (5).

Several analytical methods, employing stable isotopically labeled compounds with mass spectrometry (MS) analysis, have been developed to study the metabolism of phytonutrients (6–11). For example, Pawlosky *et al.* (6) demonstrated the utility of a liquid chromatography/particle beam (LC/PB)–MS procedure to investigate the uptake of $^2H_8$-β-carotene in a human subject. In another example, gas chromatography–combustion interfaced–isotope ratio MS was used to determine plasma concentrations of $^{13}C$-lutein in human subjects who were fed the labeled xanthophyll (7). Although this procedure has a high degree of sensitivity, it is somewhat labor intensive and requires several purification steps as well as saponification and catalytic hydrogenation before mass spectral analysis. An atmospheric pressure chemical ionization LC–MS method was used to determine the bioavailability and conversion of β-carotene to retinol in humans after oral administration of $^{13}C_{10}$-retinyl palmitate and $^{13}C_{10}$-β-carotene (8).

These study designs are limited in their scope in that the compounds were administered in purified form rather than as an integral part of a food substrate. Nutrients that are obtained from foods may be absorbed and metabolized somewhat differently compared with oral administration of purified compounds. For example, Castenmiller and co-workers (12) estimated that the bioavailability of β-carotene from fresh whole-leaf spinach was only ~5% compared with the availability of the carotenoid when given as a dietary supplement. The use of intrinsically labeled foods in metabolism studies has the potential to furnish direct information regarding the uptake and metabolism of nutrients from specific foods and/or from foods that have undergone different means of preparation. The production of plant materials for such purposes by growing them in a $^{13}CO_2$ atmosphere and other methods of labeling plants are discussed in a review (13).

In support of potential human nutrition investigations, three methods utilizing negative chemical ionization (NCI) MS were advanced for the determination of endogenous

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Abbreviations: BSTFA, *N*,*O*-bis(trimethylsilyl) trifluoroacetamide; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; ISTD, internal standard; LC/PB–MS, liquid chromatography/particle beam–mass spectrometry; NCI, negative chemical ionization; SIM, selected ion monitoring; SRM, standard reference material; TMS, trimethylsilyl; TMSOH, trimethylsilanol; UV, ultraviolet.

($^{12}$C-compounds) and $^{13}$C-labeled β-carotene, lutein, and retinol (derived from the labeled β-carotene) in plasma from a subject who was fed $^{13}$C-labeled kale. In the development of these procedures, modifications were made to two independent HPLC methods employing in-line particle-beam negative ion MS for the determination of the carotenoids and xanthophylls in plasma. The determination of the retinol isotopomers from plasma was carried out using labeled internal standards by GC–MS with NCI detection.

## MATERIALS AND METHODS

*Kale*. For this study, kale (*Brassica oleracea* cv. Vates) was grown in an airtight acrylic chamber with an atmosphere of 400 ppm $^{13}CO_2$ (99%) for 7–9 d after unfolding of the first true leaves, ~5 d after emergence of the seedlings. Growth conditions were 25°C air temperature, 10°C dewpoint, and continuous light at 800 mmol/($m^2 \cdot s$) photosynthetic photon flux density (400–700 nm) from a 50:50 mixture of 400 W metal halide and high-pressure sodium lamps. Plants were cultivated in vermiculite subirrigated with a complete nutrient solution. Only plant parts above the first true leaves were harvested for feeding studies. On the basis of the mass spectrometric analysis of the carotenoids from the labeled kale, there was >98% enrichment of $^{13}$C in β-carotene and lutein, with ~60% of that in the form of the $^{13}C_{40}$ isotopomers, which were the principal isotopes used in the quantitative determination of the nutrients in the plasma in this study.

*Human study design and plasma sample collection*. The protocol for the design of the human study was approved by the Johns Hopkins University, School of Hygiene and Public Health, Committee on Human Research. Plasma from a male subject who had consumed steamed $^{13}$C-kale (400 g) was prepared from blood that was drawn periodically from the forearm. Plasma was separated from blood cells using a clinical centrifuge at 3000 × *g*, frozen in 0.5-mL aliquots, and stored at –60°C until analyzed.

*Plasma extraction procedure*. The internal standards (Fig. 1) were obtained from commercial sources and assayed for their purity using HPLC and either LC– or GC–MS. Standards were stored at –60°C in $CH_2Cl_2$. Individual quantities of the standards were added to the plasma (400 mL) in 130 mL of diethyl ether. The amounts of the internal standards that were added to the samples were as follows: $^2H_4$-retinol (125.2 ng; Cambridge Isotope Labs, Woburn, MA), $^2H_8$-β-carotene (36.56 ng; Cambridge Isotope Labs), and β-apo-8′-carotenal (146.5 ng; (Sigma, St. Louis, MO). The samples were vortex-mixed for 30 s and then allowed to equilibrate with the internal standards for 30 min at 4°C. All solvents that were used were HPLC grade. The analytes were extracted into hexane according to a previously published method (6). The hexane extracts were combined and the solvent was evaporated under a stream of $N_2$. The samples were dissolved in 200 mL of the HPLC mobile phase (65:10:25, acetonitrile/methanol/dichloromethane plus 0.1% diisopropylethylamine) used for the β-carotene analysis and stored at –60°C until analyzed. Aliquots (50 mL) were injected onto an HPLC interfaced with a PB-MS (5989 mass spectrometer, Hewlett-Packard, Palo Alto, CA).

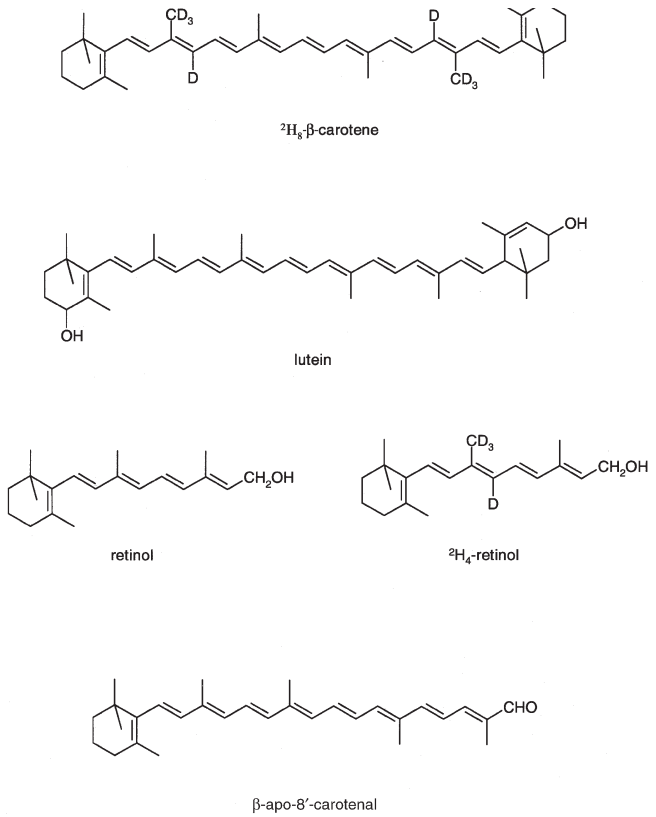


**FIG. 1.** Chemical structures of the analytes and internal standards that were investigated in the study: $^2H_8$-β-carotene, all *trans*-lutein, retinol, $^2H_4$-retinol and β-apo-8′-carotenal.

*Mass spectral analyses. (i) HPLC apparatus*. Separate HPLC procedures were used for the analysis of β-carotene and lutein. The HPLC system consisted of a Beckman Model 114M solvent delivery module equipped with a Beckman Model 421 controller that was interfaced to a Hewlett-Packard 1040M Series II ultraviolet/visible (UV/VIS) photodiode array detector. The data were stored and processed on a Hewlett-Packard 9000 Chem Station data system. Absorption spectra of analytes were recorded between 200 and 600 nm at a rate of 12 spectra/min.

*Analysis of β-carotene isotopomers by LC/PB–MS*. The principal procedures for the determination of β-carotene from plasma using PB–MS may be found elsewhere with the additional modifications that are noted here (6). Briefly, the concentrations of the endogenous β-carotene and $^{13}C_{40}$-β-carotene in the plasma were quantified using $^2H_8$-β-carotene as the internal standard. A standard reference material (SRM) of serum, which consisted of a set of samples certified for β-carotene at two different concentrations (SRM # 968c, National Institute of Standards and Technology, NIST, Gaithersburg, MD) was analyzed together with the samples and used as an internal laboratory control.

(ii) *Fraction collection and HPLC conditions for lutein analysis*. To resolve the all *trans*-lutein isomer from other naturally occurring *cis-trans* isomers of the xanthophyll present in the plasma, specific fractions of the β-carotene HPLC effluent (elution-time fractions from 5.5 to 10.5 min) containing the lutein isotopomers and β-apo-8′-carotenal (~2.5 mL) were collected. The solvent was evaporated under $N_2$ and the sample was brought up in 50 mL of the lutein mobile phase; 35 mL was injected onto a 250 × 4.6 mm, 5 µm VYDAC 201TP column (Phenomenex, Torrance, CA) using acetonitrile/methanol/hexane (86:8:5 with 0.1% diisopropylethylamine) as the mobile phase with a flow rate of 0.7 mL/min. The column temperature was maintained at 15.5°C (Thermo Haake, Paramus, NJ). A standard dilution curve of lutein was prepared using β-apo-8′-carotenal as an internal standard (ISTD) throughout a range of concentrations that incorporated typical plasma values (range: 0.023–0.19 mg/mL). Total plasma concentrations of lutein were then determined using the ratios of the UV absorbances (at 450 nm) of β-apo-8′-carotenal/lutein, which were interpolated from the standard curve ($y = 0.040x + 0.092$, $R^2 = 0.996$). The HPLC effluent was then delivered into the particle beam mass spectrometer and the concentrations of the $^{12}C_{40}$ and $^{13}C_{40}$ isotopomers were determined using the ratios of the ion abundances for $^{12}C_{40}$-lutein (*m/z* 568) and $^{13}C_{40}$-lutein (*m/z* 608).

*(iii) PB–MS conditions*. The mass spectrometer was operated in the negative ion mode using methane as the moderating gas according to a previously published procedure (6). The analytes were detected using selected ion monitoring (SIM) with the quadrupole mass filter focused to transmit the radical anions ($M^{\bullet -}$) for $^{12}C_{40}$-β-carotene (*m/z* 536), $^{13}C_{40}$-β-carotene (*m/z* 576), and the ISTD $^2H_8$-β-carotene (*m/z* 544) and for $^{12}C_{40}$-lutein (*m/z* 568) and $^{13}C_{40}$-lutein (*m/z* 608) that were produced under these conditions.

*(iv) Retinol fraction collection, derivatization, and quantification*. The HPLC fractions containing the retinol isotopomers ($^{12}C_{20}$-retinol, $^{13}C_{20}$-retinol, and $^2H_4$-retinol) were collected from the β-carotene mobile phase effluent (elution times from 5.5 to 6.5 min) for GC–MS analysis. The fractions were reduced to dryness at 40°C under a stream of $N_2$. To the residue was added 20 µL of a 1:1 mixture of *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA; Pierce, Rockford, IL) and pyridine. The solution was heated to 60°C for 30 min and the excess reagent was removed under a stream of nitrogen at room temperature. The residue was dissolved in 30 µL of isooctane containing 1% BSTFA/pyridine reagent. The plasma concentrations of $^{13}C_{20}$-retinol and the endogenous retinol were determined by obtaining the ratio of the peak area counts of the internal standard to those of the analytes. Together with the study samples and according to the procedures developed for the β-carotene analysis, the SRM 968c serum samples (NIST) were analyzed for all *trans*-retinol and included as internal controls to determine the overall accuracy of the method. A standard dilution curve of $^{12}C_{20}$-retinol was prepared throughout a range that included typical plasma concentrations of vitamin A (0.105–3.4 mg/mL) using $^2H_4$-retinol as the internal standard ($y = 0.03x - 0.04$; $R^2 = 0.999$).

*(v) GC–MS conditions*. The retinol isotopomers were analyzed on a Varian 3400 gas chromatograph (Walnut Creek, CA) interfaced to a Finnigan-Mat (San Jose, CA) TSQ-700 triple-stage quadrupole mass spectrometer. Samples (1 mL) were injected onto a 14 m × 0.25 mm DB-1701 (J&W Scientific, Rancho Cordova, CA) in the splitless mode. The injection port temperature and transfer line were maintained at 250 and 280°C, respectively. The oven was heated from 100 to 250°C at 21°C/min with helium carrier gas velocity of 70 cm/s. The retinol trimethylsilyl derivatives eluted at 8.9 min. The mass spectrometer was operated in the NCI mode with methane as the moderating gas. The quadrupole mass filter was scanned in the SIM mode for the loss of trimethylsilanol (TMSOH, 90 amu) from the respective analytes of retinol (*m/z* 268), $^2H_4$-retinol (*m/z* 272), and $^{13}C_{20}$-retinol (*m/z* 288).

## RESULTS

*Determination of β-carotene isotopomers in the plasma*. The ion current tracings depicting an illustrative example of an analysis of the three isotopomers of β-carotene ($^2H_8$-β-carotene, $^{13}C_{40}$-β-carotene, and $^{12}C_{40}$-β-carotene) from a plasma extract are given in Figure 2. Under these HPLC conditions, the carotenoids eluted at ~27 min. The somewhat earlier elution time of the $^2H_8$-β-carotene (*m/z* 544) is not unusual because under these chromatographic conditions, a slightly different chemical partitioning of the partially deuterated species takes place between the stationary and mobile phases compared with the protonated analog. The concentrations of $^{13}C_{40}$-β-carotene and $^{12}C_{40}$-β-carotene isotopomers in the plasma were calculated using the ratio of the area counts of the internal standard to that of the analyte as described previously (6). Selected time points for the determinations of the β-carotene isotopomers are given in Table 1. The levels of $^{13}C_{40}$-β-carotene ranged from below the detection limits to 0.0490 nmol/mL (Table 1). The concentration of $^{13}C_{40}$-β-carotene attained a maximum value 48 h after the subject consumed the kale. The mean value for the concentration of endogenous $^{12}C_{40}$-β-carotene in the plasma of this subject over the time period was 0.2945 ± 0.0412 nmol/mL (Table 1).

*Analysis of lutein isotopomers in the plasma*. Figure 3 depicts the UV tracing (at 450 nm) of the elution order profile of an HPLC chromatogram (C-18 VYDAC column) from a plasma extract that contains lutein and β-apo-8′-carotenal. The total concentration of all *trans*-lutein in the plasma over the time course (for the major isotopomers $^{12}C_{40}$, $^{13}C_{40}$, $^{13}C_{39}$, and $^{13}C_{38}$) was interpolated from the standard dilution curve using the ratio of peak area counts of the β-apo-8′-carotenal and lutein. The plasma concentrations for the $^{12}C_{40}$-lutein and $^{13}C_{40}$-lutein isotopomers were then determined from this preliminary value using the isotope ratios of the ions at *m/z* 568 ($^{12}C_{40}$-lutein) and *m/z* 608 ($^{13}C_{40}$-lutein) (Fig. 4). Initially, it was observed that several isomers of the

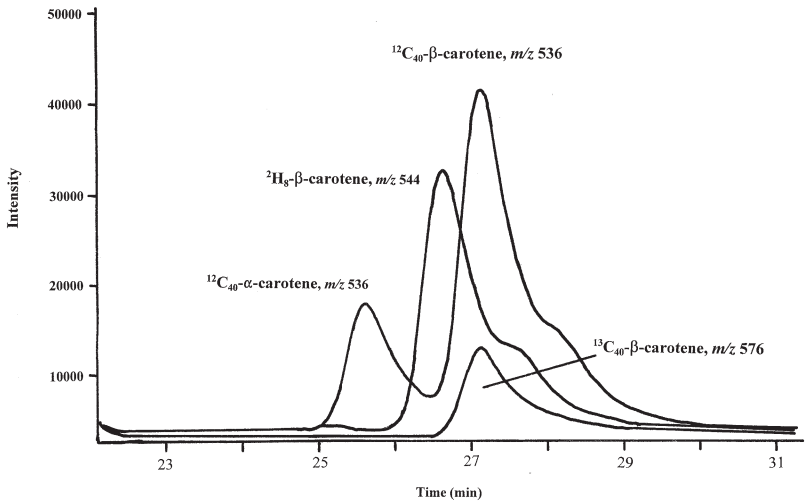


**FIG. 2.** Selected ion chromatographic tracings for $^{2}H_{4}$-β-carotene, $^{13}C_{40}$-β-carotene, and $^{12}C_{40}$-β-carotene from an 8-h plasma extract analyzed by liquid chromatography/particle beam–mass spectrometry.

xanthophyll (presumed to be various *cis-trans* isomers of lutein because of similarities in the UV profiles as well as similar chromatographic and mass spectral characteristics) eluted within a single chromatographic peak using the solvent system developed for the β-carotene analysis. One of these isomers was determined to be zeaxanthin on the basis of comparisons made with an authentic standard. The resolution of the all *trans*-lutein from the other xanthophyll isomers required an additional HPLC procedure, which was optimized to resolve the individual xanthophylls into distinct chromatographic peaks (Fig. 3). The mean plasma concentration of $^{12}C_{40}$-lutein throughout the time course was 0.1347 ± 0.0207 nmol/mL. The earliest time in which $^{13}C_{40}$-lutein was detected in the plasma was 4 h after the subject consumed the kale (Table 1) and $^{13}C_{40}$-lutein was no longer detected in the plasma after day 39.

*Determination of $^{12}C_{20}$-retinol and $^{13}C_{20}$-retinol in the plasma.* As described above, individual fraction cuts of the HPLC effluent from the β-carotene analysis were collected for the determination of retinol by GC–MS. The mean value of the plasma concentration of $^{12}C_{20}$-retinol was 1.4447 ± 0.2690 nmol/mL. The appearance of $^{13}C_{20}$-retinol in the plasma resulted from the metabolism of $^{13}C_{40}$-β-carotene. The $^{13}C_{20}$-retinol was first detected in the plasma 4 h after the subject consumed the kale (0.0052 nmol/mL). The plasma concentrations for $^{13}C_{20}$-retinol reached a maximum value at 8 h and were no longer detected beyond day 43.

## DISCUSSION

Three quantitative procedures employing MS were adapted from existing methods for the analysis of endogenous and $^{13}C$-labeled lutein, β-carotene, and retinol in the plasma of a subject who consumed $^{13}C$-labeled kale. The results reported here demonstrate the usefulness of labeled food substrates together with MS to investigate the metabolism of phytochemicals in humans. No attempt was made here to determine the bioavailability of these nutrients from the labeled kale; however, the stable isotope methods demonstrate the practicality of designing human studies to take advantage of similar approaches. A unique advantage of this study design is that it allows for the determination of carotenoids and xanthophylls derived from a single food. Such an approach may be useful, for instance, in assessing any potential effect that xanthophylls may have on β-carotene metabolism (14).

As reported previously (6), negative chemical ionization of carotenoids using methane as a moderating gas affords the superior degree of sensitivity that is required for the determination of femtomolar concentrations of these compounds in plasma. The application of this procedure has now been extended to the analysis of labeled xanthophylls, which also circulate in picomolar concentrations in the plasma. The low

**TABLE 1**
**Selected Time Course Values for the Determinations of $^{13}C_{40}$ and $^{12}C_{40}$ β-Carotene, $^{13}C_{40}$ and $^{12}C_{40}$ All *trans*-Lutein, and $^{13}C_{20}$ and $^{12}C_{20}$ Retinol from Plasma Extracts Analyzed Using the Appropriate Negative Ion Mass Spectrometry Technique[a]**

| | β-Carotene | | all *trans*-Lutein (nmol/mL) | | Retinol | |
|---|---|---|---|---|---|---|
| Hours | $^{13}C_{40}$ | $^{12}C_{40}$ | $^{13}C_{40}$ | $^{12}C_{40}$ | $^{13}C_{20}$ | $^{12}C_{20}$ |
| 0 | ND | 0.3144 | ND | 0.0307 | ND | 1.4227 |
| 4 | 0.0082 | 0.2838 | 0.0030 | 0.0650 | 0.0052 | 1.0399 |
| 8 | 0.0490 | 0.2091 | 0.1446 | 0.1613 | 0.0899 | 2.3640 |
| 24 | 0.0319 | 0.3287 | 0.0503 | 0.1621 | 0.0471 | 1.1273 |
| 48 | 0.0559 | 0.2170 | 0.0859 | 0.1276 | 0.0154 | 1.4664 |
| 432 | 0.0082 | 0.4341 | 0.0118 | 0.1577 | 0.0206 | 1.2259 |
| Mean ($n$ = 6) | | 0.2945 | | 0.1347 | | 1.4447 |
| SD (±) | | 0.0412 | | 0.0207 | | 0.2690 |

[a]ND indicates below detection limits for the method.

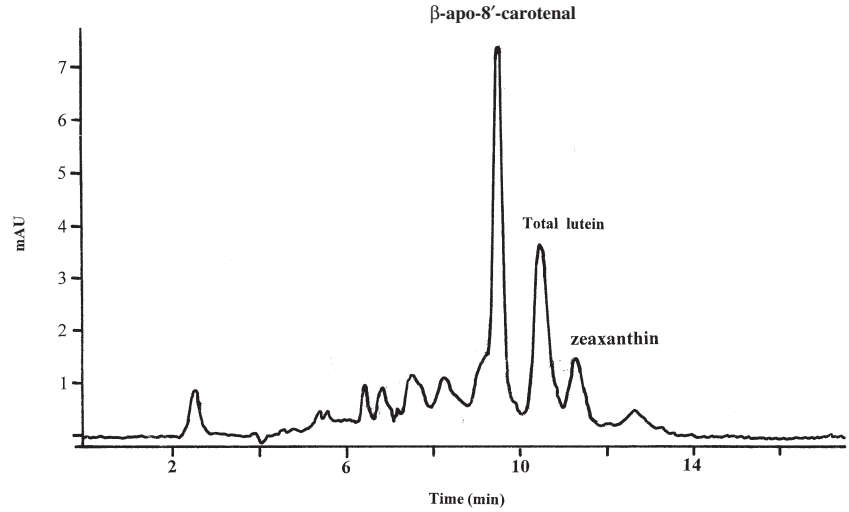


**FIG. 3.** An elution order profile on a high-performance liquid chromatography analysis of a plasma fraction extract containing all *trans*-lutein isotopomers as well as *cis-trans* isomers of lutein and β-apo-8′-carotenal. The compounds were resolved on a VYDAC column using a mobile phase consisting of 86:8:5 acetonitrile/MeoH/hexane 0.1% diisopropylethylamine 0.7 mL/min at 15°C and analyzed with a photodiode array detector (450 nm).

detection limits established for these analyses result from the highly conjugated system of double bonds that occur in these molecules, which have a high affinity for the low-energy thermal electrons that are generated in the methane plasma. This high degree of sensitivity may be required for the analysis of labeled carotenoids in plasma samples from subjects who have consumed a typical serving of labeled food. Moreover, the purification procedures and chemical workup routines do not involve an extensive degree of sample manipulation before mass spectral analysis, which also may have afforded lower detection limits (7,10).

A high degree of accuracy was noted for the determination of both β-carotene and retinol using the isotopically labeled internal standards. For example, there was only a 10% difference in the values determined using the LC/PB–MS method (0.141 mg/mL) compared with the certified value (0.157 mg/mL) for all *trans*-β-carotene in the SRM. Similarly, there was only a 7% difference in the values obtained for retinol compared with the certified concentrations. Moreover, the determinations for both β-carotene and retinol were within the 95% confidence interval established for these analytes by the Institute issuing the reference materials. In the analysis of

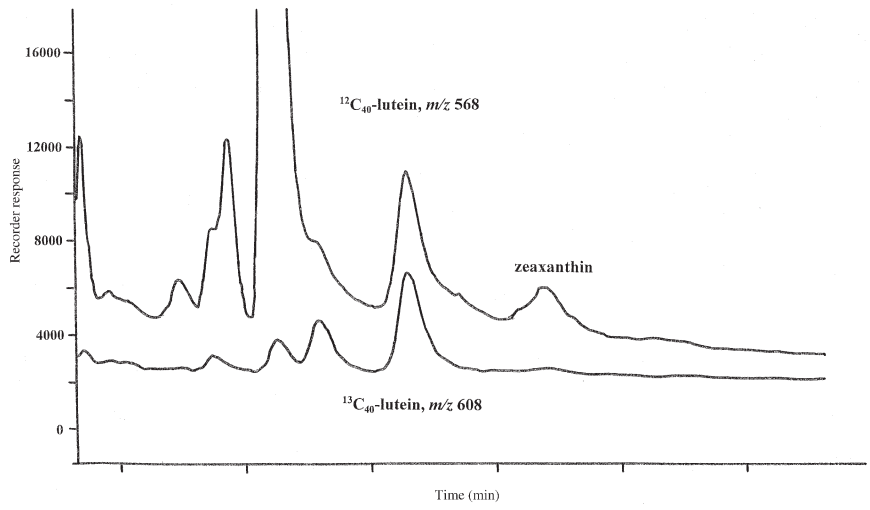


**FIG. 4.** The corresponding selected ion tracing of the plasma extract that appears in Figure 3 using liquid chromatography/particle beam–mass spectrometry analysis. The retention time for all *trans*-lutein is 10.7 min, and chromatographic peaks at *m/z* 568 and 608 represent the all *trans*-$^{12}C_{40}$-lutein and all *trans*-$^{13}C_{40}$-lutein, respectively.

the lutein isotopomers, an additional chromatographic procedure was required to resolve all *trans*-lutein from the other closely eluting *cis-trans* isomers. The resolution of these isomers was essential for establishing the precise identity of the xanthophyll and for the accurate determination of concentration of the *trans*-lutein isotopomers.

The three mass spectral procedures described herein demonstrate the practicability of adapting various analytical strategies to investigate the metabolism of phytonutrients utilizing intrinsically labeled foods. The rapid sample extraction and purification procedures coupled with the superior sensitivity of NCI MS methodology result in a quantitative method for determining trace quantities of analytes in plasma. These methods may be adapted to large-scale human or animal studies for the purpose of assessing the fate of plant nutrients in metabolism studies.

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# Comparison of the Bligh and Dyer and Folch Methods for Total Lipid Determination in a Broad Range of Marine Tissue

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**ABSTRACT:** For many studies, it is important to measure the total lipid content of biological samples accurately. The Bligh and Dyer method of extraction was developed as a rapid but effective method for determining total lipid content in fish muscle. However, it is also widely used in studies measuring total lipid content of whole fish and other tissues. Although some investigators may have used modified Bligh and Dyer procedures, rarely have modifications been specified nor has their effectiveness been quantitatively evaluated. Thus, we compared this method with that of the classic Folch extraction in determining total lipid content of fish samples ranging from 0.5 to 26.6% lipid. We performed both methods as originally specified, i.e., using the chloroform/methanol/water ratios of 1:2:0.8 and 2:2:1.8 (before and after dilution, respectively) for Bligh and Dyer and of 8:4:3 for Folch, and with the initial solvent/sample ratios of (3+1):1 (Bligh and Dyer) and 20:1 (Folch). We also compared these with several other solvent/sample ratios. In samples containing <2% lipid, the results of the two methods did not differ. However, for samples containing >2% lipid, the Bligh and Dyer method produced significantly lower estimates of lipid content, and this underestimation increased significantly with increasing lipid content of the sample. In the highest lipid samples, lipid content was underestimated by up to 50% using the Bligh and Dyer method. However, we found a highly significant linear relationship between the two methods, which will permit the correction of reported lipid levels in samples previously analyzed using an unmodified Bligh and Dyer extraction. In the future, modifications to procedures and solvent/sample ratios should be described.

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The total lipid content of biological samples is an important quantity used in many biochemical, physiological, and nutritional studies. Thus, reliable methods for the quantitative extraction of lipids from tissues are of critical importance. Natural lipids generally comprise mixtures of nonpolar components such as glycerides (primarily triacylglycerol) and cholesterol, as well as some free fatty acids and more polar lipids. Isolation, or extraction, of lipid from tissues is performed with the use of various organic solvents. In principle, the solvent or solvent mixture used must be adequately polar to remove lipids from their association with cell membranes and tissue constituents but also not so polar that the solvent does not readily dissolve all triacylglycerols and other nonpolar lipids (1). Folch *et al.* (2) were one of the first to recognize this and develop the chloroform/methanol/water phase system (the so-called "Folch" method), which, under various modifications, continues to be considered the classic and most reliable means for quantitatively extracting lipids. In the interest of economy, less exhaustive methods have been developed. By far the best known is the "Bligh and Dyer" method (3), which has become one of the most recommended methods for determining total lipid in biological tissues (4,5) and indeed has become the standard for lipid determination in many studies of marine fish (e.g., 1, 5–12) as well as for other types of samples such as milks (e.g., 13,14).

The primary advantage of the Bligh and Dyer method is a reduction in the solvent/sample ratio (1 part sample to 3 parts 1:2 chloroform/methanol followed by 1 or 2 parts chloroform) (1,3). In contrast, the Folch method employs a ratio of 1 part sample to 20 parts 2:1 chloroform/methanol, followed by several washings of the crude extract (2). Despite this solvent reduction, the Bligh and Dyer method is nevertheless thought to yield recovery of ≥95% of total lipids (1). Although the procedure was developed using cod muscle, it states (1,3) that it can be applied to any tissue containing (or modified to contain) 80% water. Hence, it has been used ubiquitously. Although the Bligh and Dyer method has undergone rigorous and favorable evaluations (e.g., 5,9,16), virtually all of these evaluations have been performed on samples containing less than 1.5% total lipid. Some studies report using a modified Bligh and Dyer method for lipid-rich samples; however, the modifications are often unspecified (e.g., 15), making the evaluation and comparison of results difficult. In other cases, investigators report the use of the Bligh and Dyer method even with samples having high lipid contents, but do not indicate that any modifications have been made. In the course of recent studies in our laboratory, we discovered that samples of a known high lipid content were greatly underestimated using the Bligh and Dyer method compared to the Folch method, although we did not detect any difference in the fatty acid composition under either method. Since much of the data published on the lipid contents of whole fish and other samples have been derived using the Bligh and Dyer method, we undertook a study to evaluate the relationship between these methods in their estimation of total lipid content.

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## MATERIALS AND METHODS

Fish and invertebrates were chosen to represent a wide range of lipid contents based on previous species estimates. A total of 36 individuals were used, which included pollock, herring, rock sole, rock fish, sculpin, octopus, and squid. Each whole animal was thoroughly ground and homogenous subsamples were taken for extraction. To increase the range of lipid contents evaluated, we also used weighed aliquots ($n$ = 9) of a homogenous mixture of ground commercial fish (originally containing 2% fat) and commercial fish oil. Weighed quantities of oil were added to produce mixtures ranging from an estimated 21 to 26% lipid. Our primary interest was to evaluate the Bligh and Dyer method compared to the Folch method, but because of the high solvent volumes used in the Folch, we also evaluated the performance of a reduced-solvent Folch using a subset of these samples. Within each method, all samples were extracted and lipid contents were quantified in duplicate.

The Bligh and Dyer extraction was performed as originally outlined using the following ratios (1,3): Briefly, 100 g sample containing (or adjusted to contain) 80 g water (as determined by oven drying separate aliquots) is homogenized with 100 mL chloroform and 200 mL methanol (monophasic system). The solution is rehomogenized with 100 mL chloroform, following which 100 mL of either distilled water (3) or weak salt solution (e.g., 0.88% NaCl or KCl) (1,9) is added. After filtration is performed under suction, the final biphasic system is allowed to separate into two layers and the lower (chloroform) phase is collected. For quantitative lipid extraction (3), the tissue residue is then rehomogenized with 100 mL chloroform, filtered, and the filtrate added to the lower phase collected. Lipid content is then determined gravimetrically after evaporating a measured aliquot of the combined chloroform phase to dryness under nitrogen (see below). As Bligh and Dyer stated (3,16), the above volumes can be scaled down, as long as the critical ratios of chloroform, methanol, and water (1:2:0.8 and 2:2:1.8, before and after dilution, respectively) and of initial solvent to tissue [(3 + 1):1] are kept identical. Thus, we followed the above procedures but reduced the scale of all components (i.e., keeping all ratios the same) for use with a smaller sample amount (4 g sample in a 40 mL conical glass centrifuge tube), to allow both centrifugation of the final biphasic system and collection of the entire lower phase for evaporation and subsequent lipid estimation. Instead of applying manual pressure (3) to the small filter cake, we performed a second chloroform wash to improve removal of residual lipid during filtration.

The Folch extractions were performed as described, using the original extraction ratio of 20 parts 2:1 chloroform/methanol to 1 part tissue, which can be done on any scale that is technically feasible (2). A weak salt solution (e.g., 0.58–0.88% NaCl or KCl) is then added to achieve a final ratio of 8:4:3 chloroform/methanol/water after including the water contained in the tissue (1,2). We also compared the original ratio against a modified version using 30 parts 2:1 chloroform/methanol to 1 part tissue (1). After verifying that the 20:1 and 30:1 solvent/sample ratios produced similar results in our samples ($n$ = 27, all <25% lipid; Fig. 1A), we analyzed the rest of the samples using only the 20:1 ratio as follows: 1.5 g tissue was homogenized with 30 mL 2:1 chloroform/methanol. Although Christie (1) reports improvement by first homogenizing with 10 mL methanol followed by 20 mL chloroform, we have tested both procedures without detecting differences (Iverson, S.J, Lang, S.L.C., and Cooper, M.H., unpublished results). The mixture was filtered and then washed several times with 2:1 chloroform/methanol, and 0.88% NaCl in water was added to the combined filtrate at a final ratio of 8:4:3 chloroform/methanol/water. Finally, we used a "reduced-solvent" Folch, where the ratios of solvent to sample were 7.5:1.0 (i.e., closer to that of the Bligh and Dyer method), but the chloroform/methanol/water ratio was kept the same (i.e., 8:4:3).

In all the above extractions (both Bligh and Dyer and Folch), the final biphasic system was centrifuged, and the entire lower phase (along with washings) was collected into a

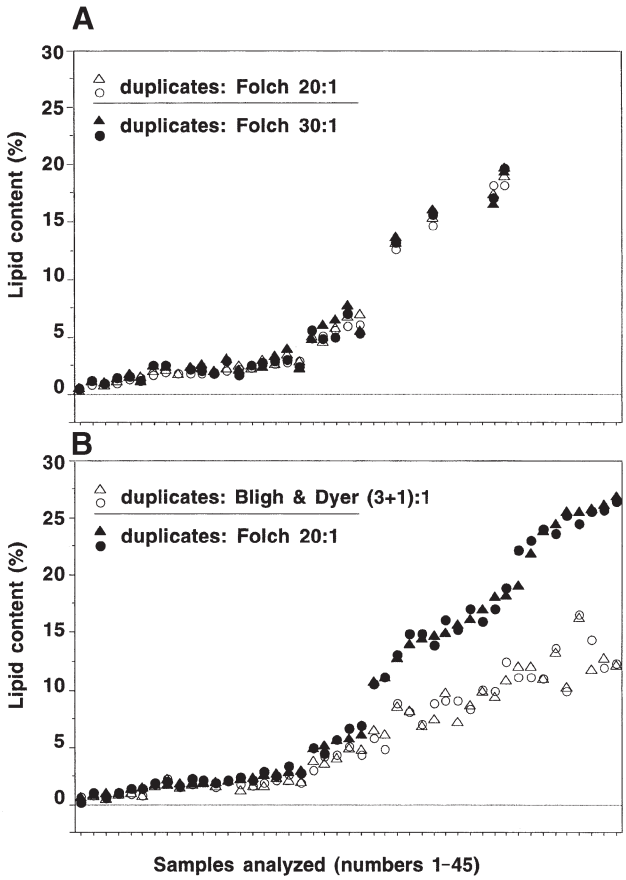


**FIG. 1.** Estimates of total lipid content determined in replicate aliquots: (A) samples ($n$ = 27) extracted using both a 20:1 and a 30:1 solvent/sample ratio Folch and (B) all samples ($n$ = 45) using the Bligh and Dyer method in comparison with the original Folch method. The last nine samples on the x-axis represent the homogenates of commercial fish and oil, which were produced to contain a range of 21–26% lipid. All samples were analyzed in duplicate in each of the extraction methods and are presented in approximate order of increasing lipid content.

preweighed glass tube and evaporated to dryness in an analytical high-speed nitrogen evaporator (24-position N-EVAP 112, Organomation Associates, Inc., Berlin, MA) fitted with stainless steel 14-in. × 19-gauge needles and equipped with a thermostatically controlled water bath maintained at 25–30°C. The nitrogen stream was continually moved so that it actively disturbed the evaporating surface of the sample until all detectable traces of solvent were gone. To remove all final traces of solvent and water, the sample tube was then wiped dry and placed in a sealed glass vacuum tube and flushed with nitrogen, and vacuum suction was applied for 5 min (BOC Edwards model RV3 vacuum pump; Crawley, West Sussex, United Kingdom). Lipid content was then determined gravimetrically. Since results of the Folch method using 20:1 or 30:1 solvent/sample ratio did not differ, we used the results from the 20:1 Folch method as the basis for comparison with and evaluation of the other extraction methods.

## RESULTS

In general, duplicate analyses within each extraction method were very consistent, although more so for Folch extractions ($n$ = 45, Fig. 1B). In samples containing <2% lipid ($n$ = 11), results for the Bligh and Dyer method did not differ from those obtained by the Folch method ($P$ = 0.150, paired $t$-test). However, for samples containing >2% lipid ($n$ = 34), the Bligh and Dyer estimates of lipid content were significantly lower than those of Folch ($P$ < 0.0001). In our nine samples of fish oil–supplemented homogenates, lipid content estimates (20.6–26.6%) using Folch extraction concurred with our estimated lipid contents (21–26%, as discussed in the Materials and Methods section); however, lipid content estimates using the Bligh and Dyer extraction were 50% lower (Fig. 1B). The next-highest lipid contents were found in herring samples ($n$ = 12, 10.7–18.6% lipid by Folch), which were estimated to be about 45% lower (6.1–11.6% lipid) using the Bligh and Dyer method.

The underestimation of lipid content by the Bligh and Dyer method increased significantly with increasing lipid content (Fig. 2A). From 0% to approximately 2% lipid, results of the two methods agreed well. However, with increasing lipid content, the deviation from the one-to-one reference line increased. We were interested in describing the predictive relationship between the two methods to allow correction of previous lipid content analyses that we had performed using the Bligh and Dyer method. Using a log–log plot, we found a highly significant linear relationship between lipid content determined by the Folch method and that determined by the Bligh and Dyer method (Fig. 2B).

The results of the reduced-solvent Folch (7.5:1.0 solvent/sample ratio) were highly correlated with both the 20:1 and 30:1 Folch ($r$ = 0.999, $n$ = 34, and $r$ = 0.987, $n$ = 27, respectively); however, the reduced-solvent method tended to underestimate lipid content as lipid content increased. In samples containing ≤3% lipid ($n$ = 19), there was no significant difference between the Folch extractions using the 20:1 vs.

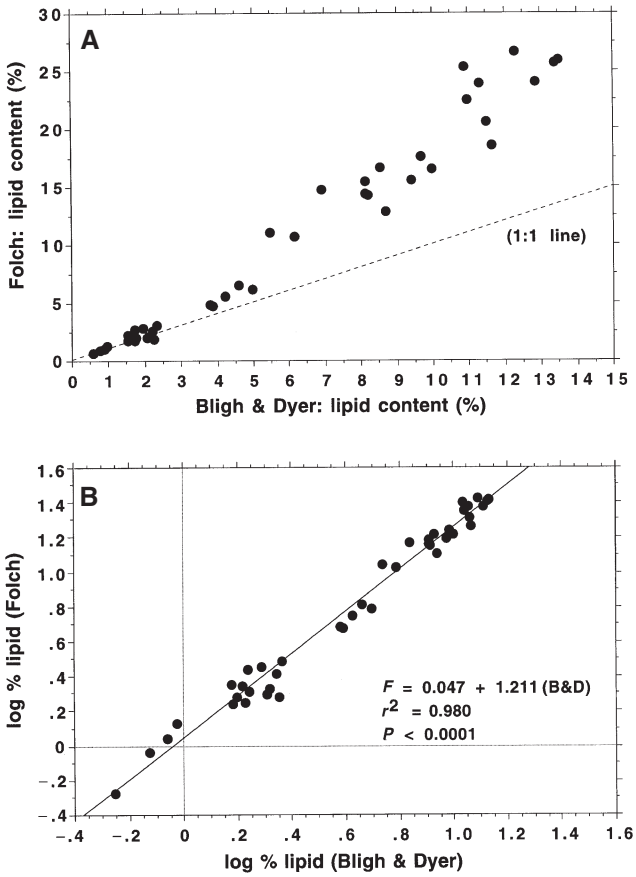


**FIG. 2.** (A) Correlation of the estimates of lipid content (duplicates averaged) in 45 samples using the Folch (20:1) vs. Bligh and Dyer methods ($r$ = 0.9834, $P$ < 0.0001); the dashed line represents the one-to-one reference line. (B) The log–log predictive relationship between estimates of lipid content using the Folch (F) vs. the Bligh and Dyer (B&D) method.

the 7.5:1 solvent/sample ratios (1.9 ± 0.16% vs. 1.9 ± 0.18% lipid, respectively; $P$ = 0.9559, paired $t$-test), but in samples containing >3% lipid ($n$ = 15), the reduced-solvent Folch significantly underestimated lipid content (10.7 ± 1.18% vs. 12.0 ± 1.30%, $P$ < 0.0001). The lipid content estimates of these same 15 samples, using the Bligh and Dyer method, were even lower at 7.2 ± 0.65% lipid. In the highest-lipid natural fish sample tested (herring), lipid content was estimated as 18.6, 16.4, and 11.6% using the 20:1 Folch, the 7.5:1.0 Folch, and the Bligh and Dyer methods, respectively.

## DISCUSSION

In the time since the Folch (2) and the Bligh and Dyer (3) methods for total lipid determination were published, there have undoubtedly been numerous modifications to both methods to improve the efficiency of lipid recovery from various tissues. However, in many publications where these methods have been used, modifications have been neither described nor validated. In other cases, investigators stated that lipids were quantified "according to" one or the other method, but they do not indicate whether any modifications were made,

implying that the methods were applied basically according to the original procedures, even though that may not have been the case. Given that many conclusions about tissue and whole-body lipid and energy values are based on published lipid contents, our purpose was to evaluate these two methods, as originally described, with the aim that investigators could evaluate previously published data and that appropriate modifications would be made and described in the future.

In numerous tests with samples containing <2% lipid, the Bligh and Dyer method has been shown to be very effective and reliable (4,5,9,16). Like other investigators (5), we found that lipid extraction using the Bligh and Dyer method produced estimates of total lipid content identical to those of Folch in samples containing <2% lipid. We also did not detect any differences in the subsequent fatty acid composition of duplicate samples extracted under either method, although this may require further investigation in very low fat samples that contain a higher phospholipid/neutral lipid ratio (e.g., alkali hydrolysis followed by methylation and fatty acid quantitation could also be used to examine any biases in total fatty acid recovery). However, in contrast to low-lipid samples, in all samples containing >2% lipid, the Bligh and Dyer method produced significantly lower estimates of lipid content, and this underestimation increased with increasing lipid content of the sample.

We have several reasons to believe that the total lipid contents of all samples were accurately determined using the Folch extraction method. First, as stated above, in low-lipid samples both the Folch and Bligh and Dyer results were identical. Second, the estimates of percent lipid in the high-lipid fish oil–supplemented homogenates, using the basic Folch extraction, agreed with our calculated lipid contents; furthermore, an increased (30:1) solvent/sample ratio Folch produced the same values. Finally, these homogenates were also analyzed for protein content (by macro-Kjeldahl), as well as dry matter (Cooper, M.H., unpublished data). The amount of dry matter not accounted for by protein and lipid in these samples was reasonably consistent with expectation at 2–4% using the lipid values obtained by Folch extractions, but was quite high (14–20%) using the lipid values obtained by the Bligh and Dyer extractions.

Bligh and Dyer (3) developed their method using fish fillets (i.e., muscle) that generally contained low levels of lipid and a high proportion of phospholipid. In whole animals and in tissue, an increase in total lipid content is due predominantly to increases in triacylglycerol. Indeed, subsets of our isolated lipid subjected to thin-layer chromatography (17) showed that the primary component in the extract was triacylglycerol (especially as lipid content increases), followed by minor amounts of more polar lipid classes. Although Bligh and Dyer (3) stated that their method could readily be applied to other biological tissues, they, as well as others, acknowledged that lipid-rich samples may require modifications. For instance, Christie (1) suggested that very lipid-rich tissues such as adipose tissue and oil seeds should be extracted first with a nonpolar solvent such as diethyl-ether or chloroform, after which the remaining lipid could be recovered effectively using Bligh and Dyer methods. However, this appears to have often gone unrecognized. The total yield of lipids may be more reduced than most investigators have suspected, especially given the widespread use of apparently unmodified Bligh and Dyer extractions for whole fish and other tissues. Even in samples containing 2–10% lipid (which is common for many marine fish and invertebrates), underestimation will still be a significant problem (e.g., Fig. 1), and this has likely been neglected.

The reduced efficiency of the Bligh and Dyer method with increasing tissue lipid contents might be explained from several standpoints. One cause of reduced lipid yield at high lipid concentrations could be the limited solubility of the predominantly nonpolar lipids, such as triacylglycerols, in the seemingly relatively polar solvent solution (1:2 vol/vol chloroform/methanol) employed in the Bligh and Dyer method, which was designed chiefly to extract phospholipid efficiently. However, although the initial solvent ratios are different in the Bligh and Dyer vs. the Folch methods, they do not result in measurably different contents of methanol in the final organic (chloroform) phase (e.g., 16). Hence, this is not likely to be a significant factor. Smedes and Thomasen (16) found that the absorption of the organic phase by the tissue was one of the main causes of incomplete lipid yield. Relatively constant amounts of the organic phase are absorbed by the tissue such that using greater volumes of organic-phase solvents reduces the fraction of the organic phase that is lost in this manner (16). When tissues with increasing lipid contents are extracted (using the same volumes of solvents), the lipid concentration in the organic phase should also increase, assuming that limits of solubility are not reached. This would result in increased loss of lipid in the fraction of organic phase absorbed by the tissue, causing a reduction in final lipid yield. Thus, in addition to maintaining critical solvent and water ratios, perhaps the most important consideration is simply the ratio of solvent to dry-weight sample (and expected fat content), as even with the Folch method, a reduced ratio produced significant underestimates of lipid content.

Our results do indicate that all methods used to estimate lipid contents were highly correlated. Fortunately, there is a highly predictable relationship between the Bligh and Dyer and Folch methods (Fig. 2B), potentially allowing correction of reported values from previous analyses that used an unmodified Bligh and Dyer extraction. It may also be the case that investigators have used a modified Bligh and Dyer extraction employing an increased solvent/sample ratio that produced reliable results and have simply not stated this. It will be important in the future that investigators specify modifications to any of these procedures, especially the precise solvent/sample ratio used. For instance, although an increase in the solvent/sample ratio (i.e., to 30:1) from the original Folch did not appear to alter the estimated lipid content significantly (Fig. 1A), we would not recommend making this assumption for tissues containing greater than 25% lipid (i.e. adipose tissue, milks of many species) unless verified. In such samples,

a further increase in the solvent/sample ratio and/or further multiple extractions may be necessary for quantitative lipid evaluation (e.g., 1), as we have found for marine mammal milks (Iverson, S.J., personal communication).

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REVIEW

# Factors Affecting the Storage and Excretion of Toxic Lipophilic Xenobiotics

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**ABSTRACT:** Lipophilic toxins have been introduced into the environment both as functional compounds, such as pesticides, and as industrial waste from incineration or the manufacture of electrical transformer components. Among these substances are compounds that are carcinogenic and that affect the endocrine system. Accidental high exposures of humans to some lipophilic toxins have produced overt disease symptoms including chloracne and altered liver function. These toxic materials have been the recent focus of international effort to reduce or eliminate classes of halogenated hydrocarbons from the environment. Evidence of the widespread distribution of lipophilic toxins in the biosphere has been obtained by analyses of human tissues and human milk. The principal route of entry of lipophilic toxins into humans is through the food chain, and most of them are stored in adipose tissue. A common route of excretion is in bile, but there is also evidence of nonbiliary excretion into the intestine. Enterohepatic circulation of many of these compounds slows their removal from the body. Substances that interrupt the enterohepatic circulation of compounds that enter the intestine by the biliary and nonbiliary routes increase the rate of their removal from the body and reduce their storage half-lives. Reduction in body fat, along with these dietary substances that interrupt enterohepatic circulation, further enhances the excretion rate. Areas for further research include optimizing regimens for body burden reductions, understanding the nature of nonbiliary excretion, and following the effects of tissue redistribution during loss of body fat.

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## BACKGROUND

Many lipophilic xenobiotic compounds enter the body and are deposited in adipose depots and other tissues. Several classes of these chemicals that are known to contribute to the risk of cancer and death enter the environment intentionally as pesticides or unintentionally as industrial by-products. Accidental exposure to high levels of some lipophilic toxins results in chronic disease affecting the liver and skin. An enormous amount of research has focused on the toxicity, carcinogenicity, developmental effects, endocrine effects, and other metabolic alterations by these compounds. For most of these compounds, a "no-effect" level has not been established. These substances have been detected in the tissues of essentially all humans and animals and may contribute to the risk of disease or altered development. The chemical stability of many of these xenobiotics has resulted in environmental accumulation and increasing concentrations in people and animals through dietary and respiratory intakes.

The presence of lipophilic toxins in the environment has received a high level international attention, scientific study, and governmental action. The awareness of the ubiquitous nature of toxic lipophiles has resulted in efforts to minimize their introduction into the environment. There also have been sporadic efforts to intervene at the level of the individual to reduce toxicity.

This paper reviews these interventions to help determine their potential utility as a complementary or alternative approach to environmental efforts to manage lipophilic toxins. The sections that follow first give a brief overview of the types of materials and the current environmental concerns. In addition, brief views of the absorption, tissue distribution, metabolism, and excretion of some representative compounds are presented to give a picture of the paths that lipophiles follow as they enter, move within, and ultimately leave an organism. Although the behavior of the compounds varies among classes and individual molecular species, it is possible to make some generalizations that help establish a basis for consideration of methods of intervention.

### Recent Efforts to Change the Environment

The United Nations (U.N.) sponsored a study of the presence and sources of lipophililic toxins in the environment and concluded that global production of 12 of these materials should be reduced or eliminated. The study led to a multinational treaty imposing reductions in the use and production of these persistent organic pollutants (POP). In May 2001, representatives of 127 countries including the United States agreed to this treaty. The treaty specifies the control of eight pesticides [aldrin, chlordane, DDT (dichlorodiphenyl-trichloroethane), dieldrin, endrin, heptachlor, mirex, and toxaphene], two industrial chemicals [polychlorinated biphenyls (PCBs), once used in electrical transformers; and hexachlorobenzene, formerly used in the manufacture of synthetic rubber and also as a fungicide], and two by-products of combustion and industrial processes (dioxins and furans). Structures illustrating the 12 POP that are the subject of the treaty are given in Figure 1. All of these substances are characterized by carbon-chlorine

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Abbreviations: Ah, arylhydrocarbon; DDE, 1,1-dichloro-2,2-bis(chlorophenyl)ethylene; DDT, dichlorodiphenyl-trichloroethane; PBB, polybrominated biphenyl; PCB, polychlorinated biphenyl; POP, persistent organic pollutant; SPE, sucrose polyester; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

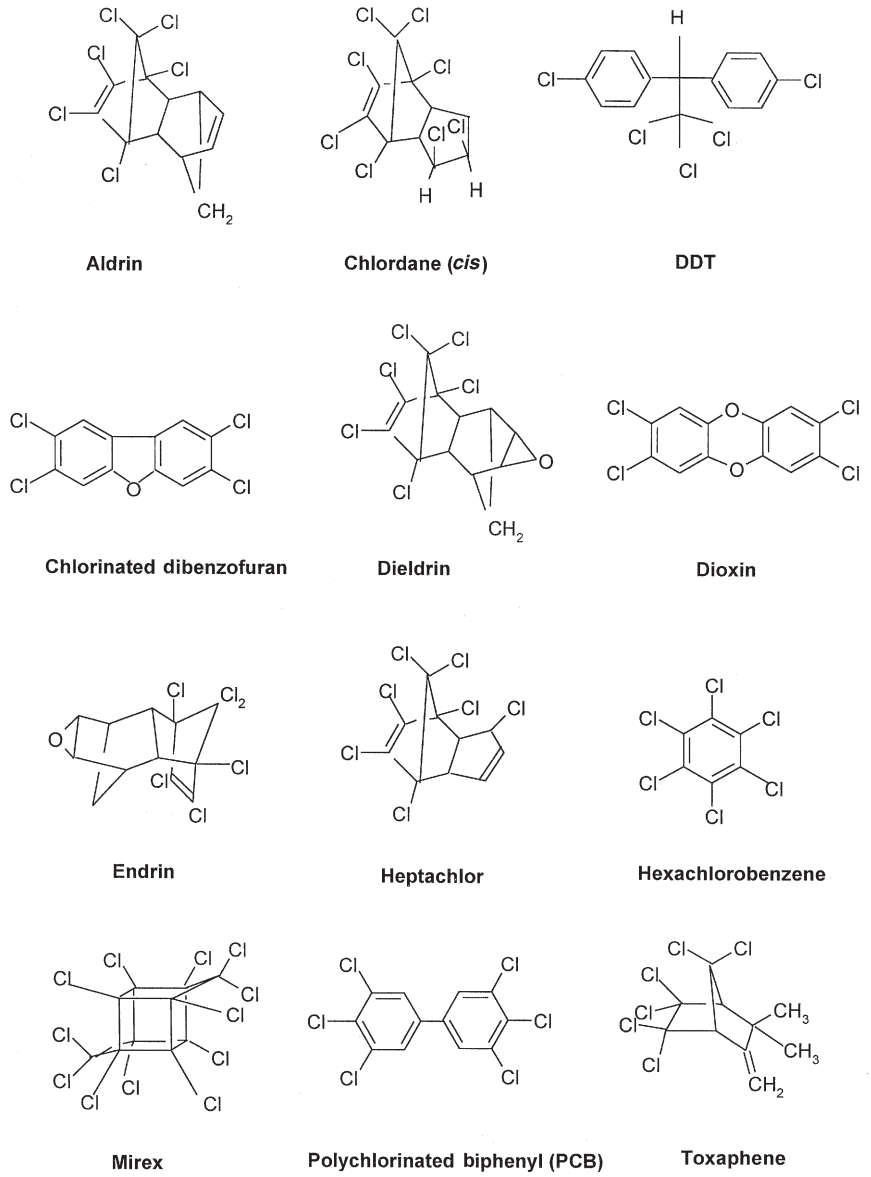



**FIG. 1.** Persistent organic pollutants included in the United Nations treaty.

bonds with stabilities that result in very long half-lives in the environment. Because of their stability and lipophilic character, these materials ascend the food chain from plants to deposition in fat depots and other tissues of higher organisms.

The principal focus for the international management of these materials in the environment has thus become that of elimination and reduction at the sources rather than intervention in individuals or populations with high levels of the materials. This approach does not affect the levels of toxic lipophiles that have already been deposited in the environment. An additional strategy is to find ways to hasten elimination of these substances from the body; to that end, studies have addressed the metabolism and excretion of many of these substances.

This interventional approach is key. The environment and the tissues of virtually all people in all countries already have a considerable burden of these persistent compounds, and it would be desirable to intervene to favorably alter their metabolic course. Finding the best ways to reduce body burdens of these materials is important since there is no consensus about the concentrations in tissues that might result in long-term detrimental health effects. For many of these compounds, a no-effect concentration has not been determined, and the possibility remains that any tissue concentration could increase the risk of disease. In addition to dealing with low-level, long-term exposure, there is a need for optimal treatment of the effects of acute exposure, such as that resulting from accidental ingestion or environmental accidents.

We review here interventions that could affect the absorption, distribution, metabolism, and/or excretion of lipophilic xenobiotic substances. These substances include the chlorinated compounds (POP) cited in the U.N. treaty as well as other lipophilic substances of known or putative toxicity.

## Lipophilic Toxins in the Environment

Numerous studies have determined the presence of various lipophilic materials in the environment and in humans. Since it is beyond the scope of this review to consider studies of lipophilic toxins in the environment, several studies are cited to illustrate the widespread distribution.

One of the most important and most studied substances that provide evidence for the widespread distribution of lipophilic toxins is human milk. A review by Jensen (1) documented the levels of organochlorine compounds including DDT and other contaminants in milk in samples from many countries. Levels in human milk are markedly higher than those in dairy milk as would be predicted from the human position at the top of the food chain. Substances other than DDT also appear in human milk. Hofvander *et al.* (2) reported measurable levels of DDT, DDE (the principal metabolite of DDT), PCBs, dieldrin, and hexachlorobenzene in human milk in Sweden. The primary concern about these toxins in human milk is their potential effect on children's health and development (3,4).

PCBs were used extensively as coolants and lubricants in electric transformers prior to 1977. Although their use was banned that year in the United States, they remain in the environment because of their resistance to chemical decomposition. Evidence for slow disappearance from the body was reported by Wolff and coworkers (5), who longitudinally followed PCB concentrations in capacitor manufacturing workers after PCB use at their manufacturing facility was discontinued. The levels of organochlorine pesticides and PCBs in butter from 23 countries were studied by Kalantzi and coworkers (6). They found a wide range of concentrations, with levels of DDT and its metabolites being highest in countries where this pesticide is still in use. Current levels of PCBs in chicken and pork in Belgium were found to be remarkably high—12% of samples contained more than 50 ng of PCBs/g of fat (7). These levels were unrelated to the contamination of animal feed cited below and were attributed to recycling of fat into animal feed.

The ubiquitous nature of POP is also seen in the very high levels of organochlorines from autopsy samples from Greenland Inuits who consumed high levels of sea mammal fat containing these substances (8). This finding is consistent with an environmental path that begins with an industrial source and ends with humans at the top of the food chain. Another view of the food chain was provided by a study of Kelly and Gobas (9). Organic pollutants in Arctic terrestial animals were traced from lichen to caribou and finally to wolf. They reported evidence for the biomagnification of hexachlorocyclohexane and tetrachlorobenzene *via* this food chain.

Environmental accidents have occurred that have contaminated geographical areas or groups of people. Polybrominated biphenyl (PBB) fire retardants were accidentally introduced into cattle feed that resulted in the contamination of Michigan dairy products and essentially all of the population of Michigan (10). Animal feed was contaminated by PCBs, dioxins, and furans in Belgium in 1999 (11). These exposures resulted in continuing surveillance programs to assess the effects of the toxins on health. In Japan in 1968, cooking oil contaminated with dioxins produced symptoms of chloracne, malaise, and joint pain that were designated as Yusho disease (12). A similar example was the tragic contamination of cooking oil with PCBs in Taiwan in 1979 (13).

Polynuclear aromatic compounds (polycyclic aromatic compounds) are another class of lipophilic toxins/carcinogens. Substances such as benzo(α)pyrene are produced in fossil fuel emissions and are carcinogenic in animals and humans (14,15). Compounds in this class are also found in meats cooked over coals or open flames. Two examples of this class of lipophilic toxins, benzo(α)pyrene and 7,12-dimethylanthracene are shown in Figure 2.

Phthalate esters are plasticizers that are present in numerous plastic/polymeric products around the globe. Phthalate esters are hepatocarcinogens in rats, presumably acting through peroxisome proliferation (16). The relevance of this effect to cancer in humans remains uncertain (17,18). Given the ubiquitous nature of this class of lipophilic compounds, the potential risks of phthalates remain a subject of concern and study (19). A commonly used phthalate ester plasticizer is presented in Figure 2.

These examples represent numerous studies of the widespread distribution of lipophilic toxins. They point out not only the global nature of the problem but also the extent of penetration into the biosphere.

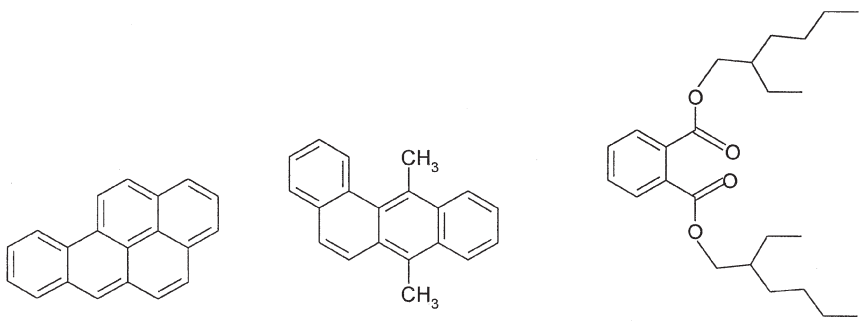



**FIG. 2.** Examples of polycyclic aromatic hydrocarbons and a commonly used phthalate ester plasticizer.

## Toxicity and Carcinogenicity

The results of studies sponsored by the United Nations and the subsequent treaty based on these studies are evidence of consensus about the risk to health of POP in the environment. Chlorinated hydrocarbon pesticides, PBBs, and the class of polycyclic aromatic hydrocarbons are included in the list of substances "reasonably anticipated to be human carcinogens" in the U.S. Department of Health and Human Services *9th Report on Carcinogens* (20). That report upgraded dioxins to the category of "known human carcinogens." Some examples of the many studies that have addressed the toxicity and carcinogenicity of lipophilic compounds are given below.

Among the first reports was that of Fitzhugh and Nelson (21), who studied the oral toxicity of DDT in rats. Mukerjee (22) reviewed the effects of dioxins, which are among the most toxic of the lipophiles, and noted the chloracne, changes in liver function, and associations of increased risk of cancer. Hexachlorobenzene promotes hepatocarcinogenesis in rats (23). Polycyclic aromatic hydrocarbons have been studied extensively and have been shown to cause DNA damage (see, e.g., 24) and to be associated with increased cancer risk (see, e.g., 14,25,26). Estrogenic effects of organochlorine compounds have been suggested to increase the risk of breast cancer, although recent studies have not supported this hypothesis (27). There are indications that other endocrine effects of these compounds may adversely affect health (28).

## Absorption, Distribution, Metabolism, and Excretion

### *Absorption*

Oral ingestion is generally the principal route of entry for lipophiles that ascend the food chain. Absorption by inhalation or through the skin is therefore of less importance when considering methods for intervening in the absorption process.

Studies of oral absorption of toxic lipophiles have utilized techniques and mechanisms associated with the absorption of dietary triacylglycerols, fat-soluble vitamins, and cholesterol. Dietary lipids enter the small intestine, are emulsified to small droplets in the presence of detergent bile salts, are hydrolyzed into more polar products (fatty acid and 2-monoacylglycerol) by pancreatic lipase, and are incorporated into mixed micelles with bile salts. Dietary fat increases the absorption of some lipophilic compounds through the formation of these mixed micelles that can effectively solubilize such compounds as cholesterol and vitamin E. Micelles transport not only the lipid digestion products but also lipophilic solutes to the enterocyte. In the enterocyte triacylglycerols are synthesized and incorporated into lipid-based particles that are stabilized with protein (chylomicrons). These chylomicrons are transported in lymph, which can be studied in thoracic duct-cannulated animals. Chylomicrons carry most lipophilic compounds that are absorbed from the intestine. They enter the blood circulation and are partially degraded to remnant particles by the action of lipoprotein lipase. Some of the components of the chylomicrons and their remnants are delivered to peripheral tissues prior to their uptake by the liver.

The nonpolar character of toxic lipophiles suggests a pathway of absorption and transport similar to that of the dietary fats and fat-soluble vitamins. Studies of the absorption of halogenated hydrocarbons are consistent with this pathway. Some studies of toxic lipophile absorption are summarized in Table 1.

### *Distribution*

Understanding the distribution of lipophilic toxins among tissues is an important area of research and has been the subject

**TABLE 1**
**Studies of the Absorption of Toxic Lipophiles[a]**

| Study design | Results | Reference |
|---|---|---|
| Lymph was collected from rats dosed with [$^3$H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin. | 30% of dose appeared in lymph chylomicrons in 24 h. | 29 |
| [$^{14}$C]DDT in sunflower oil was dosed to thoracic duct-cannulated rats. | 63% of dose was in 72-h lymph collection, and 9% in feces. DDT was transferred to other lipoproteins from chylomicrons. | 30 |
| DDT was administered to rats in corn oil or in ethanol and the appearance in lymph was measured. | DDT absorption from corn oil was twice the absorption from ethanol as the vehicle. | 31 |
| [$^{14}$C]DDT, benzo(α)pyrene, octadecane, and hexadecane were administered to rats with thoracic duct cannulae. Chylomicrons were assayed and injected into recipient rats. | The compounds appeared in the triacylglycerol phase of chylomicrons. The labeled compounds in injected chylomicrons were taken up by high density lipoprotein in recipient animals. | 32 |
| Lymphatic absorption of a moderately lipophilic PCB congener was studied in sheep. | There was little lymphatic absorption. | 33 |
| Pregnant rats were fed hexachlorobenzene with high-fat and low-fat diets. Adipose tissue was assayed. | The amount of hexachlorobenzene found in the high-fat group fat was twice that of the low-fat group. | 34 |
| [$^3$H]Benzo(α)pyrene absorption was studied in rats with cannulated bile ducts and mesenteric lymph ducts. | Low concentration in lymph relative to bile was explained by metabolism of the benzo(α)pyrene to polar metabolites in the enterocyte and absorption *via* the portal vein. | 35 |

[a]Abbreviations: DDT, dichlorodiphenyl-trichloroethane; PCB, polychlorinated biphenyl.

**TABLE 2**
**Studies of the Tissue Distribution of Toxic Lipophiles[a]**

| Study design | Results | Reference |
|---|---|---|
| Autopsy samples from Greenlanders who consumed sea mammals were analyzed. | Chlorinated pesticides and PCBs accumulated in the liver, brain, and fat. | 8 |
| Rats were injected intravenously with [$^{14}$C]-2,3,7,8-tetrachlorodibenzo-*p*-dioxin. | Radioactivity was found in liver, white and brown adipose tissue, thyroid, and adrenals. | 42 |
| Octachlorodibenzo-*p*-dioxin was dosed orally to rats. | There was little conversion of the parent compound to metabolites. Most deposition was in adipose tissue, liver, and skin. | 43 |
| Mice lacking inducible hepatic binding protein, CYP1A2, were dosed orally with dioxin, dibenzofuran, and PCB. | Dioxin and dibenzofuran uptake by the liver was reduced in the knockout animals. | 44 |

[a]For abbreviation see Table 1.

of numerous studies. Although the distribution varies greatly among the compounds of interest, some generalizations can be made. Lipophilic toxins are transported in lipoproteins (36) but may be associated with albumin (37). Uptake and retention of lipophiles and their lipophilic metabolites depends on the lipid content of the tissue or compartment (38). The amount of uptake by the liver can be determined by the induction of systems such as the arylhydrocarbon (Ah) receptor and binding to the cytochrome P450 complex as in the case of dioxins (39). As seen in a study of dioxin pharmacokinetics, the determinants of the compound's disposition include lipophilicity, liver binding elements, diffusion-limited tissue distribution, and metabolic elimination (40). Presumably these determinants apply in varying degrees to most of the lipophilic toxins. The most lipophilic substances are absorbed *via* the lymph, are carried in lipoproteins, accumulate in adipose tissue, and are taken up by the liver and other tissues. Evidence that retention in adipose tissue influences the distribution to other tissues was seen in a study by Rozman *et al*. (41). In rats, hexachlorobenzene excretion decreased with increasing body weight and fat. Studies that illustrate the uptake by fat and liver are listed in Table 2.

### *Metabolism*

The persistence of many lipophilic compounds in the body reflects resistance to metabolism and provides reason for consideration of interventions to increase their rate of elimination. The metabolism of lipophilic toxins varies among the classes and individual compounds. Individual congeners within classes have a wide range of elimination rates (45). People who ingested cooking oil contaminated with PCBs were studied to determine rates of elimination (46). There was a wide range of rates among the individual PCB congeners. In general the hexa- and heptachlorinated congeners were eliminated much more slowly than the tetra- and pentachlorinated compounds. The study was consistent with slower elimination of slowly metabolized congeners. Half-lives for pentachlorinated congeners ranged from 3 to 24 mon. Other classes of lipophilic toxins also are retained for long periods in the body. Dioxins and dibenzofurans have been estimated to have half-lives that range from 4 to 12 yr (47). Half-lives of 12.9 to 28.7 yr have been estimated for PBBs in women (48).

The conversion of lipophilic compounds to more polar species is a common metabolic path for many substances. Aryl hydroxylase catalyzes the hydroxylation of polycyclic aromatic hydrocarbons to produce species that are mobilized for transport in the blood, bile, and urine (49). PCBs and other halogenated hydrocarbons are metabolized by cytochrome P450-dependent monooxygenases to form more hydrophilic species (e.g., 50). The transformation of lipophilic xenobiotics by cytochrome P450 and other systems includes both activation and detoxification, and there have been extensive reviews of these metabolic pathways (51–54).

Hexachlorobenzene and pentachlorobenzene metabolism was studied in the rat after 13 wk of dietary exposure (55). In this study of the more polar derivatives in urine, pentachlorophenol and tetrachlorohydroquinone were formed in addition to sulfur and glucuronide derivatives.

A review of dibenzodioxins discussed the difference in metabolism of the congeners and concluded that the 2,3,7,8-chlorinated species has high toxicity due in part to affinity for the cytosolic Ah receptor protein (39). Another tetrachloro dioxin congener (1,2,7,8-tetrachlorodibenzo-*p*-dioxin) was shown to be converted to polar species in the rat (56). Glucuronides and diglucuronides of the hydroxy derivatives of the congener were identified.

Of relevance to this review are the lipophilic substances that are slowly metabolized to more hydrophilic metabolites and/or those with principal metabolites that are highly lipophilic and slowly excreted. The persistence of organic pollutants in the environment is the result of their stability and is reflected in resistance to chemical alteration by reactions including those that are enymatically catalyzed in the intact organism.

### *Excretion*

*Biliary and nonbiliary*. Lipophilic xenobiotics are excreted in feces and urine. Fecal excretion is the principal route for unchanged lipophilic compounds and their lipophilic metabolites, whereas urinary excretion is the path of more polar metabolites. Biliary excretion can account for a large portion

of the fecal elimination of many compounds; however, there is evidence that some lipophilic substances follow a nonbiliary route.

The excretion of hexachlorobenzene was studied by Ingebrigtsen and coworkers (57). In bile duct-cannulated rats dosed intragastrically with [$^{14}$C]hexachlorobenzene, only small fractions of the dose appeared in bile as the parent compound and as pentachlorobenzene (2.0 and 1.8%, respectively). The data indicated that the major part of biliary excretion of radioactivity was contained in other metabolites. Urinary excretion accounted for 2.1% of the dose during the 4 d after its administration. Urinary metabolites of hexachlorobenzene include pentachlorophenol, sulfur-containing derivatives, and glucuronides (55).

Octachlorodibenzo-*p*-dioxin was studied in rats by Birnbaum and Couture (43). The fecal route was the major pathway for elimination with minimal urinary excretion for both intravenous and oral administration of the $^{14}$C-labeled compound.

Evidence of a nonbiliary route of excretion of lipophilic toxins resulted from studies of the organochlorine pesticide, chlordecone. Excretion data obtained from humans exposed to chlordecone and rats that were administered the compound are consistent with this route as a significant excretory pathway (58,59). The authors observed that fecal excretion of chlordecone was maintained or increased when bile flow was diverted relative to that seen while bile flow was intact.

More evidence for nonbiliary intestinal excretion was reported by Rozman *et al.* (60). A study in the rhesus monkey showed that bile diversion did not alter fecal excretion of hexachlorobenzene. The presence of unchanged hexachlorobenzene in feces when the bile mainly contained metabolites was seen as evidence that nonbiliary excretion *via* exfoliation of the epithelium or exudation across the mucosa was a significant excretory route (61).

Toxic lipophiles in the lumen of the intestine from biliary and nonbiliary excretion routes are a principal target for intervention to accelerate elimination rates. Nonbiliary transport of lipophilic compounds into the lumen of the intestine is poorly understood in terms of their cellular or molecular associations, but as discussed in a later section, there is evidence of enterohepatic circulation of compounds that enter the intestinal lumen *via* a nonbiliary route. Lipophilic compounds that enter the intestine in bile also take part in enterohepatic circulation. Most of the past and current efforts to hasten the egress of toxic lipophiles from the body have focused on interruption of enterohepatic circulation to direct the compounds into fecal excretion instead of reabsorption from the intestine.

*Lactation.* Lipophilic compounds repeatedly have been observed in milk from humans and other species. An important path for the elimination of lipophilic substances from the body is through the lactation process. As discussed below, the mobilization of fat that provides a source of energy for neonates results in the accompanying transport of stored lipophiles into milk.

Patton (62) reviewed ideas about the movement of lipophilic xenobiotics from adipose tissue to the mammary gland. Fatty acid is released by hormone-sensitive lipase in the adipocyte, is transported to the mammary gland bound to albumin, and is re-esterified into triacylglycerol. It is not clear whether lipophilic xenobiotics stored in adipose tissue follow this same path to the mammary gland.

A study of the distribution of PCBs in female mice illustrates the importance of milk as an excretory route (63). The animals were treated with [$^{14}$C]PCB (2,4,5,2′,4′,5′-hexachlorobiphenyl) by intraperitoneal injection in corn oil 2 wk prior to mating. At 20 d postpartum, 98% of the dose was eliminated, and the level in the offspring was consistent with transfer of the mother's entire body burden of PCB to the pups. The level of PCB in virgin mice matched with the mothers maintained through the same time period remained essentially constant.

An example of the removal of lipophilic toxic materials from humans was reported by Schechter and coworkers (64). They followed the levels of lipophilic materials in the blood and milk of a mother who nursed twins for a period of 38 mon. Dioxin and dibenzofuran concentrations in milk decreased from 309 to 173 and from 21 to 9 ng/kg, respectively, during 30 mon. Hexachlorobenzene concentration decreased from 10.7 to 1.8 ng/g. These observations reflect the mobilization of the mother's adipose tissue to provide energy in milk, and the mobilization of stored dioxin, dibenzofuran, and hexachlorobenzene accompanying the fat.

Clearly, milk can be a major excretory route for lipophilic toxins, but the obvious consequence of this route is the introduction of the toxin into the neonate. There have been few studies that have addressed ways to affect the rate or amount of excretion of lipophilic toxins in milk.

## EFFECTS OF DIETARY ADDITIVES

The foregoing discussion is intended to give an overview as a background for the review of methods that alter the residence time and elimination rate of toxic lipophiles in an organism. These interventions include specific dietary additives, changes in energy intake, and variations in dietary fat.

### Nonabsorbable Lipids

#### *Effect on Absorption from the Diet*

Since most toxic lipophiles enter the body orally *via* food and water, the first approach for potential intervention is reduction of absorption from the intestine. As discussed by Jandacek (65) and Patton (62), a large fraction of dietary lipophiles enter the mouth and stomach dissolved in or associated with dietary triacylglycerol. Lipophiles that are adsorbed to other dietary constituents presumably associate with the dietary fat during mastication and mixing in the stomach.

In the small intestine, triacylglycerol is split into fatty acids and 2-monoacylglycerol, which form mixed micelles with bile salts and phospholipid from bile. During this

digestion process, a triacylglycerol oil phase remains mixed with an aqueous micellar phase until its digestion is complete. There is also evidence of liquid crystalline phases in the intestinal lumen during this process (66). Lipophiles are distributed among these phases but are presumably transported to the enterocyte membrane in the bile salt mixed micelles. Entry into the enterocyte and incorporation into chylomicrons presumably occur for many lipophiles. A strong affinity for an oil phase, as is the case for lipophiles with octanol–water partition coefficients of $>10^5$, results in retention of a large portion of the lipophile in the oil.

The triacylglycerol oil phase in the intestinal lumen is relatively transient. Digestion and absorption of dietary fat are efficient and fast, so that the oil phase disappears quickly. It is possible, however, to maintain an oil phase that competes with the micellar phase for lipophile solubilization. Sucrose esterified with long-chain fatty acids is not hydrolyzed by pancreatic lipase, and thereby maintains an intestinal oil phase throughout gastrointestinal transit (67). Sucrose esterified with six or more long-chain fatty acids has been termed "sucrose polyester" (SPE) and is a component of olestra (brand name, Olean®) (68).

*DDT in rats. (i) Olestra.* The effect of olestra (SPE) on the absorption of dietary [$^{14}$C]DDT was studied in the rat by Volpenhein and coworkers (69). When [$^{14}$C]DDT dissolved in soybean oil was intragastrically intubated into rats fitted with thoracic duct cannulas, 67% of the dose was recovered in a 48-h lymph collection. When the dosing oil comprised 50% soybean oil and 50% SPE, 21% of the dose was in the lymph. Data from rats that did not undergo surgery were consistent with the lymph recoveries. After dosing with the same oils used in the thoracic duct experiments, the animals received *ad libitum* semipurified diets without DDT, but which contained 20% by weight soybean oil or the same blend of 50% soybean oil and 50% SPE used in the lymphatic measurements. Animals dosed with soybean oil excreted less than 10% of the radioactivity in 72 h after its ingestion, whereas those dosed with 50% soybean oil and 50% SPE excreted more than 55% of the dose. The recovery of radioactivity from extracted tissues was consistent with the reduction in absorption by SPE. Approximately 60% of the dose appeared in total carcass fat of the soybean oil group compared with 20% in the soybean oil-SPE group. Similar results were obtained from the liver and epididymal fat pads, with recovery from the soybean oil-SPE group equal to one-third that of the soybean oil group.

These data are consistent with the nonabsorbable oil interfering with the absorption of the lipophile from the diet. The apparent mechanism is that of retention of or transport to the nonabsorbable oil, thereby diminishing the concentration of the lipophile in the micellar phase. Since micellar solubilization is obligate for the absorption of lipophiles, absorption was decreased in the presence of a nonabsorbable oil.

*(ii) Mineral oil.* A study of the effect of another nonabsorbable lipophilic material, mineral oil, on absorption from the diet did not provide clear answers. Keller and Yeary (70) tested the effects of mineral oil and sodium sulfate on the intestinal absorption of DDT in rats. Rats were intragastrically gavaged with [$^{14}$C]DDT in either soybean oil, mineral oil, 15% sodium sulfate with acacia, or water with acacia. After an hour the same formulations were intubated without DDT. This procedure was repeated 24 h later. The animals were sacrificed 48 h after the first dosing regimen, and tissues were taken for measurement. Adipose concentration of DDT was used as the principal assay. The authors concluded that the most important observation was the higher absorption observed when soybean oil was ingested. There was minimal difference among the other groups. The data may indicate the facilitation of the absorption of the pesticide by the presence of hydrolyzable dietary fat.

In contrast with the results of Keller and Yeary, another study found that mineral oil reduced markedly the appearance of DDT in lymph collected for 4 h after oral dosing (71). The peak lymphatic concentration after dosing in mineral oil was approximately one-fourth of that seen after dosing in arachis oil. DDT absorption was also determined from plasma areas under the curve in rats that did not undergo surgery. The decrease in absorption seen in the rats fed mineral oil relative to those fed arachis oil was consistent with the measurements of DDT in lymph.

*Lindane in pigs.* Morgan and coworkers (72) studied the effects of dietary additives on the absorption of the lipophilic pesticide lindane (the γ isomer of hexachlorocyclohexane). Anesthetized pigs were gavaged with lindane and various test formulations, and plasma lindane concentrations were followed for 150 min. The formulations that were compared included water (control), activated charcoal, mineral oil, and castor oil. The authors concluded that none of the treatments reliably altered the gastrointestinal absorption of the pesticide.

### *Effects on Stored Lipophiles*

*Olestra. (i) Animal studies.* The effects of three dietary lipids that are either poorly absorbed or not absorbed were studied in rats. Hexachlorobenzene elimination was measured during consumption of diets containing either olestra (SPE), squalane (30-C saturated hydrocarbon; 2,6,10,15,19,23-hexamethyltetracosane, obtained by hydrogenation of squalene), or paraffin (73). [$^{14}$C]Hexachlorobenzene was added to the diet for 4 d, and after 10 additional days the rat diets were supplemented with 8% olestra, paraffin, or squalane. After 3 wk of treatment, measurements of radioactivity in feces were made, and the excretion in each of the treatment groups was found to be three times higher than that of the control groups. Significant reductions in the tissue levels of radioactivity in the treatment groups were consistent with the excretion data. The authors suggested that in light of the absence of biliary excretion of hexachlorobenzene, nonabsorbable oils reduced the effective concentration gradient between blood and the gut lumen.

The effect of olestra on the elimination of [$^{14}$C]DDT (and/or its principal metabolite, DDE) was studied in gerbils

(74). Gerbils were given [$^{14}$C]DDT orally and maintained on chow until a log-linear fecal excretion rate was established. Excreted radioactivity increased more than twofold when 2.5 wt% olestra was added to the diet. Similar increases were seen with 5 and 10% olestra. Animals were changed to diets that provided 50–75% of normal caloric intake, and fecal excretion of radioactivity increased approximately 50% above that of control animals. When olestra was included in the reduced energy diet at a level of 10%, the average increase in fecal excretion was eightfold that of the control animals.

These studies of olestra in animals indicated that olestra could be used to interrupt enterohepatic circulation of lipophilic toxins that had been stored in the body after oral ingestion. Studies in humans discussed below have supported its potential use in removing lipophilic toxins from the body.

*(ii) Human trials.* Olestra has been studied in detoxification of dioxin in human trials. Gesau *et al.* (75) fed olestra to two subjects who had elevated levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Five different dosing regimens were used, and olestra ingestion ranged from 15 to 66 g/d. Fecal excretion of TCDD was markedly increased with olestra consumption. In one patient the increase ranged from 3- to 10-fold above that prior to olestra consumption. In the other patient the increase ranged from three- to eightfold, depending on the specific regimen.

Moser and McLachlan (76) studied olestra in three healthy volunteers with normal exposure to lipophilic pollutants. Fecal excretion of dioxins, dibenzofurans, PCBs, and hexachlorobenzene was measured after 8 d of ingestion of 25 g/d of olestra. The excretion was compared with that of an initial 3-d control phase without olestra consumption. The excretion during the olestra period was 1.5 to 11 times higher than that during the control period, depending on the specific compound. Excretion enhancement (the increase in a compound's excretion relative to its ingestion) increased for all compounds during olestra ingestion. The authors also reported the interesting observation that the excretion enhancement decreased as the octanol/water partition coefficient of the lipophile of interest increased.

*Mineral oil/hexadecane/squalane.* Mineral oil is a cut of petroleum distillates that is dominated by but not limited to long-chain hydrocarbons, including hexadecane. Paraffin/paraffin oil also includes long-chain hydrocarbon compounds. Squalane is defined in an earlier section. These materials are poorly absorbed from the intestine and maintain an intestinal lipid phase during gastrointestinal transit; however, partial absorption may occur and has been implicated in histological lesions in the liver and spleen (77,78). The absorption of trace amounts of mineral oil and its components has not prevented its use as a model lipid sink in elimination studies, but it raises issues relative to its use in long-term human therapy.

Richter (79) reviewed studies that intervened to change the rate of fecal excretion of hexachlorobenzene and cited 12 studies that utilized mineral oil in changing the excretion of hexachlorobenzene, hexachlorocyclohexane, pentachlorobiphenyl, hexabromobiphenyl, chlordecone, mirex, and DDT. Significant enhancement of the rate of excretion was seen with mineral oil in all but one of the cited studies. Five studies with squalane and four with hexadecane were also cited in Richter's review. Both compounds effected increases in the excretion of hexachlorobenzene in the studies reviewed. The review noted that hexadecane is partially absorbed and therefore is not suitable for consideration as a nonabsorbable lipid for therapeutic purposes; there is also evidence for the accumulation of dietary squalane in the liver of rodents (73,80).

Much of the work that was carried out with these poorly absorbed oils was directed by K. Rozman and T. Rozman. Table 3 summarizes the design and results from their studies and from those of other investigators.

Mineral oil (paraffin oil) has been the subject of most studies of intervention in the metabolism of lipophilic toxins. The data are clearly supportive of its interference with the reabsorption of lipophiles that enter the intestinal lumen *via* bile or a nonbiliary mechanism. Although the possibility of absorption of mineral oil limits its applicability in humans, the experiments with animals have demonstrated the validity of an intervention that is based on interruption of enterohepatic circulation of lipophiles.

## Resins

Pharmaceutical cationic ion exchange resins have been used to treat hypercholesterolemia by binding bile salts in the intestine and enhancing their excretion. Since these polymeric resins are not absorbed, bind bile salts, and have lipophilic backbones, they were considered as candidates for intervention in the enterohepatic excretion of some lipophilic toxins. The use of cholestyramine in people contaminated with chlordecone in the study discussed below was the first effective detoxification of a lipophilic toxin that was stored in the body (95).

Cholestyramine binds the pesticide chlordecone *in vitro* and enhances its excretion in rats (96). Rats were given $^{14}$C-chlordecone by stomach tube and after 7 d assigned to diets containing cholestyramine or silica gel/cellulose (control). Measurements of fecal excretion and tissue concentrations ensued. The concentration in the liver was reduced by 39% in the cholestyramine group relative to controls, in fat, by 30%, and in the brain, by 50%. Significant increases in fecal excretion and decreases in body burden were seen in the cholestyramine group relative to the control group.

The reduction of chlordecone from human subjects with a high body burden was reported by Cohn and colleagues (95). Workers in a factory that manufactured chlordecone exhibited symptoms of toxicity attributed to the pesticide. A double-blinded trial of subjects who received either placebo or 24 g/d of cholestyramine was carried out. Cholestyramine increased the fecal excretion by 3- to 18-fold. A significant reduction in the half-life of chlordecone (calculated from longitudinal measurements of blood levels) was seen with cholestyramine. Chlordecone excretion in the bile of a patient fitted with a T-tube was 19 times faster than that in feces,

**TABLE 3**
**Studies with Mineral Oil, Squalane, or Hexadecane[a]**

| Study design | Results | Reference |
|---|---|---|
| Mineral oil and hexadecane were fed to rats and rhesus monkeys that had been dosed with [$^{14}$C]hexachlorobenzene (HCB). Fecal excretion and tissue levels of $^{14}$C were measured. | Hexadecane at a level of 5% in the diet increased $^{14}$C-HCB fecal excretion 4- to 13-fold; mineral oil caused a six- to ninefold enhancement. Blood and adipose tissue levels of $^{14}$C decreased with increased excretion. | 60 |
| Rats were dosed orally with [$^{14}$C]HCB and fed diets containing 8% squalane, paraffin oil, or sucrose polyester. Fecal excretion was monitored. | The half-life, calculated 35–38 d after discontinuation of the HCB diet, was reduced to approximately one-third of that of the control group by all oils. | 73 |
| [$^{14}$C]HCB was dosed to rats in the diet. After 35 and 53 d, animals were sacrificed for analysis. Feces were analyzed. | The fast and slow half-lives of elimination were reduced by 48 and 77% by paraffin oil. There were reductions of HCB in all analyzed tissues; fat concentration was reduced more than 80% by paraffin oil. | 81 |
| [$^{14}$C]PCB (2,4,6,2′,4′-pentachlorobiphenyl) was dosed orally to rats. A control diet was compared with a diet with 8% light paraffin oil. Feces were assayed. | The paraffin oil group excreted 54% of the dose, and the control animals, 43%, during the 4 wk after discontinuation of the PCB-containing diet. | 82 |
| Rhesus monkeys were given mirex and then received a diet containing 5% mineral oil. | Fecal excretion was increased 50% 1 mon after dosing; the increase was 400% 6 mon after dosing. The long-term effect was attributed to "deep" storage in fatty tissue. | 83 |
| Rhesus monkeys were given 5% mineral oil after a dose of 2,4,5,2′,4′,5′-hexabromobiphenyl. | Fecal excretion increased 175% with the addition of mineral oil to the diet. | 84 |
| Rats dosed with [$^{14}$C]HCB were given hexadecane. The effect of bile duct ligation was tested. | Bile duct ligation, dietary hexadecane, and the combination of all treatments increased fecal excretion. The combination of treatments was more effective than each alone. | 85 |
| Orally dosed [$^{14}$C]HCB in intestinal segments was measured; the effect of hexadecane on HCB intestinal excretion in the segments was determined. | The data indicated that hexadecane increased excretion of HCB in the colon. | 86 |
| Rhesus monkeys were dosed orally with [$^{14}$C]HCB with varying doses. Bile flow was diverted, and excretion was measured in animals given diets with and without mineral oil. | Fecal excretion of HCB was raised fivefold by mineral oil. Urinary excretion was not altered by mineral oil. | 87 |
| Rhesus monkeys were dosed orally with [$^{14}$C]DDT and given a diet with 5% mineral oil. Fecal excretion and adipose tissue levels were assayed. | Mineral oil doubled the excretion rate and halved the amount of radioactivity in the adipose tissue. | 88 |
| Hexadecane was injected into ligated intestinal segments of rats previously dosed with [$^{14}$C]HCB; the effect on intestinal excretion was determined. | Intestinal excretion increased in the order jejunum > ileum > cecum and colon. The authors noted that high residency time makes the colon an important site for interaction with hexadecane. | 89 |
| Sheep were fed [$^{14}$C]HCB and then given diets containing 5% mineral oil or 5% hexadecane. Fecal excretion and adipose stores were measured. | Treatment with hexadecane and mineral oil increased fecal excretion threefold. Reductions in adipose tissue corresponded to increases in excretion. | 90 |
| Lactating goats dosed with mirex were given 5% light mineral oil. Fecal excretion, blood, and milk concentrations of mirex were measured. Mineral oil as 3% of the diet was fed to cattle that produced butterfat contaminated with DDT. | Fecal excretion of mirex from goats was increased by dietary mineral oil. There were no changes in concentrations in milk or blood during the period of mineral oil consumption. Fecal DDE excretion from cows increased with mineral oil consumption; milk concentrations did not change with mineral oil treatment. | 91 |
| Rabbits and rats were dosed with [$^{14}$C]HCB and given a diet containing 5% hexadecane. | Half-lives were of HCB in the rat (24 d) and rabbit (32 d) were similar. Hexadecane increased fecal excretion of HCB four- to fivefold. | 92 |
| Chickens with HCB or pentachlorophenol body burdens were given mineral oil, colestipol, and/or reduced caloric intake. | 5 wt% mineral oil in the diet reduced body burden to 36% of dose, compared with 63% of dose with no treatment. | 93 |
| Heptachlor epoxide was given to mink. The effect of diet restriction with a diet containing 10% mineral oil was observed. | Rapid reduction of body burden occurred with control (*ad lib* diet) animals; mineral oil and restricted diet did not accelerate the reduction relative to the control. | 94 |

[a]DDE, 1,1-dichloro-2,2-bis(chlorophenyl)ethylene. For other abbreviations see Table 1.

**Table 4**
**The Use of Resins to Intervene in Toxic Lipophile Excretion[a]**

| Study design | Results | Reference |
|---|---|---|
| Rhesus monkeys were orally dosed with [$^{14}$C]HCB, and 4% cholestyramine was added to the diet. Fecal excretion of $^{14}$C was measured. | Cholestyramine did not increase the rate of fecal excretion above that seen during a period without dietary cholestyramine. | 60 |
| [$^{14}$C]DDT was given to gerbils, and the excretion was followed after diets with and without 4% cholestyramine. | A modest increase in excretion of radioactivity was observed with added cholestyramine. | 74 |
| Rhesus monkeys dosed with 2,4,5,2′,4′,5′-hexabromobiphenyl (PBB) were given 4% cholestyramine in the diet. | Fecal excretion of the PBB increased 50% with the addition of the resin. | 84 |
| Chickens were fed HCB or pentachlorophenol and given colestipol with or without food restriction. | Marked reductions in body burdens were seen for both compounds with colestipol; greater reductions were seen when colestipol was combined with food restriction. | 93 |
| Turkeys and chickens were orally administered [$^{14}$C]dieldrin and fed resins. | Based on carcass analyses, the resins were generally ineffective in reducing the dieldrin levels. | 97,98 |
| Rhesus monkeys dosed with [$^{14}$C]-pentachlorophenol were given a diet containing 4% cholestyramine; fecal excretion was monitored. | Fecal excretion of radioactivity increased 9- to 14-fold with cholestyramine in the diet. | 99 |
| Rhesus monkeys were orally dosed with [$^{14}$C]pentachlorophenol and fed cholestyramine. | Fecal excretion of pentachlorophenol increased by as much as 40%. | 100 |
| PBBs were fed to chickens. Colestipol was fed alone or in combination with a starvation regimen. | Colestipol alone had minimal effect but reduced body burden by 70% in 21 d in combination with starvation. | 101 |
| Patients exposed to PCBs and dibenzofurans were given 8–12 g/d cholestyramine for 24 wk. | In 4 of 6 patients there was no effect of cholestyramine. Fecal excretion of PCBs increased by 36–46% in the other two. | 102,103 |
| Rats fed rice oil contaminated with PCBs and dioxins were given 5% cholestyramine alone or in combination with squalane and rice bran. | Cholestyramine alone enhanced excretion of some congeners by factors of 1.3–3.3. Combinations with rice bran and squalane somewhat enhanced the excretion. Excretion of a heptachlorodibenzodioxin congener was not affected by the resin. | 104 |
| Rats received PBB for 6 mon followed by normal diets for 4 mon. Cholestyramine was included in the diet for 6 mon. | Cholestyramine did not reduce the levels of bromine in the tissues. | 105 |

[a]For abbreviations see Tables 1 and 3.

thereby indicating the existence of an enterohepatic circulation of chlordecone.

Other trials that have studied cholestyramine and colestipol (also a bile salt-binding cationic resin) are summarized in Table 4.

## Activated Carbon

Activated carbon, which is used as an adsorbent in treatment of patients with acute poisoning, has also been studied as a treatment for stored lipophilic toxins. The rationale for its use is that of interference with enterohepatic circulation of compounds that enter the intestine *via* biliary and nonbiliary routes. The use of activated carbon in studies of lipophilic toxin metabolism is summarized in Table 5.

## Protoporphyrin

One study was carried out with dietary protoporphyrin and hemin (108). The rationale for the use of these materials was apparently based on expected low intestinal absorption of protoporphyrin and hemin and predicted high adsorption of lipophilic substances to these substances. Animals that were given the oil that contained polychlorinated dioxin and polychlorinated dibenzofuran were subsequently provided a diet that contained either 0.5% disodiumprotoporphyrin or 0.5% hemin. The protoporphyrin group excreted 2.1 times the amount of the toxins in feces relative to the control group. The group that received hemin was not different from the control group.

## Dietary Fibers/Indigestible Polysaccharides

As noted in Table 4, rice bran fiber in combination with cholestyramine slightly increased the excretion of polychlorinated dibenzofuran congeners relative to cholestyramine alone in rats with body burdens of these compounds (104). The same study included comparisons of rice bran and other fibers (burdock, corn, soybean) that were included in the diet at a concentration of 10% by weight. All of the fibers significantly increased the excretion of all measured dibenzofuran and dioxin congeners except the 1,2,3,4,6,7,8-heptachlorodibenzodioxin relative to the control group. The effect of the rice bran fiber was the greatest, with rates of excretion ranging from 1.9 to 3.3 times that of the control group.

A comparison of the effects of some indigestible polysac-

**TABLE 5**
**The Use of Activated Carbon in Detoxification of Lipophiles**

| Study design | Results | Reference |
|---|---|---|
| 3 patients with body burdens of chlordecone were given 40 g/d of activated charcoal. | Excretion rate was increased by less than twofold by the carbon. | 95 |
| Turkeys and chickens were administered $^{14}$C-dieldrin orally and fed activated carbon. | Activated carbon had no effect on the rate of elimination. | 96,98 |
| Rats received PBB for 6 mon followed by normal diet for 4 mon. Activated charcoal was included in the diet for 6 mon. | Activated charcoal did not reduce the concentration of bromine in tissues. | 105 |
| Activated carbon was fed as 5% of the diet with or after DDT exposure to rats and cows. | Activated carbon decreased DDT absorption but had no effect on rate of elimination. | 106 |
| Mice, rats, and guinea pigs were injected intraperitoneally or subcutaneously with 2,3,7,8-tetrachloro-*p*-dioxin and given chow or chow plus charcoal. | Mortality was reduced from 93 to 53% in mice; 80 to 50% in rats; 64 to 29% in guinea pigs. | 107 |

charides on the accumulation of pentachlorobenzene was performed in rats (109). Rats were fed either cellulose (control), sodium alginate, guar gum, or γ-carrageenan at a level of 5 wt% of the diet. After 2 wk they were dosed orally with 20 mg of pentachlorobenzene, maintained with the experimental diets for seven more days, and then sacrificed to assay tissues. Body weight and adipose tissue mass (as percentage of body weight) were significantly lower in each of the polysaccharide groups relative to the control. The level of pentachlorobenzene was lower in the livers, kidneys, and adipose tissue of the animals that ate the polysaccharides relative to the control group. Fecal excretion of pentachlorobenzene was higher than that from control animals in the groups fed sodium alginate and guar gum. The authors suggested that lower adipose tissue mass seen in the polysaccharide groups was in part responsible for the enhanced excretion of pentachlorobenzene.

### Amount and Type of Absorbable Dietary Fat

Contrasted with the studies of energy deprivation, Nakashima *et al.* (34) studied the effect of a high-fat diet on the elimination of hexachlorobenzene in lactating rats. Pregnant rats fed a high-fat diet transferred hexachlorobenzene more slowly in milk to pups than those fed a low-fat diet. This observation may reflect a reduced rate of mobilization of the adipose stores in the high-fat fed mothers so that the stored hexachlorobenzene also was transported more slowly.

In addition to the effects of nonabsorbable dietary fats, there is evidence that the fatty acid composition of typical dietary fats may influence the metabolism of organochlorine compounds. Umegaki reported that fish oil enhanced the metabolism of pentachlorobenzene in rats relative to the effects of lard or soybean oil (110). Fish oil not only enhanced the levels of the principal metabolite of pentachlorobenzene, pentachlorophenol but also reduced the concentration of pentachlorobenzene in fat. The authors suggest that reduced accumulation of adipose tissue in the animals fed fish oil contributed to its reduced accumulation.

Umegaki and Ikegami (111) studied the influence of dietary fatty acid on hexachlorobenzene metabolism. They reported that relative to soybean oil, fish oil and lard in the diet resulted in a lower total fat mass and higher concentrations of hexachlorobenzene in the blood, liver, and brain. The animals that received fish oil had higher urinary levels of pentachlorophenol, a principal metabolite of hexachlorobenzene. The authors concluded that fish oil increased cytochrome P450 and accelerated hexachlorobenzene metabolism, thereby increasing oxidation to more polar compounds (51).

The absorption of [$^3$H]benzo(α)pyrene was measured in rats that were fitted with bile and lymph duct cannulae and fed 50 or 500 μmol of olive oil (35). There was no effect of the level of dietary fat on the recovery of radioactivity in bile and lymph. The results were consistent with rapid conversion of benzo(α)pyrene to polar compounds in the enterocytes and absorption *via* the portal vein rather than lymph.

## CHANGES IN BODY FAT

Adipose tissue is the principal depot for accumulation of many lipophilic toxins. Therefore, reduction in adipose tissue stores (e.g., from weight loss) can release stored lipophilic toxins into the circulation and expose other sites to them. The following outcomes of this release are possible: storage in tissues that are more affected than adipose tissue by the compounds; increased metabolism resulting from increased uptake by the liver; decrease in body burden; and combinations of these events. Some studies of adipose tissue and weight reduction have examined excretion rates and tissue distribution. There also have been studies in which weight reduction was combined with use of nonabsorbable dietary lipids.

A suggestion of adipose tissue "protecting" more sensitive targets from lipophilic toxins was presented by Geyer *et al.* (112), who suggested that the toxicity of dioxin is inversely related to the amount of body fat in an animal. Based on the relationship between toxicity and body fat among and within species, the authors concluded that body fat is a reservoir that limits the exposure of target organs to toxic lipophiles. This kind of relationship underscores the importance that changes in adipose tissue mass might have on lipophilic toxin metabolism.

## Effects of Weight Loss on Elimination of Lipophiles in Experimental Animals

The study by Davison and Sell (98) of dieldrin in chickens cited in Table 4 included a regimen of severe starvation. The authors observed significant increases in excretion and complementary reductions in carcass levels of dieldrin with reduction in energy intake.

Mitjavila *et al*. (113) studied the effect of restricted food intake on DDT metabolism in the rat. Animals received DDT daily *via* intragastric gavage. They were then subjected to 3 d of starvation followed by 14 d of a fat mobilization period with a diet limited to 2.5 g food/d/rat. Tissue compositions of DDT and DDE were assayed. The half-life of DDT was calculated to be 5 d under these conditions, markedly less than estimates of 2 mon from studies of *ad libitum* feeding. Observation of the animals led the authors to conclude that the rat liver is capable of metabolizing large quantities of DDT that are mobilized by reduction of adipose stores of the pesticide.

In the study by Polin *et al.* (93) (cited in Table 4) of chickens with body burdens of hexachlorobenzene and pentachlorophenol, the effect of reduction of food intake was studied. Treatment with food restriction at 50% of *ad libitum* intake reduced the body burden to 37% of the initial dose of hexachlorophene, compared with a value of 63% for the *ad libitum* diet control group. The analogous figures for pentachlorophenol reduction were 25 and 70% of the dose, respectively. Chickens that were fed 10 wt% colestipol or 10 wt% mineral oil showed a further reduction to 19% of the original dose for each treatment.

As already noted in the summary of the study by Mutter *et al*. (74), caloric deprivation with and without dietary olestra in gerbils increased the rate of excretion of DDT/DDE. This increase was approximately 50% above that from control animals that received an *ad libitum* diet when no other treatment was included. The combination of dietary olestra with caloric deprivation markedly increased the excretion rate to approximately eight times that of the excretion from fed control animals.

A combination of mineral oil and food restriction was studied in the elimination of PBBs from chickens in the study by Polin *et al.* cited in Table 4 (101). Both mineral oil and food restriction treatments resulted in reductions in body burdens greater than those seen in control animals at relatively low doses of the PBBs (0.1 and 1.0 ppb in the diet). At higher dose levels of PBBs, there were minimal effects in terms of percent reduction.

Restricted caloric intake was ineffective in a study of rats with a body burden of PBBs (105). The animals were fed a mixture of PBBs for 6 mon followed by a diet free of PBBs for 4 mon. Reduction in energy intake did not reduce the level of PBBs in adipose tissue.

The study of an interesting lactation process was reported by Polischuk and coworkers (114), who measured the concentration of chlorinated organic compounds in polar bears undergoing an extensive fast and lactating. They found that the concentration of PCBs, chlordanes, and chlorobenzenes in milk lipids increased markedly during the fasting period. The concentrations of DDT and hexachlorocyclohexane did not have a consistent pattern.

## Effect of Weight Loss on Elimination in Humans

Two subjects who were part of the Biosphere 2 project near Tucson, Arizona, participated in measurements of blood levels of PCBs and DDE (115). In the conditions of a closed ecological space and self-sufficient food supply, men and women lost 18 and 10% of body weight during 2 yr, respectively, with most of the loss in the first 6–9 mon. The blood concentrations of the toxicants were reported to increase during the first 12–18 mon and then decrease. The initial increase is consistent with the mobilization of adipose tissue, and the subsequent decrease may reflect reduced body stores.

The effect of modest weight loss on the concentration of hexachlorobenzene and DDT in the breast milk of women with relatively low levels of the contaminants was reported (116). There was no change in the milk concentration of these compounds with a mean weight loss of 4.1 kg.

## Effect of Weight Loss on Tissue Distribution in Experimental Animals

Dale *et al.* (117) studied the effect of caloric restriction in rats that received 200 ppm DDT added to chow for the duration of the study. One group was sacrificed after 90 d of *ad libitum* feeding. A second group was sacrificed after a sequence of 90 d of *ad libitum* followed by 10 d of 50% reduction in normal energy intake. A third group followed the regimen of 90 d of *ad libitum* feeding, 10 d of 50% caloric deprivation, and 40 d of *ad libitum* feeding. A fourth group followed a regimen of *ad libitum* feeding for 140 d before sacrifice. The levels of DDT and its metabolite DDE in tissues were measured. The concentration of DDT and DDE increased in the tissue and the lipid fractions of fat, brains, plasma, livers, and kidneys of animals that were energy-deprived at 100 d relative to those sacrificed at 90 d. Although excretion of DDT and metabolites increased during energy deprivation, the excretion rate did not prevent the increase in concentration of DDT and its metabolites in the examined tissues.

Findlay and deFreitas (118) studied the effect of lipid utilization on the mobilization of DDT in pigeons. Homing pigeons were dosed with [$^{14}$C]DDT in corn oil for 16 d. Some of the birds were maintained at 6°C without food until they had lost 18–20% of their body weight and then sacrificed. The distribution of radioactivity among tissues was measured in the birds and compared with that from a group that was not stressed by the weight loss regimen. The total amount of DDT in the fat in the stressed birds was reduced relative to the unstressed birds at all doses of DDT that were administered. The concentration of DDT in the fat lipids was two- to threefold higher in the stressed birds. The total DDT in breast muscle

increased 172 to 262% in the stressed birds, and the concentration increased 174 to 235%.

Weight loss in female rats that were dosed orally with hexachlorobenzene and restricted to 30% of normal intake for 7 d resulted in a marked increase in hexachlorobenzene in all tissues (119). The levels in the brain and liver were elevated 367 and 496%, respectively.

The effects of restricted feeding on pentachlorobenzene in rats was reported by Umegaki *et al.* (120). The animals that were eating *ad libitum* or with intake restriction received a single dose of pentachlorobenzene in soybean oil. Fecal excretion of dietary pentachlorobenzene was decreased by restriction of feed intake to 50 and 25% of *ad libitum* consumption. Its accumulation in liver, brain, and fat was also reduced by dietary restriction. The authors concluded that the reduced level in these tissues was the result of increased metabolism by the liver resulting from a decrease in adipose tissue mass and mobilization of the compound.

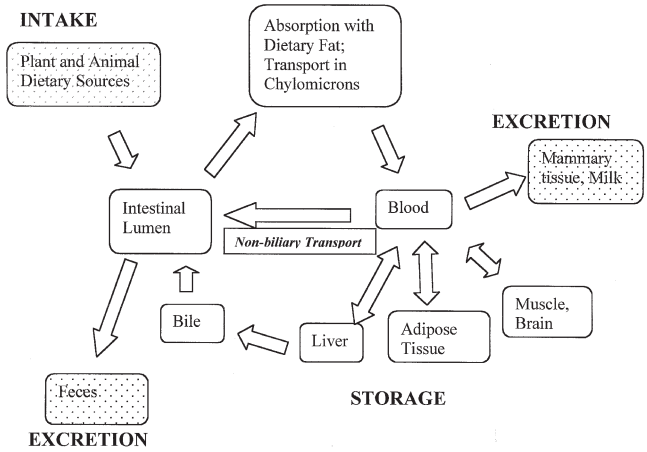
A study of the effect of fasting on the estrogenic effect of DDT and β-hexachlorocyclohexane was studied in mice (121). The animals were injected with the the halogenated compounds and then fasted for 2 d. Uterine weights of fasted and fed animals were compared with those from animals injected with vehicle (control animals). The uterine weights of the animals injected with β-hexachlorocyclohexane were greater than those from fed animals or from fasted control animals. Fasting did not alter the estrogenic effect of DDT. The authors concluded that lipolysis accompanying fasting releases β-hexachlorocyclohexane with subsequent stimulation of estrogen target tissues. The data indicated that DDT was mobilized in a different manner.

There is evidence that energy restriction may make an animal more vulnerable to the toxic effects of lipopohiles. In rats fed hexachlorobenzene, the restriction of energy intake to 50% of a control diet resulted in an increase in liver hypertrophy and hepatocyte foci (23).

## DISCUSSION

### Summary of Lipophilic Toxin Metabolism

The research discussed in the previous sections provides a general view of the metabolism of toxic lipophiles. This view is summarized in the diagram in Scheme 1. Oral ingestion is the principal entry route for most of these compounds. Dietary fat, which facilitates the absorporption of lipophilic xenobiotics, may act as a carrier for the compounds. They are incorporated into chylomicrons, transported to peripheral tissues and the liver, and may exchange with circulating lipoproteins. They are carried in the blood by lipoproteins. The way that they are metabolized varies among the classes of compounds and among the individual congeners within the classes. Metabolism of very lipophilic compounds converts them into more hydrophilic molecules such as glucuronides or hydroxylated derivatives. Molecules that are very hydrophilic are excreted in urine whereas more lipophilic compounds are transported in bile. In addition, there is evidence that lipophilic compounds that are excreted in feces may be of nonbiliary origin. The nature of this excretory route is unclear, but presumably it results from the sloughing of intestinal cells that accumulate lipophilic substances and/or from a hypothetical secretion directly out of the cells. It is clear from several studies that reabsorption of lipophilic compounds from the intestine occurs with subsequent enterohepatic circulation. It is also evident that enterohepatic circulation occurs for compounds both of biliary origin and of nonbiliary origin.




SCHEME 1

There is convincing evidence that many lipophilic xenobiotics and their more lipophilic metabolites are stored in adipose tissue. Uptake by other tissues and organs also occurs, in some cases resulting from induction of and affinity for enzyme systems such as aryldehydrogenase, and in other cases from the lipid content of the tissue.

Lipophilic xenobiotics are mobilized from adipose tissue when the adipose stores are depleted through reduction in energy intake. The fate of these mobilized molecules varies with the compound and with other conditions, including interventions. Some are taken up by other tissues and organs, including muscle and brain. There is some evidence that toxic effects increase with the transport of compounds from adipose tissue to more sensitive organs. Mobilization generally increases biliary and nonbiliary transport into the intestine, which in turn enhances fecal excretion and enterohepatic circulation.

Mobilization of lipophiles from adipose stores accompanies the transport of triacylglycerol from adipose tissue into milk during lactation. Milk fat formed during lactation can remove a large fraction of a body burden of many lipophilic xenobiotics. There are obvious implications of this process for the health of infants ingesting breast milk.

### Summary of Interventions That Alter Storage and Elimination of Lipophilic Toxins

Interventions that can potentially alter the storage and rates of elimination of lipophilic toxins have generally been

directed toward interruption of their enterohepatic circulation. Poorly absorbed materials that adsorb or dissolve lipophilic compounds in the intestinal lumen can reduce absorption that returns them into the circulation and tissues. Nonabsorbable substances that bind lipophiles, such as lipids (olestra, mineral oil), fiber, resins, and activated carbon increase elimination rates in a number of studies, and these results are consistent with interruption of enterohepatic circulation. Nonabsorbable lipids and activated carbon also reduce the initial absorption of dietary lipophilic xenobiotics by the same mechanism of adsorption/dissolution and fecal excretion.

Another approach has been the combination of substances that reduce enterohepatic circulation with a reduction in body fat stores. This combination has been more effective than separate regimens of weight loss or nonabsorbable binders of lipophilic compounds. In addition, there is some evidence that weight loss alone may cause redistribution of toxic compounds to tissues that are more sensitive than adipose tissue. Interruption of the enterohepatic circulation combined with weight loss may reduce this undesirable effect of weight loss alone.

To date there have been no studies of the effects of a high-energy or high-fat diet in combination with substances that interfere with enterohepatic circulation. This regimen could potentially provide a safe method for reducing the body burden of lipophilic xenobiotics by minimizing concentrations in sensitive tissues. The observation by Nakashima *et al.* (34) of a high-fat diet reducing excretion of hexachlorobenzene in milk lends support to this approach.

## Conclusions and Direction for Further Work

There have been numerous attempts to alter the storage and elimination of lipophilic xenobiotics both in animal models and in humans. It is clear that clinically significant changes in elimination rates can be achieved by regimens that reduce the enterohepatic circulation of toxic lipophiles and their metabolites. There have not, however, been systematic approaches that address the combination of regimens to optimize the conditions for the safe and rapid elimination of stored toxic lipophiles.

Many lipophilic substances are known to be toxic and/or carcinogenic, have poorly understood or unknown "no-effect" levels in humans, and are distributed widely in the environment and the biosphere. This scenario warrants continued efforts to develop safe and achievable regimens for reducing body burdens of toxic lipophiles.

In spite of the large amount of work on the elimination of toxic lipophiles, there remain unaddressed areas that warrant further research. An optimal regimen for toxic lipophile removal from the body has not been systematically developed to provide guidance for removal of toxins in situations of acute and of chronic exposure. A better understanding of the nature of nonbiliary excretion in the intestine may provide new ways to control the rate of elimination of toxic lipophiles. There have only been a few studies that have been directed to understand the effect of dietary regimens on the important area of the transport of toxic lipophiles in milk. Since toxic lipophiles are suspected to influence early childhood development, there is a clear need to understand the relationships of lactation, fat mobilization, and toxin transport. Given the known detrimental effects of persistent toxic lipophiles and their widespread distribution in the environment, there is a need to gain better understanding of influences on their elimination from the human body, the top of the food chain for lipophile accumulation.

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# Plasma Kinetics of a Cholesterol-Rich Emulsion in Young, Middle-Aged, and Elderly Subjects

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**ABSTRACT:** Low density lipoprotein (LDL) plasma concentration is increased in the elderly. In this group, the incidence of coronary artery disease (CAD) is greater and LDL remains an important risk factor for CAD development. In this study, the plasma kinetics of a cholesterol-rich emulsion that binds to LDL receptors was studied in 10-subject groups of the elderly (70 ± 4 yr), middle-aged (42 ± 5 yr) and young (23 ± 2 yr). All were normolipidemic, nonobese, nondiabetic subjects who did not have CAD. The emulsion was labeled with $^{14}$C-cholesteryl oleate and injected intravenously into the subjects. Blood samples were drawn at regular intervals over 24 h to determine the plasma decay curve of the emulsion radioactive label and to estimate its plasma fractional clearance rate (FCR, in $h^{-1}$). FCR of the emulsion label was smaller in elderly compared to young subjects (0.032 ± 0.035 and 0.071 ± 0.049 $h^{-1}$, respectively; mean ± SD, $P < 0.05$). FCR of the middle-aged subjects (0.050 ± 0.071 $h^{-1}$) was intermediate between the values of the elderly and young subjects, although not statistically different from them. A negative correlation was found betweeen the emulsion FCR and subjects' age ($r = –0.47$, $P = 0.008$). We conclude that aging is accompanied by progressively diminished clearance of the emulsion cholesterol esters and, by analogy, of the native LDL.

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The concentration of low density lipoprotein (LDL) is raised in aged subjects (1,2). Because the incidence of coronary artery disease (CAD) markedly increases in this population and the positive correlation between LDL cholesterol levels and CAD persists in the elderly (3,4), it is important to investigate the mechanisms of LDL elevation. The plasma concentration of LDL is determined by the balance between the lipoprotein production rate and the rates of removal from the plasma. LDL production comprises the hepatic synthesis of the precursor lipoprotein VLDL (very low density lipoprotein) and VLDL catabolism with generation of LDL. Removal from the plasma and LDL uptake by the body tissues are mediated by cell membrane receptors that recognize apolipoprotein B100 (apo B100), which is virtually the only apolipoprotein existing in LDL (5).

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Abbreviations: apo, apolipoprotein; CAD, coronary artery disease; LDE, cholesterol-rich emulsion that resembles the lipidic portion of LDL; LDL, low density lipoprotein.

Grundy *et al.* (6), followed by Ericksson *et al.* (7) and Millar *et al*. (8), showed that plasma removal rate of LDL apo B100 is diminished in older compared with younger subjects, confirming the conclusions of the meta-analysis by Miller (1). The study by Grundy *et al.* (6) also found a greater LDL production rate in the elderly, but this was not confirmed in the two other studies. Therefore, the aging process apparently courses along with diminished removal from the plasma of the LDL particles.

Previously, we have studied the metabolic behavior of a cholesterol-rich emulsion that roughly resembles the LDL lipidic structure, which we termed LDE (9). LDE is made without protein, but when injected into the blood stream it acquires apo E and other circulating apo; hence, it is taken up by the cells through the LDL receptors. Those receptors recognize not only apo B but also apo E, which is not found in the LDL fraction (10).

Emulsions can be a practical and efficient tool to study lipid metabolism. The utility of LDE to test LDL metabolism in subjects was shown in dyslipidemia (11) and in cancer patients (12), where its plasma kinetic behavior was as predicted for the native lipoprotein. Those experiments showed that it was feasible to test with LDE the LDL receptor function in subjects. In the current study, LDE labeled with radioactive cholesterol esters was used to verify whether the mechanisms that remove cholesterol from the blood stream indeed become less efficient with aging.

## MATERIALS AND METHODS

*Subjects.* The participants in the study were selected from the outpatient clinics of the Heart Institute of the São Paulo University Medical School Hospital to make up three study groups, each with 10 volunteers: (i) Young subjects: aged 20 to 24 yr (mean ± SD: 23 ± 2 yr), six males and four females. (ii) Middle-aged subjects: aged 36 to 54 yr (mean ± S.D: 42 ± 5 yr), all male. They had no clinical evidence of CAD. (iii) Elderly subjects: aged 65 to 79 yr (mean ± SD: 70 ± 4 yr), six males and four females. Absence of CAD was confirmed by cinecoronary angiography.

None of the participants was obese, addicted to alcohol consumption, or had liver, renal, metabolic, inflammatory, or neoplastic disease. All had total plasma cholesterol <240 mg/dL, LDL cholesterol <160 mg/dL, and triglycerides <250

mg/dL. The experimental protocol was approved by the Ethical Committee of the Heart Institute, and written informed consent was given by all participants. The safety of the radioactive dose intravenously injected into the patients was assured according to the regulations of the International Commission on Radiological Protection (13), as described in our previous study (14).

*Plasma lipids and apo.* Commercial enzymatic methods were used to determine total cholesterol (Boehringer-Mannheim, Penzberg, Germany) and triglycerides (Abbott Park, IL). HDL cholesterol was determined by the same method used for total cholesterol after lipoprotein precipitation with magnesium phosphotungstate. VLDL cholesterol and LDL cholesterol were calculated by the formula of Friedewald (15). Plasma apo A-I and B were assayed by radial immunodiffusion (Lipo-Partigen R-Apo A-I and Nor-Partigen R-Apo B plates, Behring, Marburg, Germany).

*LDE preparation.* LDE was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphatidylcholine, 1 mg triolein, and 0.5 mg cholesterol. $^{14}$C-cholesteryl oleate was added to the mixture. Emulsification of lipids was done by prolonged ultrasonic irradiation in aqueous media, and the procedure of two-step ultracentrifugation of the crude emulsion with density adjustment by addition of KBr to obtain LDE microemulsion was carried out by the method of Ginsburg *et al*. (16), modified by Maranhão *et al.* (11). LDE was dialyzed against saline solution and passed through a 0.22 µm filter for injection into the patients.

*LDE plasma kinetics.* The participants were fasting for 12 h at the beginning of the test at approximately 9 AM, but they were allowed two standard meals during the study, at approximately 12:30 and 7 PM. LDE containing 37 kBq $^{14}$C-cholesteryl oleate and of 5–6 mg of total lipid mass in a volume of 500 µL aqueous solution was intravenously injected in a bolus. Plasma samples were collected over a 24-h period, in intervals of 5 min and 1, 2, 4, 6, 8, 12, and 24 h. Aliquots (1.5 mL) of blood plasma were extracted with chloroform/methanol (2:1, vol/vol) (17), and the solvent phases were transferred into counting vials and dried under a nitrogen stream. Radioactivity was quantified in a scintillation solution (PPO/dimethyl POPOP/Triton X-100/toluene, 5 g/0.5 g/333 mL/667 mL) using a Beckman LS100C spectrometer.

*Estimation of fractional catabolic rate (FCR).* FCR of the LDE $^{14}$C-cholesteryl oleate was calculated according to the method described by Matthews (18) as FCR, where $a_1$, $a_2$, $b_1$, and $b_2$ were estimated from biexponential curves obtained from the radioactivity remaining in plasma after injection, fitted by the least squares procedure, as $y = (a_1 \cdot e^{-b_1 x}) + (a_2 \cdot e^{-b_2 x})$ where $y$ represents the radioactivity plasma decay.

*Statistical analysis.* The differences in the FCR of LDE, triglycerides, and apo were evaluated by analysis of variance, whereas the Kruskal–Wallis test was used for cholesterol (total and fractions) data analysis. In all analyses, a difference of $P < 0.05$ was considered statistically significant.

## RESULTS

Table 1 shows the plasma lipid and apo data of the two study groups. Total and LDL cholesterol values were about 30% greater in the elderly and middle-aged subjects than in younger individuals ($P < 0.05$). VLDL and HDL cholesterol did not differ among the groups, nor did the values of triglycerides, apo B, and apo A1.

Figure 1 shows the plasma decay curves of the LDE cholesteryl ester radioactive label. It is apparent that the curve of the older subjects was slower than that of the younger ones and that the decay curve of the middle-aged subjects tended to run between that of the two other groups. In fact, the older subjects had a roughly 50% diminution of the emulsion cholesteryl ester FCR compared with the younger group ($0.032 \pm 0.035$ h$^{-1}$ and $0.071 \pm 0.035$ h$^{-1}$, respectively; mean ± SD, $P < 0.05$). The group of middle-aged subjects showed FCR values ($0.050 \pm 0.071$ h$^{-1}$) between the groups of younger and elderly subjects, although not statistically different from either.

Figure 2 shows the negative correlation that was found between the emulsion cholesteryl ester FCR and age ($r = -0.47$, $P = 0.008$).

## DISCUSSION

In this study, while the plasma total and LDL cholesterol values were greater in the elderly and middle-aged than in the young subjects, all the other lipid and apo values were similar in the three groups.

In the studies by Ericksson *et al.* (7) and Grundy *et al.* (6), LDL kinetics were evaluated by $^{125}$I labeling of the apo B100 component and reinjecting it into the subjects. Because apo B100 is not an exchangeable protein and thus remains attached to the LDL particles, its decay curve mirrors the plasma decay curve of the lipoprotein. The use of the autologous lipoprotein is obligatory because of the risk of transmission to recipient subjects of HIV, hepatitis, or other infectious agents by the heterologous preparation. Since LDL is not a homogeneous fraction, comprising subfractions with different compositions, sizes, and densities that show wide interindividual variations, it is conceivable that these factors

**TABLE 1**
**Plasma Lipids and Apolipoproteins in the Young, Middle-Aged, and Elderly Groups of Subjects ($n = 10$ in all groups)**

| | Young | Middle-aged | Elderly |
|---|---|---|---|
| Cholesterol (mg/dL) | | | |
| Total | 170 ± 24 | 200 ± 45* | 203 ± 37* |
| HDL | 48 ± 9 | 50 ± 12 | 52 ± 12 |
| LDL | 99 ± 21 | 134 ± 31* | 127 ± 38* |
| VLDL | 23 ± 10 | 25 ± 17 | 24 ± 7 |
| Triglycerides (mg/dL) | 112 ± 49 | 126 ± 85 | 119 ± 44 |
| Apolipoproteins (g/L) | | | |
| Apo A1 | 1.65 ± 0.38 | 1.59 ± 0.22 | 1.67 ± 0.39 |
| Apo B | 1.37 ± 0.34 | 1.27 ± 0.31 | 1.24 ± 0.27 |

*Significance at the 0.05 level. HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; Apo, apolipoprotein.

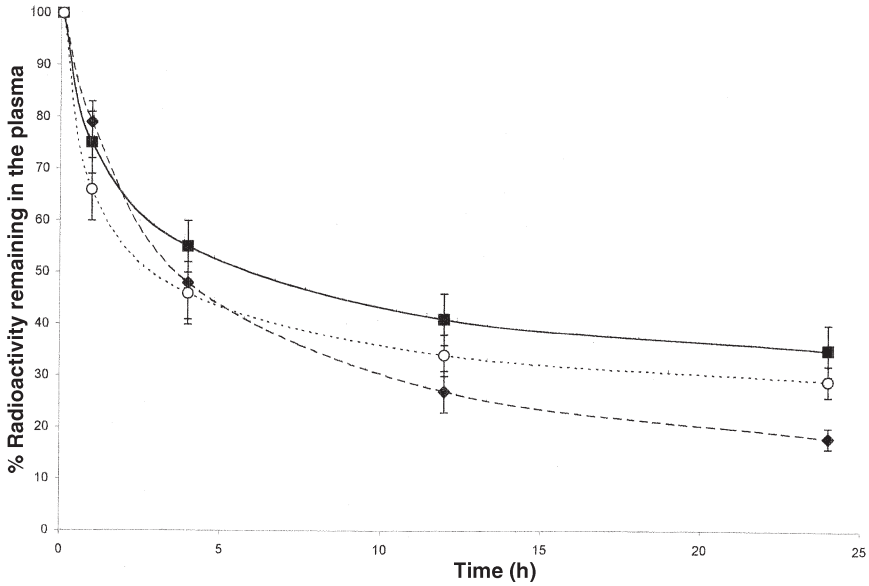


**FIG. 1.** Decaying curves of the emulsion $^{14}$C-cholesteryl oleate obtained from young (♦), middle-aged (○) and elderly (■) subjects ($n$ = 10 in all groups). The labeled emulsion was intravenously injected in a bolus, and blood samples were drawn in preestablished intervals over 24 h for measurement of the radioactivity in a scintillation solution. Points and bars are mean ± standard error of the mean.

may influence the plasma kinetics of autologous $^{125}$I apo B100-labeled LDL when subjects are compared with each other. In fact, smaller, denser LDL subfractions were shown to have poorer affinity for the LDL receptors. Regarding the elderly, it has been shown that LDL from older subjects possessed a higher proportion of cholesterol (19), and significant differences have been found in fatty acid composition of LDL between young and older subjects (20). It could then be possible that the delayed LDL clearance found in the elderly (1,6,7) could be attributed not only to decreased function of the LDL receptors that remove the lipoprotein from the plasma but also to age-related changes in LDL composition. Those changes could presumably decrease the affinity of LDL particles for the receptors, likewise leading to decreased clearance. In the study by Millar *et al.* (8), in which an intravenous infusion of deuterated leucine was used to label apo B100, decreased LDL removal rates were also found. As in the studies mentioned, the possibility that LDL status in the elderly could be implicated in the defect could not be excluded, although Ericksson *et al.* (7) found no differences in LDL isolated from the elderly compared with those from young subjects regarding uptake by lymphocytes.

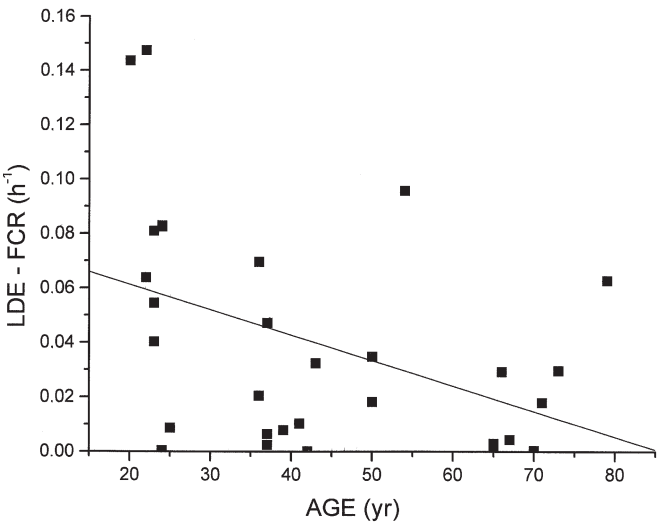


**FIG. 2.** Correlation between the fractional catabolic rate (FCR) of the emulsion radioactive cholesteryl esters and the age of subjects ($n$ = 30, $r$ = –0.47, $P$ = 0.008).

The lipid structure of LDL can be artificially modeled by protein-free emulsions that mimic the lipid physical behavior of native LDL (16,21). LDE particles are composed mainly of a phosphatidylcholine monolayer surrounding a cholesteryl oleate core also containing small amounts of unesterified cholesterol and triolein. When injected into the bloodstream, the metabolic behavior of LDE resembles that of endogenous LDL in rats (9) and in human subjects (11). Similar to triglyceride-rich emulsions (14,22), LDE is capable of assimilating apo from the plasma lipoproteins or from ones free in the plasma. *In vitro* experiments have confirmed that LDE is able to acquire various exchangeable apo, mainly apo E. These apo E molecules endow LDE with the capacity to be recognized and taken up by LDL receptors. In competition studies in cell incubates, it was shown that LDE is dislocated from its cell binding sites by native LDL but not by HDL, as expected, confirming that both LDL and the artificial emulsion share common binding sites. Relative to native LDL, LDE is more rapidly removed from plasma (23). This may be related to the differences in apo profiles on the particle surface. In fact, apo E has been shown to possess several times

greater affinity for the receptor than the unique LDL apo, namely, apo B100 (5). Adequacy of LDE to test LDL metabolism has been proven in clinical studies, wherein the plasma kinetic results obtained with LDE were as expected for native LDL according to the literature. In this respect, removal of LDE was slower in patients with familial hypercholesterolemia (11), wherein LDL receptor function was defective and accelerated in patients with acute myeloid leukemia (12) because in neoplastic cells LDL receptors were upregulated (24). The fact that LDE was removed from the plasma faster than native LDL is an additional methodological advantage because it shortens the period required for performance of the plasma kinetics test.

In our study, by using LDE as an investigation tool we expanded the findings of slowed LDL apo B100 removal, showing also that the removal of cholesterol carried in an LDL emulsion model was delayed. Because we injected the same standard preparation into all study subjects, it can be stated that the delayed emulsion clearance is indeed attributable to a deficiency in the mechanisms of removal from the plasma in the elderly rather than to compositional differences in the LDL fraction between elderly and young subjects.

In both native LDL and the emulsion model, the cholesterol ester moiety tended to shift partially to other lipoprotein classes by the action of the cholesteryl ester transfer protein (see Ref. 25 for a review). Therefore, our results of cholesterol ester-labeled LDE FCR are not strictly specific for the emulsion particles' removal from the plasma, as are those of the other studies mentioned here (5–7), wherein the plasma kinetics of labeled apo B100 were determined to assess the native LDL kinetics. In considering LDE as a tool to model native LDL to explore this metabolism, the negative correlation found between LDE FCR and age implies that with increasing age there is a progressive slowing down of the removal from plasma of this lipoprotein class.

As discussed by Miller (1), decline in the ability to clear LDL from bloodsteam might not be an inevitable consequence of aging in humans, but might result from potentially avoidable environmental factors or lifestyle features related to the aging process. In this regard, we have recently noticed that the LDE clearance is much greater in exercise-trained compared with sedentary subjects (26). If confirmed in the elderly, this observation might lead to novel insights in atherosclerosis prevention.

In conclusion, our study indicates that not only LDL apo B100, as reported by others, but also cholesterol packed in an LDL model emulsion is slowly removed from the plasma in the elderly. This removal deficiency is already manifested in middle-age subjects, who show clearance rates between those of the young and the elderly subjects.

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# Incorporation of α-Tocopherol in Marine Lipid-Based Liposomes: *in Vitro* and *in Vivo* Studies

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**ABSTRACT:** Liposomes made from a natural marine lipid extract and containing a high polyunsaturated n-3 fatty lipid ratio were envisaged as oral route vectors and a potential α-tocopherol supplement. The behavior of vesicles obtained by simple filtration and of giant vesicles prepared by electroformation was investigated in gastrointestinal-like conditions. The influence of α-tocopherol incorporation into liposomes was studied on both physical and chemical membrane stability. Propanal, as an oxidation product of n-3 polyunsaturated fatty acids, was quantified by static headspace gas chromatography when α-tocopherol incorporation into liposome ratios ranged from 0.01 to 12 mol%. Best oxidative stability was obtained for liposomes that contained 5 mol% α-tocopherol. Compared to the other formulas, propanal formation was reduced, and time of the oxidation induction phase was longer. Moreover, α-tocopherol induced both liposome structural modifications, evidenced by turbidity, and phospholipid chemical hydrolysis, quantified as the amount of lysophospholipids. This physicochemical liposome instability was even more pronounced in acid storage conditions, i.e., α-tocopherol incorporation into liposome membranes accelerated the structural rearrangements and increased the rate of phospholipid hydrolysis. In particular, giant vesicles incubated at pH 1.5 underwent complex irreversible shape transformations including invaginations. In parallel, the absorption rate of α-tocopherol was measured in lymph-cannulated rats when α-tocopherol was administrated, as liposome suspension or added to sardine oil, through a gastrostomy tube. α-Tocopherol recovery in lymph was increased by almost threefold, following liposome administration. This may be related to phospholipids that should favor α-tocopherol solubilization and to liposome instability in the case of a high amount of α-tocopherol in the membranes. A need to correlate results obtained from *in vitro* liposome behavior with *in vivo* lipid absorption was demonstrated by this study.

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For several years, n-3 polyunsaturated fatty acids (PUFA) have been investigated for their roles in numerous metabolic pathways and regulation processes. At the membrane level, n-3 PUFA play a role in cellular communication (1) and regulation of the extracellular milieu (2,3). At the physiological level, PUFA are associated with a variety of health benefits, such as alleviated symptoms of rheumatoid arthritis (4) and a reduced risk of coronary heart disease. In this latter case, PUFA influence parameters such as plasma triglyceride level (5), lipoprotein metabolism (6,7), and platelet function (8,9). With the aim to elucidate PUFA benefits, dietary fat modification has been undertaken and is now considered to be an effective tool for modifying physiological parameters (10,11) or cell membrane fatty acid composition (12). Among PUFA, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) seem to play a major role (1,9,12). Nevertheless, these unsaturated lipids are susceptible to oxidation, the mechanism of which has been thoroughly studied (13,14). As lipid oxidation products are implicated in a number of pathological events (14,15), the presence of antioxidants in biological membranes is essential for lipid integrity. Among the natural antioxidants, α-tocopherol, the main active form of vitamin E, is known to be retained in the membrane and to suppress natural and model membrane oxidation (16,17). It acts by inhibiting the propagation step of the free-radical autoxidation mechanism by reacting with various free radicals (18). In parallel, evidence has accumulated for a protective effect of vitamin E against risk of cancers (19,20) and cardiovascular disease (21,22). In particular, the results of prospective cohort studies and intervention trials have demonstrated that high intake of vitamin E can reduce cardiovascular risk (23).

Nevertheless, absorption of vitamin E depends on various parameters, such as the individual's ability to absorb fat (24) and the identity of the lipids co-ingested (24,25). Up to 70% absorption of α-tocopherol by humans has been reported in the literature (26), but this value seems to be overestimated (24); 50% absorption rate should be considered. Because of increasing interest in its protective effects as well as other beneficial effects, many vitamin E preparations have become widely available. The most marketed form is as gelatin capsules. However, as bile salts are required for its absorption (27), vitamin E administration as a dietary supplement should preferably be taken with food, which consumers may not know. Thus, novel forms of vitamin E preparations have been formulated, i.e., oil, liposome and cyclodextrin preparations (28), self-emulsifying systems (29), or emulsion fat globules (30). However, a normal emulsion preparation is bulky and

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; OD, optical density; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid.

suffers from cracking and creaming on storage. Thus, there is an increasing interest in the use of more stable systems.

In this context, we envisaged using liposomes based on a natural lipid mixture extracted from a marine organism, in association with α-tocopherol. First, α-tocopherol incorporation should improve chemical stability of the liposome suspensions. Second, in the case of an oral dosage form, lipid vesicles should provide an adequate binding surface for pancreatic phospholipase $A_2$, and the lysophospholipids generated by phospholipid hydrolysis should favor α-tocopherol absorption. Indeed, fat-soluble vitamins, including vitamin E, are better absorbed in the presence of surfactants or when in emulsified vehicles compared to oily preparations (31). Thus, the purpose of this study was (i) to select the most efficient amount of α-tocopherol to incorporate into PUFA-rich liposome membranes so as to prevent lipids from oxidizing when vesicles are submitted to conditions similar to those of the stomach, i.e., physiological temperature and acid medium, and (ii) to correlate the *in vitro* physicochemical behavior of the obtained liposomes with α-tocopherol recovery in rat lymph. This study was also carried out to determine whether the lipids (chemical and physical forms) associated with α-tocopherol administration affected the bioavailability of the vitamin. For this purpose, α-tocopherol intestinal absorption following liposome administration and sardine oil-based preparation were compared.

## MATERIALS AND METHODS

*Materials.* The natural marine lipid mixture used for liposome preparation was supplied by Phosphotech (Nantes, France). Total lipids contained mainly phospholipids (69 wt%), among which phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the major classes, 68 and 23 wt%, respectively (32,33). EPA and DHA represented 14 and 31% of PUFA (48% of total fatty acids), respectively (32). Sardine oil was supplied by Pronova Biocare (Sandefjord, Norway). Fatty acid composition of sardine oil determined by gas chromatography (GC) indicated 40% PUFA with 7% EPA and 26% DHA. In marine lipids as well as in sardine oil, α-tocopherol was initially present at 0.01 mol%. HEPES, TRIS, and EDTA were purchased from Sigma (St. Louis, MO). Two buffer solutions were used for liposome preparations: HEPES (10 mM HEPES, 145 mM NaCl, pH 7.4) and TRIS (1 mM TRIS, 10 mM EDTA, pH 7.4). α-Tocopherol (Fisher Scientific, Elancourt, France) was used without further purification. The solvents were of analytical grade or distilled when needed.

*Liposome preparations.* α-Tocopherol-associated liposome suspensions were prepared using the total marine lipid extract. α-Tocopherol and marine lipids were dissolved in chloroform/methanol (2:1, vol/vol) and blended in ratios ranging from 0 to 12 mol%. Organic solvents were removed under a nitrogen stream followed by a lyophilization step overnight. The liposome suspensions were obtained, after a hydration step, by filtration of the lipid suspension through polycarbonate membranes of 5-μm pore diameters (Millipore Corp., Bedford, MA) as described in Reference 34. In the absence of α-tocopherol, this method led to a mixture of vesicles characterized by a variable number of lamellae and a mean diameter equal to 6.3 μm (33,34). Similar results were obtained in the presence of α-tocopherol (results not shown). Adjustments of liposome suspensions to required pH were carried out using HCl (10 N) or NaOH (10 N) solutions.

Giant vesicles were prepared using the electroformation procedure described in Reference 34. α-Tocopherol (5 mol%) was added to the marine lipids. The electrical conditions used were: 10-Hz electric field applied increasingly from 50 to 800 mV $mm^{-1}$ amplitude in 60 min. Then the chamber was left overnight at 10 Hz and 800 mV $mm^{-1}$. Vesicle dispersion was promoted by the application of a low-frequency field (4 Hz).

*Liposome characterization.* Turbidity measurements [optical density (OD) at 400 nm] were performed at 37°C in a thermostated cell support using a PerkinElmer lambda Bio 20 spectrophotometer to follow liposome stability as a function of time. Micromanipulation experiments were carried out on giant vesicles as described in Reference 34. Direct visualization of liposome behavior upon acid stress was performed using a phase-contrast microscope (Axiovert 135 with a water immersion ×40/0.75 objective; Zeiss, Jena, Germany).

*Liposome chemical hydrolysis.* Liposome dispersions (1.5 mg $mL^{-1}$) incubated at 37°C in neutral (pH 7.4) and acid (pH 1.5) solutions for 3 and 24 h were tested for diacylphospholipids and lysophospholipids. The different lipid classes were assayed after lipid extraction using the procedure of Folch *et al.* (35) and two-dimensional thin-layer chromatography (34). The different spots were scraped off and analyzed for total phosphorus (36).

*Lipid oxidation.* Propanal (secondary oxidation product of n-3 PUFA) was analyzed by static headspace GC using a DB1701 column (30 m × 32 mm; J&W Scientific, Folsom, CA) (34). Liposomes (1 mg) were incubated up to 48 h at 37°C in 1 mL HEPES solution at pH 1.5 or pH 7.4. The quantity of propanal formed upon lipid peroxidation was determined from a calibration curve using a pure aldehyde standard solution.

*α-Tocopherol analysis.* α-Tocopherol present in the liposome membrane and in the lymph was quantified by high-performance liquid chromatography (HPLC) on the corresponding lipid extracts. The HPLC system consisted of an HPLC pump (Spectra system P4000) and an automatic sample injector (Spectra system AS3000). A column (Ultrasphere ODS, 25 × 0.46 cm, Beckman) was used with methanol as mobile phase (flow rate of 1 mL $min^{-1}$). α-Tocopherol was detected by fluorescence spectrophotometry (excitation wavelength 292 nm; emission wavelength 330 nm). Quantification of α-tocopherol was carried out using a calibration curve made with pure α-tocopherol standard solutions.

*Animals and surgical procedures.* Official French regulations for the care and use of laboratory animals were followed. Male Wistar rats weighing 250–300 g were obtained from Elevage Janvier (Saint-Berthein, France). They were housed for 1 wk in a controlled environment, with constant

temperature and humidity. They were fed a fat-free diet (UAR, Epinay, France) and allowed free access to water until 24 h before the surgery. Under pentobarbital anesthesia (0.05 mg $g^{-1}$ body wt), a polyethylene catheter (i.d. 0.86 mm, o.d. 1.27 mm; Biotrol, Paris, France) was inserted into the main thoracic lymph duct of each rat for collecting the lymph (37,38). After surgery, the rats were placed in individual restraining cages in a warm environment with tap water freely available. A few hours after the surgical procedure, 0.4 g of lipids (liposome suspension or sardine oil) was administered through a gastric feeding tube followed by 1 mL water in the case of sardine oil. The lymph was collected for 24 h without fractionation in tared tubes maintained in an ice bath. During the collection period, lymph flow averaged 0.6 mL $h^{-1}$. Total lipids of lymph were immediately extracted using the method of Folch *et al.* (35), and α-tocopherol content was quantified. At least five cannulated rats were used for each lipid ingestion condition studied.

*Statistical analysis.* Differences between the two groups were tested for significance using Student's *t*-test. Data were expressed as mean values ± standard deviations.

## RESULTS AND DISCUSSION

*Influence of α-tocopherol on chemical stability.* Oral administration of lipid-soluble molecules in liposomal form provided an alternative route for active compound delivery that might be more efficient. By virtue of their lipid composition, liposomes could be effective for PUFA delivery because phospholipase $A_2$ was able to hydrolyze the phospholipids present in the marine lipid-based membranes even after a 3-h incubation in acid medium that mimicked the gastric residence time (33). However, a significant amount of propanal as secondary oxidation product of n-3 fatty acids was detected after 24 h of storage (33,34). In the present study, all oxidation experiments were performed at 37°C, which corresponded to the physiological temperature. Figures 1A and 1B present lipid oxidation as a function of time. Liposomes containing different ratios of α-tocopherol were incubated in neutral (Fig. 1A) and acid (Fig. 1B) pH media. A significant increase in the rate of propanal formation was observed at pH 7.4 and at low α-tocopherol concentration (0.01 mol%) after a 10-h induction period. This increase corresponded to the propagation phase of the free-radical autoxidation mechanism. Less than 12% of the potentially oxidizable n-3 PUFA was degraded in 24 h; 80 nmol of propanal per μmol of total lipids was the maximum propanal amount that could be produced (Fig. 1B). This result agrees with the fact that liposomes containing high amounts of PUFA can demonstrate great oxidative stability (39). Antioxidant properties of phospholipids have been reported (40,41). They may arise from (i) amine groups of PC and PE that would act *via* a nucleophile reaction as decomposers of hydroperoxides to alcohols (40) and (ii) the selective distribution of PUFA at the *sn*-2 position in marine lipids. In addition, liposome oxidative stability could also result from phospholipid conformation in the membrane (42) and/or the presence of additional lipids, such as cholesterol. Through the condensing effect of cholesterol on the bilayer arrangement over the lipid phase transition temperature (43), PUFA present in the marine lipid-based membranes could be less accessible to the attack of free radicals and/or oxygen. In Figure 1A, pro- and antioxidant properties of α-tocopherol are illustrated for different vitamin amounts added up to 12 mol%. For 2 mol% ratio, the induction period lasted 16 h. At 10 h, for 0.01 mol% α-tocopherol concentration, the rate of propanal formation decreased. When α-tocopherol content was increased up to 5 mol%, aldehyde formation was prevented for 38 h. Thus, the antioxidant activity of α-tocopherol was influenced by its concentration within the membrane, in agreement with previous studies (18,39). The antioxidant activity of tocopherols is

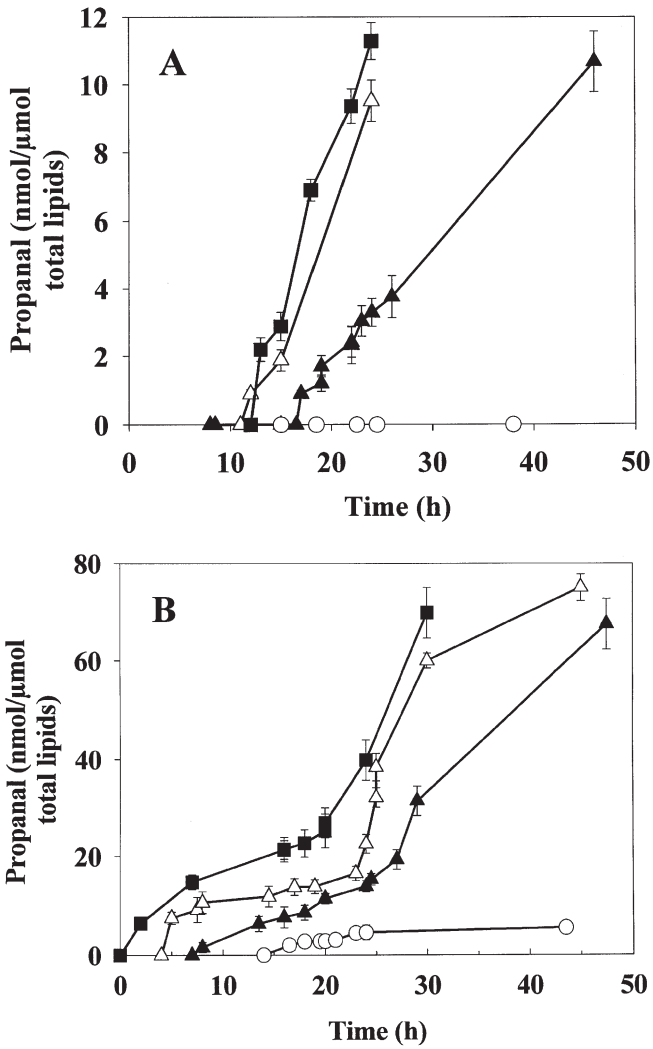


**FIG. 1.** Time-course oxidation curves showing propanal concentration (nmol/μmol total lipids) determined by headspace capillary gas chromatography as a function of α-tocopherol membrane concentration: 0.01 mol% (△), 2 mol% (▲), 5 mol% (○), 12 mol% (■), for liposomes incubated at pH 7.4 (A) and pH 1.5 (B). Lipid peroxidation was studied on liposomes prepared by filtration on 5-μm diameter filters ($[lip]_{tot}$ = 1 mg · $mL^{-1}$) incubated at 37°C. All the experiments were performed in triplicate with a standard deviation of ±8%.

mainly due to their ability to donate their phenolic hydrogens to lipid free radicals. The α-tocopheroxyl radical formed should be inactive toward stable molecules and should limit its reaction only to donation of hydrogen(s) to radicals and to radical–radical coupling (18). Moreover, an antioxidant synergy between α-tocopherol and phospholipids was reported that could involve amino groups in the regeneration of α-tocopherol by hydrogen transfer (41). For the 12 mol% α-tocopherol ratio (Fig. 1A), the vitamin antioxidant effect vanished and propanal formation was similar to that measured for 0.01 mol%. High concentrations of α-tocopherol favor side reactions, and can lead to a pro-oxidant effect in vegetable oils (18) and liposomes (39). The pro-oxidant effect of α-tocopherol was related to high concentrations of its tocopheroxyl radicals, which could be involved in reaction mechanisms described in Reference 18. Reversible reactions of the tocopheroxyl radicals with unperoxidized lipids and with lipid hydroperoxides were suggested to occur in membranes.

To be effective as an oral drug delivery system, liposomes have to clear the obstacles presented by gastrointestinal digestive fluids, especially pH variations from 1–2 (in the stomach) to 7–8 (in the intestine). Thus, the influence of α-tocopherol incorporation into the liposomes incubated in an acid medium (pH 1.5) for times up to 48 h was investigated (Fig. 1B). At a low α-tocopherol concentration (0.01 mol%), after a 5-h induction phase, the propanal formed increased to about 15% of the maximum aldehyde production. Then, the oxidation process held stable for about 15 h and then rapidly reached the maximum value of 80 nmol/μmol of total lipids. A similar profile was obtained for 2 mol% α-tocopherol, although the induction phase was slightly longer and the curve was shifted toward lower propanal values. For 5 mol% α-tocopherol incorporated into liposome membranes, the induction phase lasted 12 h. The pro-oxidant role of α-tocopherol at 12 mol% was again demonstrated when liposomes were stored under acid conditions. On the whole, the pro- and antioxidant activities of α-tocopherol for liposomes incubated in an acid medium were similar to those evidenced in a neutral medium. However, medium acidification acted to shorten the induction phase and increase the amount of propanal produced, whatever the α-tocopherol incorporation ratio. These results are in agreement with the fact that lipid peroxides decompose faster at high temperatures and low pH in the presence of transition metals (18). Moreover, marine lipid-based liposomes placed in acid conditions are subjected to vesicle aggregation and complex supramolecular and/or morphological changes as a function of time (see below; Ref. 34). These structural rearrangements could influence lipid oxidative stability (39,44).

Chemical degradation of liposome diacylglycerophospholipids was assayed looking at lysophospholipid levels, i.e., lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) resulting from carboxy ester hydrolysis (Table 1). At low α-tocopherol concentration (0.01 mol%), 1-d storage at 37°C favored hydrolysis, as evidenced by a significant increase in lysophospholipid amounts. The hydrolysis process was favored by acid medium conditions. We already showed that hydrolysis in the marine lipid-based liposomes was more pronounced in acid medium than in neutral medium at 25°C (34) in agreement with ester cleavage being catalyzed in the presence of acid (45). In addition, the hydrolysis process was significantly amplified when physiological pH and temperature conditions were combined (33). The influence of 5 mol% α-tocopherol incorporation on chemical vesicle behavior was studied because this concentration corresponded to the maximal oxidative stability observed for the marine lipid-based liposomes (Fig. 1A,B). α-Tocopherol presence slightly increased the amount of LPC and LPE of liposomes stored in neutral medium, whereas phospholipid degradation was more pronounced in acid storage conditions. The influence of α-tocopherol incorporation on liposome membrane properties has been widely reported in the literature. The role of vitamin E on membrane behavior appears to be very dependent on the lipid composition. For PC-based liposomes, the best stability, assayed by the release of an entrapped water-soluble dye, was obtained for 15 mol% α-tocopherol and was similar to that observed with 37 mol% cholesterol (46). In this case, α-tocopherol decreased membrane fluidity for lipids over their phase transition temperature as reported for cholesterol (43). However, the condensing effect of α-tocopherol progressively vanished as fatty acid unsaturation increased (47). In this study, in which the PUFA content of the lipids used was high, α-tocopherol could have favored lipid hydrolysis by increasing membrane permeability to protons when liposomes were incubated in acid medium. As a consequence, the ester cleavage rate could be increased by increasing the amount of catalyst. The fact that the presence of α-tocopherol also accelerated lipid hydrolysis under neutral storage conditions remained unexplained.

*Influence of α-tocopherol on structural stability.* The influence of 5 mol% α-tocopherol incorporation on liposome morphological stability was studied for two types of vesicles,

**TABLE 1**
**Relative Composition (wt%) of Choline and Ethanolamine Species: PC, PE, LPC, and LPE of Liposomes Containing 0.01 and 5 mol% of α-Tocopherol After Incubation at 37°C in HEPES Buffer and Acid Medium[a]**

| α-Tocopherol (mol%) | pH | Incubation time (h) | PC | PE | LPC | LPE | Others[b] |
|---|---|---|---|---|---|---|---|
| 0.01 | 7.4 | 0 | 67.7 | 23.1 | 0.8 | 1.8 | 6.6 |
| | | 24 | 65.6 | 20.4 | 2.1** | 5.5** | 6.5 |
| 5 | | 3 | 65.9 | 21.2 | 3.0** | 3.2** | 6.7 |
| | | 24 | 63.8 | 18.3* | 4.9** | 6.7** | 6.3 |
| 0.1 | 1.5 | 3 | 60.8* | 15.8** | 8.1** | 9.2** | 6.1 |
| | | 24 | 56.3** | 14.5** | 10.1** | 9.1** | 10.0 |
| 5 | | 3 | 56.2** | 13.1** | 11.9** | 11.7** | 7.1 |
| | | 24 | 41.5** | 10.0** | 28.4** | 13.2** | 6.9 |

[a]All experiments were performed at least twice with a standard deviation of 4%. *Significantly different from initial suspensions ($P \leq 0.05$). **Significantly different from initial suspensions ($P \leq 0.01$). Abbreviations: *P*, probability in the Student's *t*-test; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine.
[b]Other phospholipids (degraded or intact species), i.e., phosphatidylinositol, phosphatidylserine, sphingomyelin.

i.e., giant oligolamellar liposomes prepared by electroformation and multilamellar vesicles obtained by simple filtration. Liposome micromanipulations were performed to change the outside environment of isolated giant vesicles. Figures 2A–D show a typical shape transformation sequence observed by phase-contrast microscopy when the medium outside the vesicle was acidified from 7.4 to 1.5. Vesicle diameter drastically decreased during the first 5 min. Simultaneously, granulations appeared in the liposome internal volume. No significant membrane rearrangements were observed after 5 min. Neutralization of the external medium did not reverse the structural rearrangements (results not shown). The possible roles of pH in liposome membrane rearrangements were already discussed (34). The present results pointed out the specific influence of α-tocopherol on structural stability. α-Tocopherol incorporation into liposomes accelerated the kinetics of membrane rearrangements compared to the behavior of α-tocopherol-free liposomes (34).

Figure 3 shows the evolution of turbidity with time of α-tocopherol-associated liposomes obtained by the filtration technique and stored at two different pH values. The turbidity remained stable for about 1 d, for liposomes containing 0.01 mol% α-tocopherol and stored at pH 7.4. In contrast, turbidity slowly decreased with time for a 5 mol% α-tocopherol ratio. Incorporation of the lipophilic vitamin into the bilayer altered the liposome stability, suggesting a modification in membrane characteristics with time. When liposomes were placed in an acid medium (pH 1.5), turbidity drastically increased, indicating vesicle aggregation as confirmed by optical microscopy observation (results not shown). Increasing the α-tocopherol amount in the liposomes seemed to enhance the aggregation phenomenon, as suggested by the turbidity values. Whatever the α-tocopherol concentration, turbidity decreased with time. This turbidity pattern was already observed for α-tocopherol-free liposomes (33), although the decrease was more pronounced in the presence of 5 mol% vitamin E. Study of α-tocopherol-free liposomes showed that the turbidity decrease could be interpreted in terms of membrane rearrangements and small aggregate formation (33). These structural modifications were also probably implied in the behavior of α-tocopherol-associated liposomes. In the physiological digestion process, liposomes stay about 2–3 h in acid medium (stomach) before moving to a neutral environment (intestine). To mimic this change in pH, an α-tocopherol-associated liposome sample previously stored under acid conditions for 3 h was further neutralized. Medium neutralization induced a decrease of suspension turbidity correlated, at least partly, with a dispersion of the aggregated liposomes. However, in contrast with results observed for α-tocopherol-free liposomes (33), the resulting OD was slightly lower than that of the untreated sample, suggesting the occurrence of enhanced membrane rearrangements. On the whole, in acid conditions, it seemed that the incorporation of 5 mol% α-tocopherol reflected the morphologic instability already observed for vesicles in neutral conditions.

Membrane chemical asymmetry between the monolayers may be responsible, at least partly, for structural rearrange-

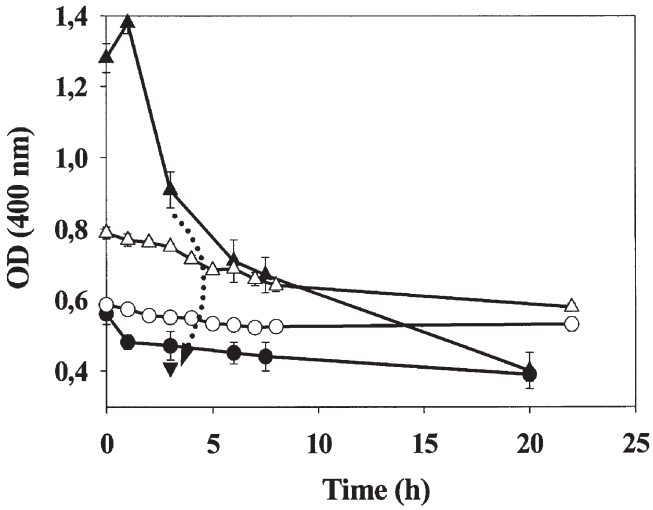


**FIG. 3.** Variations of optical density (OD) at 400 nm as a function of time for α-tocopherol-associated liposomes incubated at different pH values and 37°C: 0.01 mol% α-tocopherol, pH 7.4 (○); 0.01 mol% α-tocopherol, pH 1.5 (△); 5 mol% α-tocopherol, pH 7.4 (●); 5 mol% α-tocopherol, pH 1.5 (▲). Liposomes were prepared by the filtration technique using 5-μm pore diameter filters ($[lip]_{tot}$ = 0.25 mg · $mL^{-1}$). Acidification was performed using HCl (10 N) solution. A sample corresponding to 5 mol% α-tocopherol-associated liposomes stored during 3 h in acid medium (pH 1.5) was neutralized with NaOH (10 N) solution (▼). All the experiments were performed in triplicate with a standard deviation of ±10%.

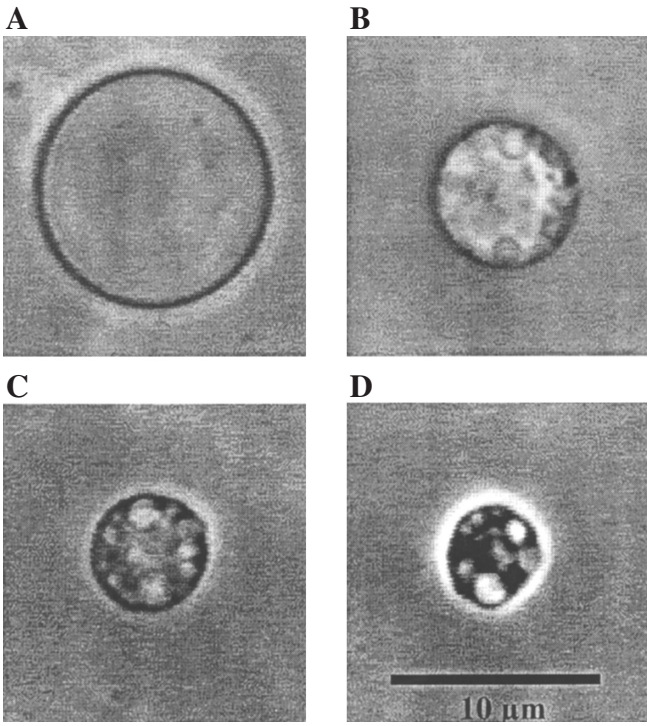


**FIG. 2.** Morphological changes observed by phase-contrast microscopy of a 5 mol% α-tocopherol-associated giant vesicle. The vesicle was swollen in TRIS buffer at pH 7.4 (A) and transferred in acid TRIS solution (pH 1.5). The incubation time in the low-pH medium was 30 s (B), 1 min (C), and 5 min (D). The temperature was 25°C. The bar corresponds to 10 μm.

ments such as invaginations or buddings that can occur both in multilamellar vesicles and giant unilamellar liposomes (48,49). In our case, these rearrangements were actually observed by optical microscopy for giant vesicles and evidenced by turbidimetry for filtered liposomes associated with 5 mol% α-tocopherol and stored in acid medium. The morphologic instability observed may be correlated with high amounts of lysophospholipids and/or increased membrane permeability to water and protons due to α-tocopherol presence in PUFA-rich membranes. The difference in the rearrangement kinetics between filtered liposomes and giant vesicles may arise from a higher lamellarity in filtered liposomes and the existence of aggregated vesicles.

In vivo *α-tocopherol lymphatic absorption.* Vitamin E absorption depends on an individual's ability to absorb fat (24,50). Fractional absorption of α-tocopherol is often overestimated (24). Rats can be considered as a good model for humans as far as digestion processes are concerned. Rat lymphatic absorption rates ranging from 40 to 65% of total ingested α-tocopherol have been found (51,52). Whether α-tocopherol absorption is influenced by unsaturation of the administered oil remains controversial (53–55). However, α-tocopherol absorption is largely influenced by the co-ingested lipids (25,56) as well as by α-tocopherol concentration (53). In this work, the intestinal absorption of α-tocopherol could not be precisely determined because lymph recovery was not quantitative. Thus, efficiencies of the liposome form or sardine oil to deliver α-tocopherol were only compared and discussed on the lymphatic vitamin concentration basis.

α-Tocopherol was not detected in lymph of rats fed a fat-free diet. Figure 4 shows that liposomes facilitated α-tocopherol uptake after oral delivery to rats compared to sardine oil ingestion. The vitamin recovery in lymph increased twofold after liposome administration compared to sardine oil ingestion when both were associated with low α-tocopherol concentration (0.01 mol%). This result was in agreement with α-tocopherol recovery in liver being highest after oral ingestion of liposomes rather than oil (25). It was reported that high levels of linoleic acid in diets (54) and linolenic acid in the form of fatty acids (55) or natural oils (53) decreased the absorption rate of vitamin E. In this study, the PUFA amount in sardine oil was about twice that in liposomes. This could partly explain the lower recovery of α-tocopherol in rats fed sardine oil. Nevertheless, liposome phospholipids might promote vitamin uptake in rats. Indeed, phospholipase $A_2$ was able to catalyze *in vitro* marine phospholipid hydrolysis even when liposomes were previously submitted to an acid treatment (33). Consequently, the higher amount of lysophospholipids may facilitate α-tocopherol incorporation into bile salt–lipid mixed micelles, in agreement with lymphatic absorption of α-tocopherol enhanced by LPC (56). Thus, the relative α-tocopherol lymphatic absorption obtained in rats fed sardine oil or liposomes may account for different parameters, in particular, the lipid composition (PUFA amount, phospholipid presence, etc.). However, when α-tocopherol was increased up to 5 mol% in both ingested lipid forms, α-tocopherol lymphatic recovery was three times higher in rats

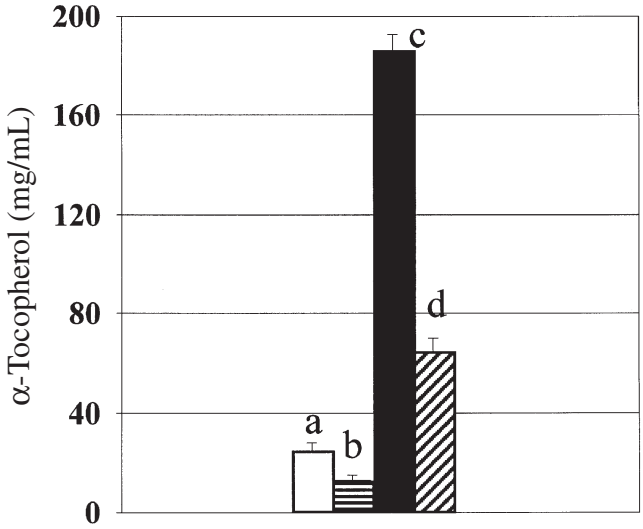


**FIG. 4.** α-Tocopherol lymphatic recovery (mg · $mL^{-1}$ lymph) in rats after oral administration of different lipid forms: α-tocopherol-associated liposomes, 0.01 mol% (empty bar) and 5 mol% (solid bar), and sardine oil containing α-tocopherol, 0.01 mol% (striped bar) and 5 mol% (hatched bar). Liposomes were prepared by the filtration technique using 5-μm pore diameter filters. Lipid ingestion by rats corresponded to 0.4 g liposome suspension or oil. Marine lipids and sardine oil initially contained 0.01 mol% of α-tocopherol. Data are means ± standard deviations of at least five rats. Letters a, b, c, and d indicate statistical difference, $P < 0.05$.

fed liposomes compared to rats fed sardine oil. In other words, increasing the α-tocopherol/lipid ratio led to higher absorption efficiency from liposomes than from oil. In the case of liposomes, this result may account for physicochemical membrane instability of 5 mol% α-tocopherol-associated vesicles compared with vesicles containing only 0.01 mol% α-tocopherol, as pointed out by the *in vitro* studies. Membrane destabilization, suggested by turbidity measurements and visualized by optical microscopy in the case of giant liposomes, associated with increased formation of lysophospholipids might favor liposome solubilization by bile salt and thus, α-tocopherol incorporation into the mixed micelles. A similar threefold increase of α-tocopherol absorption also has been measured for a self-emulsifying vitamin E preparation tested and compared to soft gelatin vitamin E capsules (29). The extent of absorption under fasting conditions has been found satisfactory. Because bile salts are required for vitamin E absorption, vitamin administration should be associated with food to ensure the best vitamin bioavailability. Moreover, results concerning the kinetic behavior of α-tocopherol administered in oil and in liposome forms have suggested that liposome formulation might result in longer persistence of the vitamin in plasma compared to oil preparation (28). This result was obtained after oral administration to heifers and has to be verified in rats and humans.

α-Tocopherol (5 mol%) incorporated into the membranes acted on the physicochemical behavior of the liposome structures. α-Tocopherol prevented the lipid membranes from peroxidation by increasing the induction phase and reducing the

propanal production even in drastic conditions such as low pH. However, it induced structural rearrangements and favored chemical hydrolysis in liposomes, especially when the suspensions were stored in acid conditions. This last phenomenon presumably occurred through increased membrane permeability. Membrane integrity was not totally maintained. *In vivo* α-tocopherol lymph recovery evidenced that liposomes acted by improving the vitamin uptake compared to a classical fish oil administration. The extent of α-tocopherol absorption from liposome formulation may arise from lysophospholipids generated through phospholipase $A_2$ hydrolysis and vesicle physicochemical instability due to α-tocopherol incorporation. These results could be useful with a view to promoting α-tocopherol absorption in bile-duct-obstructed patients or in the case of chronic cholestatic liver disease. Moreover, combination of α-tocopherol in liposomes may also enable administration of mixtures of lipid-soluble vitamin and PUFA in a single dose.

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# Novel Vitamin E Derivative with 4-Substituted Resorcinol Moiety Has Both Antioxidant and Tyrosinase Inhibitory Properties

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**ABSTRACT:** A novel vitamin E derivative, (6″-hydroxy-2″,5″,7″,8″-tetramethylchroman-2″-yl)methyl 3-(2′,4′-dihydroxyphenyl)propionate (TM4R), which has a chromanoxyl ring and 4-substituted resorcinol moieties, was synthesized; and its inhibitory effects on tyrosinase, antioxidant ability, and lightening effect of ultraviolet B (UVB)-induced hyperpigmentation were estimated. TM4R showed potent inhibitory activity on tyrosinase, which is the rate-limiting enzyme in melanogenesis. The scavenging activities of TM4R on 1,1-diphenyl-2-picrylhydrazyl and hydroxyl radicals were found to be nearly the same as those of α-tocopherol. Furthermore, an efficient lightening effect was observed following topical application of TM4R to UVB-stimulated hyperpigmented dorsal skin of brownish guinea pigs. These results suggest that TM4R may be a candidate for an efficient whitening agent, possibly by inhibiting tyrosinase activity and biological reactions caused by reactive oxygen species.

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A number of factors determine the color of mammalian skin and hair. The most important factor is the degree and distribution of melanin pigmentation. Compounds that inhibit melanin synthesis are useful not only in cosmetics as skin-whitening agents but also as a remedy for disturbances in pigmentation. Tyrosinase (phenol oxidase) is known to be a key enzyme for melanin biosynthesis in plants, microorganisms, and mammalian cells (1). Therefore, many tyrosinase inhibitors have been tested in cosmetics and pharmaceuticals as a way of preventing overproduction of melanin in epidermal layers (2).

Also, various types of stimulation, including exposure to ultraviolet (UV) radiation, induce lipid peroxidation in the skin, which may in turn cause damage to epidermal cells, leading to postinflammatory hyperpigmentation. One of the biggest causative agents of hyperpigmentation is probably oxidative stress caused by UV (3). To date, however, research on the regulation of melanogenesis has focused on factors that affect tyrosinase, the rate-limiting enzyme in the melanogenic pathway, including research on chemicals that inhibit tyrosinase function. Furthermore, considering the importance of counteracting oxidative stress caused by UV as a means to prevent skin damage, it is important to design a multifunctional skin-whitening agent with both antioxidant and anti-tyrosinase abilities.

Oral intake of vitamin E (α-tocopherol) has been reported to be effective for the treatment of facial hyperpigmentation (4). Also, α-tocopheryl ferulate, which is a compound of α-tocopherol and ferulic acid connected by an ester bond, has been reported to suppress melanogenesis and inhibit biological reactions induced by reactive oxygen species (5). α-Tocopherol is known to act as an inhibitor of oxidative attack of free and membrane-bound unsaturated fatty acids and also scavenges active oxygen species such as superoxide anion radicals, singlet molecular oxygen, and hydroxyl radicals (6) and thus has been proposed to improve facial hyperpigmentation (4). The antioxidant properties of vitamin E are based on the ability of the chromanoxyl ring to interact with different free radicals (7).

Also, 4-substituted resorcinols have been reported as a potent tyrosinase inhibitor (8), and their structure–activity relationships and inhibition mechanisms were examined in detail (9–11).

Based on these findings, we designed and synthesized a novel vitamin E derivative linking two biologically active moieties (chromanoxyl ring and 4-substituted resorcinol), named (6″-hydroxy-2″,5″,7″,8″-tetramethylchroman-2″-yl)methyl 3-(2′,4′-dihydroxyphenyl)propionate (TM4R) (Fig. 1). The antioxidant and tyrosinase inhibitory activities and skin-lightening effect on UVB-induced pigmentation of TM4R were evaluated.

## EXPERIMENTAL PROCEDURES

*Materials.* 2-Carboxy-2,5,7,8-tetramethylchroman-6-ol [(*R,S*)-Trolox] was purchased from Aldrich Chemical Co. (Milwaukee, WI). 3-(2′,4′-Dihydroxyphenyl)propionic acid (DPPacid) was purchased from Fluka Chemie AG (Buchs, Switzerland). *N,N′*-Dicyclohexylcarbodiimide (DCC) and 4-dimethylamino-

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Abbreviations: COSY, correlation spectroscopy; DCC, *N,N′*-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMSO, dimethylsulfoxide; DOPA, dihydroxyphenylalanine; DPPacid, 3-(2′,4′-dihydroxyphenyl)propionic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ESR, electron spin resonance; HMBC, heteronuclear multiple bond connectivity; HR–FAB–MS, high resolution–fast atom bombardment–mass spectrometry; $IC_{50}$, concentration at which 50% inhibition occurs; NBA, 3-nitrobenzyl alcohol; NMR, nuclear magnetic resonance; TM, 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol; TM4R, (6″-hydroxy-2″,5″,7″,8″-tetramethylchroman-2″-yl)methyl 3-(2′,4′-dihydroxyphenyl)propionate; Trolox, 2-carboxy-2,5,7,8-tetramethylchroman-6-ol; UV, ultraviolet; UVB, ultraviolet B.

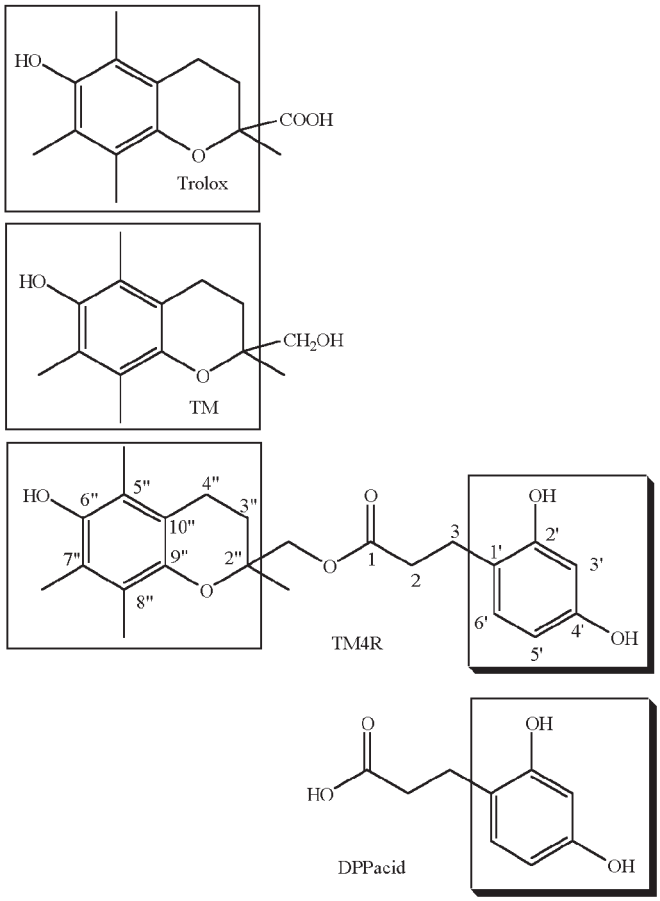



**FIG. 1.** The chemical structures of Trolox, TM, TM4R, and DPPacid. The boxed part with shadow: 4-substituted resorcinol skeleton. The boxed part without shadow: chromanoxyl ring moiety. Trolox, 2-carboxy-2,5,7,8-tetramethylchroman-6-ol; TM, 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol; TM4R, (6″-hydroxy-2″,5″,7″,8″-tetramethylchroman-2″-yl)methyl 3-(2′,4′-dihydroxyphenyl)propionate; and DPPacid, 3-(2′,4′-dihydroxyphenyl)propionic acid.

pyridine (DMAP) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Kojic acid and arbutin were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). All other reagents were of analytical grade.

*Spectral analysis.* UV spectra were measured with a Beckman DU 640 spectrophotometer. High resolution–fast atom bombardment–mass spectrometry (HR–FAB–MS) was done with a JMS-HX 110A instrument (Jeol, Tokyo, Japan) with a xenon fast atom bombardment gun with 3-nitrobenzylalcohol (NBA) as the matrix. Nuclear magnetic resonance (NMR) spectra were recorded at 25°C on a JMS-HX 110A FT-NMR spectrometer (Jeol) using acetone-$d_6$ as solvent and tetramethylsilane as internal standard. Electron spin resonance (ESR) spectra were measured using a Jeol JES-FE1XG spectrometer operating at 9.5 GHz with 100 kHz field modulation, a microwave power of 5mW, and a modulation amplitude of 0.1 mT.

*Preparation of 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol (TM).* TM was prepared as described (12).

*Preparation of TM4R.* To a solution of TM (1.50 g), DPPacid (1.15 g), and DMAP (0.238 g) in dry benzene was added DCC (1.30 g), and the solution stirred for 3 h at 50°C. After leaving overnight, the solvent was evaporated and the solid residue was macerated with acetone. The acetone-soluble fraction was filtered, and the crude mixture was separated by column chromatography on silica gel with hexane/diethyl ether (1:4, vol/vol) as the eluent. Thus, pure TM4R was obtained as colorless oil (625 mg yield), subsequent to identification by NMR and MS analysis.

TM4R: colorless oil, HR–FAB–MS (NBA): $[M]^+$ *m/z* 400.1886 ($C_{23}H_{26}O_6$ requires 400.1885). $^1$H NMR (acetone-$d_6$): δ = 1.25 (3H, *s*, 2″-$CH_3$), 1.76 (1H, *m*, 3″-$CH_{2a}$), 1.90 (1H, *m*, 3″-$CH_{2b}$), 2.04 (3H, *s*, 8″-$CH_3$), 2.09 (3H, *s*, 5″-$CH_3$), 2.13 (3H, *s*, 7″-$CH_3$), 2.59 (2H, *m*, 4″-$CH_2$), 2.63 (2H, *m*, 2-$CH_2$), 2.83 (2H, *m*, 3-$CH_2$), 4.05 (1H, *d*, *J* = 11 Hz, 2″-$CH_{2a}$-O), 4.10 (1H, *d*, *J* = 11 Hz, 2″-$CH_{2b}$-O), 6.25 (1H, *dd*, *J* = 2.3, 8.2 Hz, 5′-CH), 6.39 (1H, *d*, *J* = 2.3 Hz, 3′-CH), 6.89 (1H, *d*, *J* = 8.2 Hz, 6′-CH). $^{13}$C NMR (acetone-$d_6$): 11.8 (5″-$CH_3$), 12.0 (8″-CH), 12.7 (7″-$CH_3$), 20.8 (4″-$CH_2$), 22.3 (2″-$CH_3$), 26.1 (3-C), 29.4 (3″-C), 35.1 (2-C), 68.6 (2″-$CH_2$O), 73.9 (2″-C), 103.3 (C-3′), 107.1 (5′-C), 117.5 (10″-C), 118.5 (1′-C), 120.4 (5″-C), 122.4 (8″-C), 123.0 (7″-C), 131.0 (6′-C), 145.2 (9″-C), 146.4 (6″-C), 156.5 (2′ -C), 157.6 (4′-C), 173.1 (1-C).

*Tyrosinase assays.* Mushroom tyrosinase [EC 1.14.18.1] activities were determined by using L-tyrosine (13) or DL-dihydroxyphenylalanine (DL-DOPA) (9) as the substrate, as previously described. L-Tyrosine oxidation assay was done as follows: mushroom tyrosinase solution (0.05 mL, 625 U/mL; Sigma Chemical Co. St. Louis, MO), 1.0 mL of L-tyrosine (5.52 mM), 0.35 mL of McIlvaine buffer (pH 6.8), and 0.1 mL of dimethylsulfoxide (DMSO) with or without sample were mixed and then incubated at 37°C. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm up to the appropriate time (usually not longer than 20 min). DL-DOPA oxidation assay was done as follows: mushroom tyrosinase solution (0.1 mL, 625 U/mL), 0.7 mL of DL-DOPA buffer solution (2.0 mM), 0.1 mL of McIlvaine buffer (pH 6.8), and 0.1 mL of DMSO with or without sample were mixed and incubated at 25°C. The absorbance was measured at 475 nm between the incubation time of 0.35 and 0.45 min. The extent of inhibition by the addition of samples is expressed as the percentage necessary for 50% inhibition ($IC_{50}$). Michaelis constant ($K_m$), maximal velocity ($V_{max}$), and inhibitor constant ($K_i$) of tyrosinase were determined by Lineweaver-Burke's plot using various concentrations of DL-DOPA. Kojic acid and arbutin were used as positive standards.

*Estimation of hydroxyl radical scavenging activity.* Hydroxyl radical scavenging activities were estimated by the ESR spin-trapping method (14). Seventy-five microliters of 5 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Mohr's salt) solution, 50 μL of DMSO with or without compounds, and 20 μL of 0.1 M 3,3,5,5-tetramethyl-1-pyrroline-*N*-oxide were mixed. Then, 75 μL of 5 mM $H_2O_2$ was added to the resulting solution and shaken on a vortex mixer. After 60 s, ESR spectra were measured. Mn(II) doped in MgO was used as standard.

*Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH).* Free radical scavenging activity of each compound was assayed using a stable free radical, DPPH, according to the method previously reported (12) with modification. Thus, the reaction mixture contained 0.5 mL of 60 μM DPPH in ethanol and 0.5 mL of sample solution in ethanol. After the reaction was carried out at room temperature for 20 min, the free radical–scavenging activity of each compound was quantified by the decolorization of DPPH at 514 nm.

*Pigmentation assays.* UVB-induced hyperpigmentation was elicited on the backs of brownish guinea pigs (Kyudo Co., Ltd., Saga, Japan) using a modification of the method of Yokota *et al.* (15). Animal care was in accordance with institution guidelines. Guinea pigs were gently tethered without anesthesia and four separate areas (2.2 × 2.2 cm) on the back of each animal were exposed to UVB radiation (Toshiba FL40S-BLB; Tokyo, Japan). The total energy dose of UVB was 1350 mJ/cm$^2$ per exposure. Groups of eight animals were used in each experiment. The animals were exposed to UVB radiation once a day for 3 d. Then, 0.25 M sample solution (base solution: propylene glycol/ethanol 1:6) was topically applied to the irradiated areas (20 μL/cm$^2$) five times per week for five successive weeks, while the base solution without sample was applied to the other area as a control. Each sample-applied area was assigned using the table of random sampling numbers. Once every week from the beginning of sample application, the degree of pigmentation was assessed as the absolute value of ΔL*, calculated by the L* value measured with a chromameter (Minolta CR-200; Osaka, Japan) as follows: ΔL* = L* (at each day measured) – L* (at day 0). An increase in the absolute value of ΔL* values indicated the UVB-induced pigmentation of the skin. Kojic acid (16) and arbutin (17) were used to compare with TM4R. Differences between mean values were assessed for statistical significance by using the two-tailed paired Student's *t*-test.

## RESULTS

*Synthesis of TM4R.* TM4R was synthesized in two steps from (*R,S*)-Trolox as shown in Scheme 1. The carboxyl group of Trolox was reduced with $LiAlH_4$ to produce the corresponding TM at 80% yield. Condensation of TM with DPPacid in the presence of DCC and a catalytic amount of DMAP in benzene affords TM4R at 25% yield. The structure of TM4R, and its assignments of $^1$H and $^{13}$C NMR were confirmed by HR–FAB–MS, $^1$H-$^1$H COSY (correlation spectroscopy), $^{13}$C-$^1$H COSY, and HMBC (heteronuclear multiple bond connectivity).

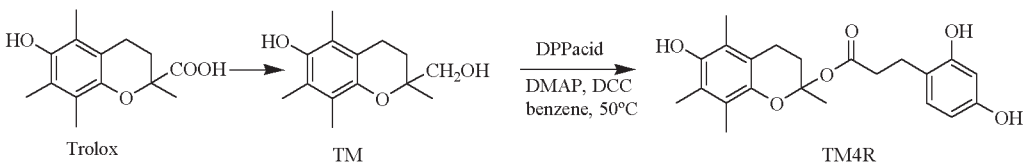
*Inhibitory effect by TM4R on tyrosinase.* The mushroom tyrosinase inhibitory activities of TM4R and some other compounds were determined using L-tyrosine or DL-DOPA as substrate (Table 1). When L-tyrosine was used as a substrate, TM4R and DPPacid showed stronger inhibitory activities than those of kojic acid (18) and arbutin (19), which are known tyrosinase inhibitors. On the other hand, TM, Trolox, and α-tocopherol did not show any tyrosinase-inhibitory activities. Therefore, potent tyrosinase-inhibitory activity of TM4R should be caused by 4-substituted resorcinol moiety, which is the structure common to TM4R and DPPacid (Fig. 1). The Lineweaver-Burke plot of TM4R for DL-DOPA as a substrate on mushroom tyrosinase is shown in Figure 2. TM4R and DPPacid increased the $K_m$ values of tyrosinase in a dose-dependent manner but did not change the $V_{max}$ values and, thus, were the competitive inhibitors with $K_i$ values of 2.74 and 11.5 μM, respectively (Table 1). These results supported our previous notions about the structure–activity relationship, that the 4-substituted resorcinol moiety is most important to reveal the potent tyrosinase inhibitory activity, and that the mode of inhibition is competitive (9). It should be added that, unexpectedly, condensation of DPPacid with the hydrophobic chromanoxyl ring to TM4R increased its inhibitory activity.

**TABLE 1**
**Effects of Compounds on Mushroom Tyrosinase[a]**

| Compound | Substrate: L-Tyrosine $IC_{50}$ (μM) | Substrate: DL-DOPA $IC_{50}$ (μM) | DL-DOPA $K_i$ (μM) | DL-DOPA Type of inhibition |
|---|---|---|---|---|
| TM4R | 0.325 | 23 | 2.74 | Competitive |
| DPPacid | 3.02 | 62 | 11.5 | Competitive |
| TM | >423 | ND | ND | ND |
| Trolox | >400 | ND | ND | ND |
| α-Tocopherol | >232 | ND | ND | ND |
| Kojic acid | 8.66[a] | 17[b] | 11.8[a] | Mixed[a] |
| Arbutin | 306 | 104,000 | ND | ND |

[a]ND, not determined; TM4R, (6″-hydroxy-2″,5″,7″,8″-tetramethylchroman-2″-yl)methyl 3-(2′,4′-dihydroxyphenyl)propionate; DPPacid, 3-(2′,4′-dihydroxyphenyl)propionic acid; TM, 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol; Trolox, 2-carboxy-2,5,7,8-tetramethylchroman-6-ol; $IC_{50}$, concentration at which 50% inhibition occurs.
[b]Obtained from data of Reference 9.

*Antioxidant activity.* The free radical–scavenging activities of TM4R and some other antioxidants were determined by the use of a stable free radical, DPPH, and hydroxyl radical (Table 2). Determination of the reducing activity of the DPPH radical was carried out by spectrophotometer, and the $IC_{50}$ value of TM4R was 3.9 μM, which was nearly the same as those of α-tocopherol, Trolox, and TM (Table 2). Taking no activity of DPPacid into consideration, the chromanoxyl ring moiety should be important for radical-scavenging activity of TM4R (Fig. 1). Determination of the hydroxyl radical–




**SCHEME 1**

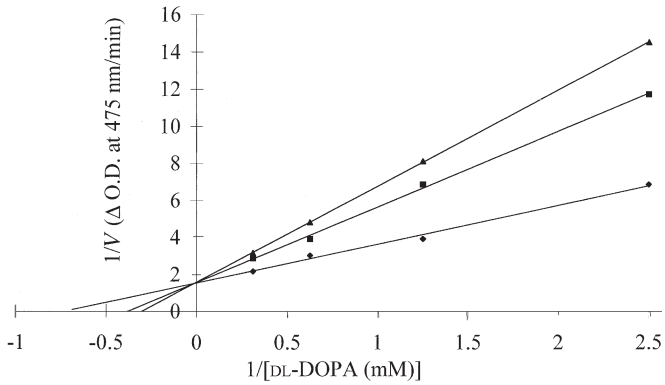



**FIG. 2.** Lineweaver-Burke plots of mushroom tyrosinase and DL-DOPA in the absence or presence of TM4R. ♦ Control, ■ 1.88 μM, ▲ 3.75 μM. O.D., optical density; DOPA, dihydroxyphenylalanine; for other abbreviation see Figure 1.

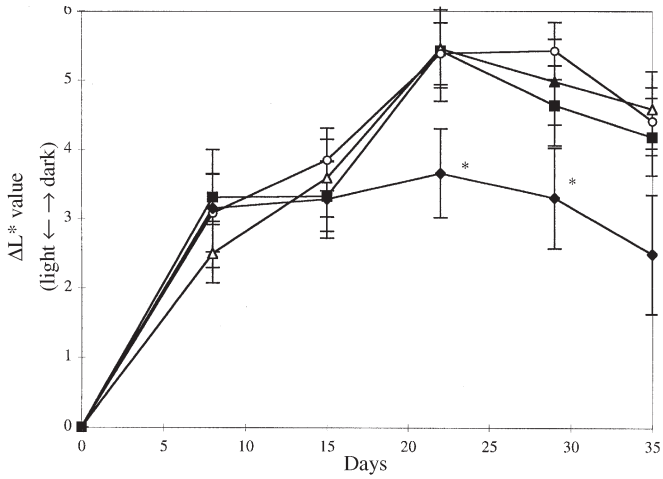



**FIG. 3.** The degree of pigmentation (ΔL* value) before and after daily topical applications (five times per week) of base solution [propylene glycol/ethanol (1:6)] (control, ○), TM4R (0.25 M in base solution, ♦), kojic acid (0.25 M in base solution, (■), or arbutin [0.25 M in base solution, ▲). The applications were continued for 35 d. Data are expressed as mean value ± standard error of the mean ($n$ = 8). L* values at day 0 are 49.9 ± 2.7 (control), 48.9 ± 3.4 (TMR), 50.1 ± 2.9 (kojic acid), and 49.9 ± 2.8 (arbutin). Student's $t$-test was used for the statistical analysis of the data. (*$P < 0.05$, vs. control). See Figure 1 for abbreviations.

scavenging activity of TM4R was carried out by the ESR spin-trapping method, and its scavenging activity at 1.0 and 10 mM (91 and 100%) was nearly the same as that of α-tocopherol (Table 2).

*Pigmentation assays.* To study mammalian melanogenesis *in vivo*, and particularly to evaluate the effects of chemical and physical agents on skin pigmentation, guinea pigs have been widely used (15) because they have similar pigmentary systems and their skin bears a morphologic resemblance to human skin (20). An increase in the absolute values of ΔL*, shown in Figure 3, were indicated by the UVB-induced pigmentation of the skin before and after up to 35 d of daily topical applications of TM4R, kojic acid, and arbutin. The ΔL* values of the only TM4R-treated skins of the eight guinea pigs (3.66 ± 0.64 at 22 d, 3.30 ± 0.73 at 29 d) were significantly lower than those of the base solution-treated skins (5.38 ± 0.44 at 22 d, 5.42 ± 0.41 at 29 d). The ΔL* values of arbutin- and kojic acid-treated skins were not significantly different from those of the control treatment (Fig. 3). It should be noted that both arbutin and kojic acid have been reported to show skin-whitening effects (16,17). However, it is not easy to relate our data precisely to those of earlier reports because of differences in test methods (e.g., dose of UV radiation, the applied concentration, etc.). During the course of these experiments, TM4R never induced erythema.

**TABLE 2**
**Antioxidant Activity of the Compounds**

| Compound | Hydroxyl radical (%)[a] 1.0 mM | 10 mM | DPPH [$IC_{50}$ (μM)][b] |
|---|---|---|---|
| TM4R | 91 | 100 | 3.9 |
| TM | ND | ND | 3.2 |
| Trolox | ND | ND | 3.3 |
| α-Tocopherol | 70 | 93 | 3.9 |
| DPPacid | ND | ND | No activity at 50 μM |

[a]Hydroxyl radical–scavenging activity was expressed as % inhibition concentration of hydroxyl radicals generated by Fe(II) and $H_2O_2$ (Fenton reaction).
[b]The $IC_{50}$ values were calculated from regression lines where the abscissa represented the concentration of tested compound and the ordinate the average percent reduction of DPPH radical from three separate tests. For abbreviations see Table 1.

## DISCUSSION

In this study, we designed and synthesized a novel vitamin E derivative, TM4R, linking two biologically active moieties (4-substituted resorcinol moiety for tyrosinase inhibitory activity and chromanoxyl ring moiety for antioxidant activity). TM4R showed more potent tyrosinase inhibitory activity than kojic acid, which is known as a potent tyrosinase inhibitor. The scavenging activities of TM4R on DPPH and hydroxyl radicals were found to be nearly the same as those of α-tocopherol, which is known as a potent antioxidant. Furthermore, an efficient lightening effect was observed following topical application of TM4R to UVB-stimulated hyperpigmented dorsal skin of brownish guinea pigs.

4-Substituted resorcinols have been reported as potent tyrosinase inhibitors (8), and their structure–activity relationships and inhibition mechanisms were examined in detail (9–11). Therefore, potent tyrosinase inhibitory activity of TM4R should be caused by 4-substituted resorcinol moiety. Unexpectedly, condensation of DPPacid with hydrophobic chromanoxyl ring to TM4R increased its inhibitory activity (Table 1). For 4-substituted resorcinol-type inhibitors, the resorcinol moiety may bind to the binuclear active site and its side chain may be associated with the hydrophobic protein pocket close to the active site (21). In other words, in addition to the stabilizing effect of resorcinol moiety to the binding site, the side chain of the 4-substituted resorcinol-type inhibitor seems to relate to its binding affinity by the enzyme. On the basis of the above assumptions, it is reasonable to conclude

that TM4R is harder to enfold in the protein pocket than DPPacid owing to the higher hydrophobicity of the side chain of TM4R (chromanoxyl ring) than that of DPPacid (carboxyl group). As a result, condensation of DPPacid with a hydrophobic chromanoxyl ring to TM4R increases its inhibitory activity. This assumption may give a hint to the interaction of 4-substituted resorcinol inhibitor with the tertiary structure of the enzyme, but this remains unclear because the structure of tyrosinase has not yet been established. It should be noted that mushroom tyrosinase differs somewhat from that from other sources (22,23), but this fungal source was used for the entire experiment because it is readily available. Also, the tyrosinase inhibitory activity has been assessed only in a test tube assay, not in a cell-based assay using, e.g., melanocyte cultures.

Vitamin E is regarded as the major lipid-soluble antioxidant preventing oxidative attack of membrane lipids and other membrane compounds. The chromanoxyl ring of vitamin E is responsible for the radical-scavenging activity, and, in particular, the hydroxyl group at the C-6 position is essential for this activity (7). TM4R exhibited a scavenging activity against a stable free radical, DPPH, and hydroxyl radical to the same extent as those of α-tocopherol, Trolox, and TM. Taking no activity of DPPacid into consideration, the chromanoxyl ring moiety should be important for radical-scavenging activity of TM4R. It is known that when human skin is exposed to UV, various reactions are induced (24). The skin tissue, especially membrane phospholipids, is known to be damaged by UV-induced active oxygen. With less melanin, DNA damages to keratinocytes following UV irradiation are more severe, and the risk of UV carcinogenesis may increase (25). Thus, whitening agents, if they lighten the basal skin color in addition to hyperpigmented spots, might increase the risk of UV carcinogenesis. However, topical application of α-tocopherol and its derivatives has been shown to protect against UV-induced chronic cutaneous damage including cancer formation (26), wrinkle formation (27), and acute cellular and cutaneous reactions such as immune suppression (28), growth arrest, and cell death (29). Taken together, our results suggest that TM4R, one of the vitamin E derivatives, could be a good candidate for a skin-whitening agent and could also prevent skin damage by UV.

Finally, it is worth adding that melanin formations, caused enzymatically and nonenzymatically, are considered to be deleterious to the color quality of plant-derived food. Therefore, TM4R should be useful as a food additive, acting as an antioxidant and tyrosinase inhibitory agent in food, as dodecyl gallate acts (30).

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# β-Oxidation of Conjugated Linoleic Acid Isomers and Linoleic Acid in Rats

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**ABSTRACT:** To assess the oxidative metabolism of conjugated linoleic acid (CLA) isomers, rats were force-fed 1.5–2.6 MBq of [1-$^{14}$C]-linoleic acid (9*c*,12*c*-18:2), -rumenic acid (9*c*,11*t*-18:2), or -10*trans*,12*cis*-18:2 (10*t*,12*c*-18:2), and $^{14}CO_2$ production was monitored for 24 h. The animals were then necropsied and the radioactivity determined in different tissues. Both CLA isomers were oxidized significantly more than linoleic acid. Moreover, less radioactivity was recovered in most tissues after CLA intake than after linoleic acid intake. The substantial oxidation of CLA isomers must be considered when assessing the putative health benefits of CLA supplements.

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In animals, conjugated isomers of linoleic acid (CLA) have been reported to have beneficial effects on a range of health parameters, including cancer (1), body composition (2), diabetes (3), immune function (4), and atherogenesis (5,6). However, the last is still controversial as results in mice (7) indicate a proatherogenic effect of CLA, whereas previous data on hamsters and rabbits suggest a preventive effect.

Few studies on the effects of CLA in humans have been published. Vessby and Smedman (8) reported a reduction of fat mass and an increase in urinary excretion of isoprostanes, suggesting enhanced lipid peroxidation (9). On the other hand, Zambell *et al.* (10) reported that CLA had no effect on body composition and energy expenditure in healthy women.

In food, CLA is present as rumenic acid, i.e., 9*cis*,11*trans*-18:2 (9*c*,11*t*-18:2), but synthetic materials are mixtures containing different isomers, 10*trans*,12*cis*-18:2 (10*t*,12*c*-18:2) being one of the major ones. This isomer seems to have specific metabolic effects, mainly on body composition and on desaturase activities (11,12) and related gene expression (13).

However, the effective dosage of CLA is not clearly known. The oxidative metabolism of CLA isomers represents a metabolic pathway that may reduce the bioavailability of CLA for further effects. In the present work, we compared the metabolic oxidation of 9*c*,11*t*- and 10*t*,12*c*-18:2 in rats. The data show that a substantial portion of both CLA isomers is oxidized more than linoleic acid (9*c*,12*c*-18:2).

## MATERIAL AND METHODS

Male rats (Janvier, Le Genest Saint Isle, France) weighing 259 ± 6 g (mean ± SEM) were used. The animals were housed under controlled conditions of temperature (22 ± 1°C) and relative humidity (55–60%). A 12-h light–dark cycle (lights on 7:00 A.M.–7 :00 P.M.) was maintained. The animals were fed *ad libitum* with commercial pellets (Extralabo, Provins, France) and had free access to tap water. The day before the experiment at 5:00 P.M., they had access to only 10 g of commercial pellets, for the researchers to get animals at the same fasting status. All the experiments started at the same time (9:00 A.M.). Animal maintenance and handling were performed according to the French guidelines for animal studies (Authorizations A21200 and 3273).

[1-$^{14}$C]Linoleic acid (2.20 GBq · mmol$^{-1}$) was purchased from NEN (Le Blanc Mesnil, France). The detailed synthesis of [1-$^{14}$C]-9*c*,11*t*- (1.97 GBq · mmol$^{-1}$) and [1-$^{14}$C]-10*t*,12*c*-CLA (2.00 GBq · mmol$^{-1}$) isomers is described elsewhere (14). Each fatty acid was dissolved in triolein (Sigma Chemicals, L'Isle d'Abeau, France) and then administered by gastric tubing.

Immediately after intubation, the rats were placed in an airtight Plexiglas metabolic chamber, as described previously (15). Briefly, the $^{14}CO_2$ expired was trapped in a bottle containing Carbosorb (Packard, Groningen, the Netherlands). Air flow (950 mL · mmol$^{-1}$) was provided by a peristaltic pump.

Without interrupting the bubbling of the expired air through the trapping agent, 1 mL was removed every 30 min during the first 6 h of the experiment, hourly during the next 10 h, and hourly again from 18 to 24 h. As the density of the trapping agent increased during the experiment, the weight of each sample and of the bottle at each sampling time was measured to determine the exact radioactivity expired.

Scintillation cocktail (9 mL; Permafluor E, Packard) was added to each sample, and the radioactivity was determined using a Tri Carb 2000 CA liquid scintillation counter (Packard).

At the end of the 24-h experimental period, the animals were anesthetized. Blood was withdrawn into a heparinized syringe. Tissues (brain, heart, liver, gastrocnemian muscle, lung, kidneys, spleen, adrenals, testes, and epididymal adipose tissue)

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Abbreviations: CLA, conjugated linoleic acid; GBq, gigaBecquerel; MBq, megaBecquerel.

were removed, blotted on filter paper, and weighed. The gastrointestinal tract was divided into two parts, as previously described (15). The first part included the stomach and the small intestine. The second part was the large intestine. The carcass of each animal was weighed before homogenization.

Three finely minced portions (30–80 mg) of each tissue finely minced, and five portions of the carcass (50–100 mg) as well as the two parts of the gastrointestinal tract were digested overnight at 50°C using 1 mL of Soluene (Packard). The radioactivity of the samples was then determined by liquid scintillation counting as described previously, after addition of Hionic Fluor (Packard) scintillation cocktail. The radiaoctivity in blood and urine was determined as described (15).

*Statistical analysis.* Data are presented as means ± SEM of three independent determinations. Analyses of variance were carried out using the SAS software (Cary, NC). *P* values of <0.05 were considered significant.

## RESULTS

The weights of rats before administration of the radiolabeled fatty acids were similar. The radioactivity administered to the animals was 2.55 ± 0.04, 1.52 ± 0.04, and 1.57 ± 0.02 GBq for 9*c*,12*c*-, 9*c*,11*t*-, and 10*t*,12*c*-18:2, respectively. At the end of the experiments, 85–95% of the ingested radioactivity was recovered.

$^{14}CO_2$ production was similar for both CLA isomers. At the end of the 24-h experimental period, 71.8 and 70.3% of the dose of radioactivity from 9*c*,11*t*- and 10*t*,12*c*-18:2, respectively, were found in $^{14}CO_2$. These values were significantly higher than that obtained with 9*c*,12*c*-18:2 (60.3%, $P < 0.05$). The cumulative $^{14}CO_2$ production over 24 h is shown in Figure 1. The three curves exhibit a similar pattern with asymptotic profiles. They reached a plateau about 8 h after feeding the labeled fatty acids. The difference between CLA isomers and linoleic acid is borderline significant from 1.5 to 2 h after administration. The *P* values were less than 0.05 from 2.5 h after admistration to the end of the experiment.

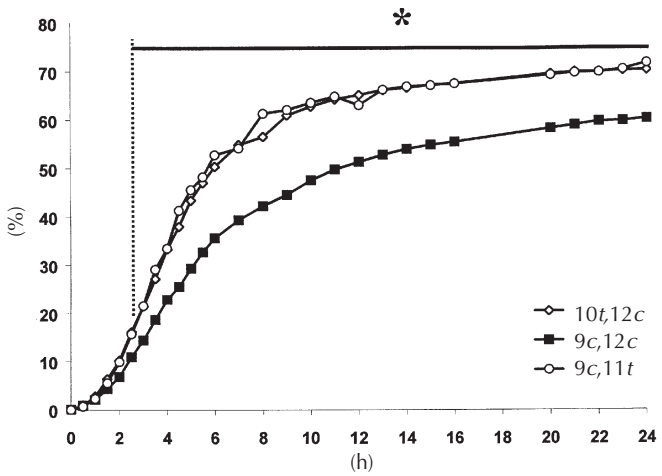


**FIG. 1.** $^{14}CO_2$ recovery after oral administration to the fasting rats of [1-$^{14}$C]-linoleic acid (9*c*,12*c*), -rumenic acid (9*c*,11*t*), or -10*trans*,12*cis*-18:2 (10*t*,12*c*). Data were obtained from three male Wistar rats for each fatty acid. Results are expressed as means of the percentage of radioactivity administered, recovered as $^{14}CO_2$, ± SEM. Asterisk (*) indicates period during which the $^{14}CO_2$ production was significantly different between conjugated linoleic acid (CLA) isomers (9*c*,11*t* and 10*t*,12*c*) and the corresponding values for linoleic acid (9*c*,12*c*) ($P < 0.05$).

The incorporated radioactivity per 100 g of tissue and as a fraction of the radioactivity administered at 24 h after the oral administration of the labeled fatty acids is presented in Table 1. In most tissues, the radioactivity recovered was similar whatever the fatty acid administered. However, the incorporation of radioactivity was different between linoleic acid and both the CLA isomers in brain, heart, adrenals, testes, and carcass (Table 1).

## DISCUSSION

In animal models, CLA isomers have been reported to have beneficial effects on some physiological parameters related to health. Their efficacy in humans is still controversial (10). However, the mechanisms by which these fatty acids may act, as well as their metabolic fate, is still unknown. As with linoleic acid, CLA isomers are converted by desaturation and elongation pathways to conjugated 18:3, 20:3, and 20:4 fatty acids (16–18). Besides these conversions to longer and more unsaturated metabolites, the incorporation of CLA in tissues is generally low. Another possible metabolic pathway involves oxidation, either complete or partial. A 16:2 conjugated fatty acid isomer has been detected and identified in rat tissues fed pure CLA isomers (20).

Using radiolabeled CLA, we compared the oxidative metabolism and tissue incorporation of the two major CLA

**TABLE 1**
**Recovery of Radioactivity (% of the administered dosage) per 100 g of Tissue 24 h After Oral Administration of the [1-$^{14}$C]-Radiolabeled Fatty Acids to Fasting Rats[a]**

| | 9*c*,12*c*-18:2 (linoleic acid) | 9*c*,11*t*-18:2 (rumenic (acid) | 10*t*,12*c*-18:2 | Standard error |
|---|---|---|---|---|
| Brain | 0.33[a] | 0.23[b] | 0.29[c] | 0.009 |
| Carcass | 1.19[a] | 0.72[b] | 0.90[a,b] | 0.096 |
| Heart | 1.88[a] | 0.77[b] | 0.94[b] | 0.091 |
| Liver | 2.27 | 1.39 | 1.78 | 0.252 |
| Gastrocnemius | 0.82 | 0.36 | 0.41 | 0.118 |
| Stomach + small intestine | 0.21 | 0.11 | 0.13 | 0.065 |
| Large intestine + feces | 0.25 | 0.50 | 0.22 | 0.121 |
| Lung | 1.19 | 1.45 | 1.53 | 0.228 |
| Kidney | 1.55 | 1.10 | 1.38 | 0.111 |
| Spleen | 1.66 | 1.04 | 1.44 | 0.192 |
| Blood | 0.32 | 0.25 | 0.34 | 0.040 |
| Adrenals | 4.72[a] | 3.21[b] | 2.38[b] | 0.289 |
| Testes | 0.88[a] | 0.24[b] | 0.30[b] | 0.020 |
| Adipose tissue | 1.73 | 1.91 | 1.51 | 0.278 |
| Urine | 1.78 | 1.31 | 2.00 | 0.184 |

[a]Results are expressed as mean ± SEM of three independent determinations. Values having a different roman superscript in rows are statistically significant ($P < 0.05$).

isomers in semifasting rats. Our data showed that CLA isomers produced more $^{14}CO_2$ than linoleic acid. This pattern was close to what we reported for α-linolenic acid, where 70% of the ingested radioactivity was recovered in $CO_2$ (15). As CLA seemed to be as well absorbed as linoleic acid (19), this difference between linoleic acid and CLA may be due to a higher metabolic utilization by cellular oxidation systems.

In the present study, the radioactivity incorporated in different tissues was similar after intragastric feeding of the three fatty acids. Some differences were observed only in brain, carcass, heart, adrenals, and testes, in which linoleic acid seemed to be better incorporated than CLA isomers. Moreover, our data do not explain why 9*c*,11*t*-18:2 is generally incorporated more than 10*t*,12*c*-18:2 into tissues (20).

When added to the diet, CLA have been reported to induce different physiological effects in several animal models. The mechanisms by which CLA act are not understood. The present data indicate that the catabolism of ingested CLA has to be taken into account with regard to their tissue bioavailability and emphasize that CLA are lipids that may also contribute to energy production.

Further studies have to be carried out to know the CLA metabolic pattern in humans.

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# Dietary α-Linolenic Acid Lowers Postprandial Lipid Levels with Increase of Eicosapentaenoic and Docosahexaenoic Acid Contents in Rat Hepatic Membrane

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**ABSTRACT:** This study was designed to examine the effects of dietary n-3 and n-6 polyunsaturated fatty acids (PUFA) on postprandial lipid levels and fatty acid composition of hepatic membranes. Male Sprague-Dawley rats were trained for a 3-h feeding protocol and fed one of five semipurified diets: one fat-free diet or one of four diets supplemented with 10% (by weight) each of corn oil, beef tallow, perilla oil, and fish oil. Two separate experiments were performed, 4-wk long-term and 4-d short-term feeding models, to compare the effects of feeding periods. Postprandial plasma lipid was affected by dietary fats. Triacylglycerol (TG) and total cholesterol levels were decreased in rats fed perilla oil and fish oil diets compared with corn oil and beef tallow diets. Hepatic TG and total cholesterol levels were also reduced by fish oil and perilla oil diets. Fatty acid composition of hepatic microsomal fraction reflected dietary fatty acids and their metabolic conversion. The major fatty acids of rats fed the beef tallow diet were palmitic, stearic, and oleic. Similarly, linoleic acid (LA) and arachidonic acid in the corn oil group, α-linolenic acid (ALA) and eicosapentaenoic acid (EPA) in the perilla oil group, and palmitic acid and docosahexaenoic acid (DHA) in the fish oil group were detected in high proportions. Both long- and short-term feeding experiments showed similar results. In addition, microsomal DHA content was negatively correlated with plasma lipid levels. Hepatic lipid levels were also negatively correlated with EPA and DHA contents. These results suggest that n-3 ALA has more of a hypolipidemic effect than n-6 LA and that the hypolipidemic effect of n-3 PUFA may be partly related to the increase of EPA and DHA in hepatic membrane.

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The study of risk factors for cardiovascular disease has focused on the concentration and composition of plasma lipids and lipoproteins present during the fasting state (1–3). However, the fed state represents a major portion of the body's metabolic time, and the alimentary state is determined by typical eating patterns. Moreover, several studies have linked alterations in postprandial lipemia with coronary vascular disease (4–6). The study on postprandial lipemia will be valuable to elucidate the effect of dietary components on fat metabolism.

Dietary fats containing different fatty acids alter the extent and character of postprandial lipid metabolism (7–9). Chronic feeding of polyunsaturated fatty acids (PUFA), both n-3 and n-6, reduce postprandial lipemia produced by a meal containing other fatty acids. Williams *et al.* (10) reported that moderate n-3 fatty acid supplementation reduced postprandial triacylglycerol (TG) response following a standard meal. However, in these studies, fish or fish oil (FO) rich in long-chain n-3 fatty acids, such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), was provided as an n-3 PUFA source, and vegetable oil rich in linoleic acid (LA; 18:2n-6) was used as an n-6 PUFA source, without considering the degree of unsaturation and chain length. So, it would be more reasonable to provide vegetable oils rich in either LA or α-linolenic acid (ALA; 18:3n-3) for comparison of the effect of n-3 and n-6 PUFA. But a study on the comparison of the effects of LA and ALA has not been performed, and little information exists on the relative effectiveness among n-3 fatty acids.

Plasma TG and lipoproteins are synthesized mainly in the liver. Microsomes contain fatty acid desaturases and the enzymes catalyzing the synthesis of phosphatidic acid, thus synthesizing new membrane lipids. The fatty acid component in the membrane serves as a modulator of the biological processes such as eicosanoid production and activation of membrane-bound enzymes (11–13). Therefore, the study on the fatty acid composition of the cell membrane is thought to be important for understanding the effect of dietary fats on plasma lipids.

Perilla oil (PO), a commonly used cooking oil in Korea, contains as much as 60% ALA in its total fatty acid composition, which makes it suitable for an ALA source. Contrary to the numerous studies on FO with long-chain highly unsaturated fatty acids, little is known about the effectiveness of ALA in lowering plasma lipids. The purpose of this study is to compare the effect of dietary PO on postprandial plasma lipid level and the fatty acid composition of hepatic membrane lipid with other fats.

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; BT, beef tallow; CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FF, fat free; FO, fish oil; HDL-C, high density lipoprotein-cholesterol; LA, linoleic acid; PO, perilla oil; PUFA, polyunsaturated fatty acid; TC, total cholesterol; TG, triacylglycerol.

## MATERIALS AND METHODS

*Animals and diets*. All animal procedures were approved by the Laboratory Animal Center of Seoul National University. Two separate experiments were conducted to compare the effects of dietary fat feeding periods. Sprague-Dawley rats, supplied by the Laboratory Animal Center of Seoul National University, were individually caged throughout the entire experimental periods and had free access to water.

In experiment I, weanling rats were fed one of the five experimental diets for 4 wk and sacrificed. In order to facilitate control of diet intake, all animals were adapted to a meal-eating regimen in which one meal allowed access to food for a 3-h period (9:00 A.M.–12:00 P.M.). The amount of basal diet was determined according to the lowest consumption of rat (in the experimental block) the previous day; it was kept constant among the experimental groups.

In the second part of this study, animals were fed the diet containing 5% corn oil (CO) until they weighed about 140 g and then they were divided into five groups. For 7 d prior to the experimental period of fat supplementation, all rats were adapted to the 3-h feeding regimen, receiving a fat-free (FF) diet so that they would have similar fatty acid status after exhausting stored fatty acids. The diet was prepared by mixing 1% (w/w) CO with the basal diet to prevent essential fatty acid deficiency. The basal diet contained the following percentages of ingredients according to weight: casein 20, sugar 34.85, corn starch 34.85, α-cellulose 5, AIN mineral mixture 4, AIN vitamin mixture 1, and DL-methionine 0.3. After that, rats were fed one of the five experimental diets for 4 d: the FF diet, or one of four diets supplemented with 10% (w/w) each of CO, beef tallow (BT), PO, and FO. The fatty acid compositions of dietary fats are shown in Table 1. To prevent autoxidation, α-tocopherol was supplemented in PO (0.015%) and FO (0.019%) based on peroxidizability index (14). In addition, meals were offered daily and diets were stored in the freezer under $N_2$ gas to minimize the opportunity for peroxidation of lipids.

**TABLE 1**
**Fatty Acid Composition of Dietary Fats[a]**

| Fatty acid | Beef tallow | Corn oil | Perilla oil | Fish oil |
|---|---|---|---|---|
| 14:0 | 3.96 | — | — | 3.58 |
| 15:0 | — | — | — | 1.36 |
| 16:0 | 28.36 | 11.85 | 6.80 | 25.66 |
| 16:1 | 2.79 | — | — | 5.50 |
| 18:0 | 20.87 | — | 2.07 | 6.65 |
| 18:1 | 44.02 | 28.94 | 15.23 | 13.83 |
| 18:2n-6 | — | 57.29 | 13.67 | 1.62 |
| 18:3n-3 | — | 0.93 | 61.49 | 1.08 |
| 20:0 | — | 0.54 | — | — |
| 20:1 | — | 0.45 | 0.48 | 1.52 |
| 20:2n-6 | — | — | 0.25 | — |
| 20:5n-3 | — | — | — | 6.47 |
| 22:6n-3 | — | — | — | 32.72 |
| ΣSFA | 53.19 | 12.39 | 8.87 | 37.25 |
| ΣMUFA | 46.81 | 29.39 | 15.71 | 20.85 |
| Σn-6 | — | 57.29 | 13.92 | 1.62 |
| Σn-3 | — | 0.93 | 61.49 | 40.27 |
| n-6/n-3 | — | 61.60 | 0.23 | 0.04 |

[a]Values are expressed as percentage of total fatty acids. ΣSFA, sum of saturated fatty acids; ΣMUFA, sum of monounsaturated fatty acids; Σn-6, sum of n-6 fatty acids; Σn-3, sum of n-3 fatty acids; n-6/n-3, ratio of n-6 to n-3 fatty acids.

*Preparation of samples*. Animals were sacrificed by decapitation at 90–120 min after completion of the final meal. Blood was collected in a heparinized tube and centrifuged at 1,500 × *g* for 20 min to separate the plasma. Plasma for high density lipoprotein-cholesterol (HDL-C) analysis was obtained by precipitating non-HDL with dextran sulfate and magnesium sulfate followed by centrifugation. Livers were removed, finely minced, and homogenized in 5 vol of ice-cold homogenizing buffer solution (154 mM KCl, 50 mM Tris-HCl, 1 mM EDTA buffer, pH 7.4). The homogenate was centrifuged at 10,000 × *g* at 4°C for 20 min, followed by recentrifugation of the supernatant at 105,000 × *g* at 4°C for 1 h. The resulting pellet was considered the microsome and resuspended in cold storage buffer (homogenizing buffer/glycerol, 80:20). The entire fractionation procedure was conducted at 0–4°C.

*Lipid analyses*. Lipids were extracted from livers of rats in experiment I by the method of Folch *et al.* (15). The extracted lipids were evaporated under nitrogen and weighed to determine the total fat content of the liver. TG and cholesterol contents in the liver extract and plasma were assayed by using commercial enzymatic kits (Youngdong Pharm. Co., Seoul, Korea). To analyze the fatty acid composition of hepatic microsomal fraction, lipid extraction and transesterification were carried out simultaneously by the method described by Lepage and Roy (16). Fatty acid methyl esters were measured by gas chromatography (Hewlett-Packard model 5890) using an EC wax packed capillary column (EC-1 0.32 mm × 30 m; Alltech, Deerfield, IL) equipped with an HP GC ChemStation data system, an autosampler, and a flame-ionization detector. The fatty acids were identified by comparison of retention time of standard esters under the same conditions, and the composition of fatty acids of each fraction was calculated as a percentage of total area.

*Statistical analysis*. All statistical analyses were carried out using analysis of variance and Duncan's multiple range test. A $P$ value of $<0.05$ was selected as a limit of statistical significance. The statistical program used was SAS package (Cary, NC).

## RESULTS

*Body weight and liver lipid contents.* No significant differences in diet intake, final body weight, or weight increase rate were noted among fat-feeding experimental groups. Diet intakes were not different within a group because animals were individually caged and adapted to the 3-h feeding regimen. All groups consumed the same amount of carbohydrate each day because food intakes were matched. The lipid content of liver is shown in Table 2. Even though there was no significant difference in total lipid content of liver tissue, hepatic TG and total cholesterol (TC) levels were affected by the di-

**TABLE 2**
**Effect of Dietary Fats on Lipid Contents in Rat Liver[a]**

| Group | Total lipid | Triacylglycerol | Total cholesterol |
|---|---|---|---|
| FF | 70.40 ± 6.49 | 4.64 ± 0.25[a] | 2.80 ± 0.10[a] |
| BT | 72.62 ± 4.90 | 4.56 ± 0.21[a] | 2.71 ± 0.13[a] |
| CO | 70.55 ± 3.78 | 4.52 ± 0.24[a] | 2.52 ± 0.08[a,b] |
| PO | 60.09 ± 2.40 | 3.38 ± 0.43[b] | 1.81 ± 1.10[c] |
| FO | 61.89 ± 3.71 | 2.74 ± 0.56[b] | 2.26 ± 0.15[b] |

[a]Expressed as mg/g liver and mean ± standard error of the mean ($n$ = 9). Values with different roman superscripts (a, b, and c) are significantly different at $P < 0.05$ by Duncan's multiple range test. Weanling Sprague-Dawley rats were fed each of the experimental diets for 4 wk by a 3-h feeding protocol. FF, fat-free diet; BT, 10% beef tallow diet; CO, 10% corn oil diet; PO, 10% perilla oil diet; FO, 10% fish oil diet.

etary treatment. Hepatic TG content was low in PO- and FO-fed groups compared with other groups. The TC content was the lowest in the PO group followed by the FO group; other groups showed no significant differences.

*Postprandial plasma lipid levels.* The postprandial plasma lipid was affected by dietary fat as shown in Table 3. In experiment I, the concentration of plasma TG was lowest in the FO group, followed by other groups in the order of PO, BT, and CO. The FF diet group showed an intermediate level between the FO and PO groups. TC content was low in the PO and FO groups compared with groups fed other fats. HDL-C concentration showed a similar tendency to that of TC. However, the relative ratios (HDL/TC) were not significantly different among the dietary fat groups. Similar plasma lipid profiles were found even in a 4-d feeding trial, with the lowest plasma TG and TC levels in FO fed, followed by PO fed and BT fed groups.

*Fatty acid composition of rat liver membrane.* Fatty acid composition of hepatic microsomal fraction is shown in Table 4 as the percentage of total fatty acids. It reflected the fatty acid composition of dietary fats. The major fatty acids in rats fed the BT diet were palmitic, stearic, and oleic. Similarly, LA in the CO group, ALA in the PO group, and palmitic acid and DHA in the FO group were detected in high proportion. In addition, fatty acids not detected in dietary fat itself were shown in hepatic microsomal fractions in substantial amounts. In the CO group, a high content of arachidonic acid (AA; 20:4, n-6) was detected, while low AA content was shown with the increase of EPA and DHA in the PO group. The PO and FO groups fed n-3 fatty acid sources showed significantly high contents of total n-3 PUFA, and the CO group showed high contents of total n-6 PUFA. Similar fatty acid composition was found in a 4-d feeding (experiment II), although the extent of difference was not so great as in experiment I.

## DISCUSSION

Numerous studies have documented that long-chain n-3 fatty acids, such as those present in FO, lower plasma lipid levels in both the fasting and postprandial states (7–9,17). Consistent with these findings, the present study demonstrates that consumption of FO markedly reduced postprandial lipid levels in both long- and short-term feeding experiments, indicating that the hypolipidemic effect of FO was apparent even in a 4-d feeding.

Another n-3 PUFA source, PO, was also effective to reduce postprandial lipemia. Plasma TC and HDL-C levels were similar to that of the FO group. Moreover, the extent of TG reduction was greater than in the CO group, although that was not so great as the FO group. In contrast to many studies on fasting plasma lipid levels, there have been only a few studies on the comparison of n-6 and n-3 fatty acids in the postprandial state. Long-chain n-3 PUFA such as EPA and DHA resulted in less postprandial plasma triglyceridemia compared with the n-6 LA diet (18,19). Thus, it can be suggested that all the n-3 PUFA (ALA, EPA, and DHA) have beneficial effects on plasma TG compared with n-6 PUFA (LA).

In addition, long-term experiment I showed low levels of plasma HDL-C in the FO and PO groups compared with the CO and BT groups. Parks and Rudel (20) demonstrated that the decreased plasma TC levels in n-3 fatty acid diets result in decreased synthesis of nascent HDL and apoprotein A-1. Thus, the high plasma HDL-C levels in the CO and BT groups seem to be related to the significantly high plasma TC levels.

In contrast to the lower TC level of the PO group in experiment I, a significant difference was not observed between PO and CO groups in experiment II. This discrepancy might be ascribed to the duration of feeding. Ihara *et al.* (21) compared the effect of diets high in LA or ALA on fasting lipid metab-

**TABLE 3**
**Effect of Dietary Fats on Plasma Lipid Levels[a]**

| | Long-term | | | | Short-term | | | |
|---|---|---|---|---|---|---|---|---|
| | | Cholesterol | | | | Cholesterol | | |
| Group | Triacylglycerol | TC | HDL | HDL/TC | Triacylglycerol | TC | HDL | HDL/TC |
| FF | 74.31 ± 3.37[c,d] | 61.51 ± 2.75[b] | 32.97 ± 1.52[a,b] | 0.56 ± 0.01[a] | 64.96 ± 4.16[c] | 57.18 ± 2.55[b] | 36.18 ± 2.66 | 0.61 ± 0.02[a] |
| BT | 122.78 ± 8.75[b] | 71.05 ± 2.71[a] | 34.53 ± 2.53[a] | 0.45 ± 0.05[b] | 95.69 ± 3.28[b] | 66.48 ± 2.97[a,b] | 37.82 ± 1.81 | 0.58 ± 0.01[a,b] |
| CO | 144.95 ± 10.63[a] | 77.36 ± 2.10[a] | 38.45 ± 1.58[a] | 0.52 ± 0.01[a,b] | 139.46 ± 6.33[a] | 73.67 ± 5.05[a] | 42.27 ± 1.90 | 0.53 ± 0.02[b] |
| PO | 90.02 ± 6.85[c] | 52.05 ± 2.47[c] | 28.06 ± 1.94[b] | 0.51 ± 0.04[a,b] | 106.09 ± 6.76[b] | 63.70 ± 2.43[a,b] | 36.77 ± 1.27 | 0.56 ± 0.01[a,b] |
| FO | 62.92 ± 6.59[d] | 54.65 ± 3.08[b,c] | 27.98 ± 2.15[b] | 0.50 ± 0.02[a,b] | 42.96 ± 2.82[d] | 56.84 ± 3.21[b] | 36.34 ± 2.05 | 0.61 ± 0.01[a] |

[a]Expressed as mg/dL plasma and mean ± standard error of the mean ($n$ = 9). Values with the different roman superscripts (a, b, c, and d) are significantly different at $P < 0.05$ by Duncan's multiple range test. Sprague-Dawley rats were fed each of the experimental diets for 4 wk in long-term and 4 d in short-term feeding. TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol; and HDL/TC, ratio of HDL to TC. For other abbreviations see Table 2.

**TABLE 4**
**Effect of Dietary Fats on Fatty Acid Composition in Hepatic Microsomal Fraction[a]**

| | Long-term | | | | Short-term | | | |
|---|---|---|---|---|---|---|---|---|
| Fatty acid | FF | BT | CO | PO | FO | BT | CO | PO | FO |
| 14:0 | 0.95 ± 0.02$^{a,b}$ | 0.99 ± 0.04$^{a}$ | 0.83 ± 0.06$^{b}$ | 0.68 ± 0.05$^{c}$ | 0.52 ± 0.03$^{d}$ | 1.64 ± 0.06$^{a}$ | 1.44 ± 0.14$^{a,b}$ | 1.40 ± 0.06$^{b}$ | 0.71 ± 0.03$^{c}$ | 0.71 ± 0.03$^{c}$ |
| 16:0 | 25.22 ± 0.43$^{b,c}$ | 28.38 ± 0.53$^{a}$ | 24.95 ± 0.88$^{b,c}$ | 23.7 ± 0.45$^{c}$ | 26.93 ± 1.22$^{a,b}$ | 26.69 ± 0.56$^{a}$ | 25.87 ± 0.41$^{a,b}$ | 24.06 ± 0.83$^{b}$ | 21.8 ± 0.87$^{c}$ | 20.57 ± 0.32$^{c}$ |
| 16:1 | 3.44 ± 0.19$^{a}$ | 2.51 ± 0.17$^{b}$ | 1.24 ± 0.10$^{d}$ | 1.23 ± 0.08$^{d}$ | 1.8 ± 0.08$^{c}$ | 2.63 ± 0.18$^{a}$ | 3.05 ± 0.31$^{a}$ | 1.09 ± 0.12$^{b}$ | 0.83 ± 0.09$^{b}$ | 1.06 ± 0.06$^{b}$ |
| 18:0 | 19.61 ± 0.27$^{a,b}$ | 20.54 ± 0.42$^{a}$ | 18.43 ± 0.38$^{b}$ | 21.08 ± 0.75$^{a}$ | 15.09 ± 1.23$^{c}$ | 22.06 ± 0.35$^{a}$ | 22.13 ± 0.42$^{a}$ | 21.31 ± 0.68$^{a}$ | 22.26 ± 0.33$^{a}$ | 19.85 ± 0.45$^{b}$ |
| 18:1 | 12.53 ± 0.27$^{b}$ | 13.53 ± 0.47$^{a}$ | 7.02 ± 0.23$^{c}$ | 6.92 ± 0.24$^{c}$ | 7.64 ± 0.28$^{c}$ | 10.47 ± 0.21$^{b}$ | 13.71 ± 0.58$^{a}$ | 8.22 ± 0.39$^{c}$ | 6.38 ± 0.19$^{d}$ | 6.05 ± 0.13$^{d}$ |
| 18:2 | 6.88 ± 0.25$^{c}$ | 5.65 ± 0.17$^{d}$ | 15.36 ± 0.53$^{a}$ | 13.74 ± 0.59$^{b}$ | 2.18 ± 0.15$^{e}$ | 6.11 ± 0.14$^{c}$ | 5.51 ± 0.17$^{c}$ | 14.43 ± 0.75$^{a}$ | 11.60 ± 0.55$^{b}$ | 2.62 ± 0.09$^{d}$ |
| 18:3 | 1.38 ± 0.26$^{b}$ | 0.57 ± 0.21$^{b}$ | 1.25 ± 0.19$^{b}$ | 6.73 ± 0.48$^{a}$ | 0.88 ± 0.14$^{b}$ | 3.81 ± 0.37$^{b}$ | 0.48 ± 0.09$^{d}$ | 3.26 ± 0.39$^{b}$ | 6.71 ± 0.38$^{a}$ | 2.18 ± 0.10$^{c}$ |
| 20:2 | 5.10 ± 0.49$^{b}$ | 8.42 ± 0.38$^{a}$ | 2.07 ± 0.47$^{c}$ | 2.29 ± 0.41$^{c}$ | 2.91 ± 0.25$^{c}$ | 7.38 ± 0.47$^{a}$ | 2.30 ± 0.33$^{c}$ | 4.65 ± 0.57$^{b}$ | 2.45 ± 0.36$^{c}$ | 5.10 ± 0.55$^{b}$ |
| 20:4 | 20.67 ± 0.44$^{b}$ | 13.72 ± 0.54$^{d}$ | 26.02 ± 0.37$^{a}$ | 7.75 ± 0.51$^{e}$ | 18.67 ± 0.86$^{c}$ | 19.79 ± 0.48$^{b,e}$ | 20.24 ± 0.7$^{b}$ | 22.76 ± 1.44$^{a}$ | 16.82 ± 0.71$^{d}$ | 17.37 ± 0.38$^{c,d}$ |
| 20:5 | 0.04 ± 0.02$^{c}$ | 0.19 ± 0.06$^{c}$ | 0.02 ± 0.02$^{c}$ | 12.43 ± 0.29$^{a}$ | 5.51 ± 0.38$^{b}$ | 0$^{c}$ | 0.22 ± 0.02$^{c}$ | 0.04 ± 0.04$^{c}$ | 8.07 ± 0.42$^{a}$ | 3.85 ± 0.27$^{b}$ |
| 22:6 | 3.52 ± 0.16$^{d}$ | 4.59 ± 0.28$^{c}$ | 2.90 ± 0.05$^{d}$ | 6.31 ± 0.28$^{b}$ | 20.91 ± 0.73$^{a}$ | 2.68 ± 0.10$^{d}$ | 3.69 ± 0.19$^{c}$ | 2.88 ± 0.08$^{c,d}$ | 5.89 ± 0.40$^{b}$ | 21.11 ± 0.49$^{a}$ |
| ΣSFA | 46.21 ± 0.59$^{a,b}$ | 48.64 ± 0.76$^{a}$ | 44.21 ± 0.72$^{b}$ | 44.19 ± 1.43$^{b}$ | 39.67 ± 1.21$^{c}$ | 49.99 ± 0.96$^{a}$ | 50.34 ± 1.03$^{a}$ | 43.03 ± 1.83$^{b,c}$ | 44.03 ± 0.43$^{b}$ | 40.68 ± 0.67$^{c}$ |
| ΣMUFA | 15.96 ± 0.38$^{a}$ | 16.15 ± 0.52$^{a}$ | 8.26 ± 0.27$^{c}$ | 8.15 ± 0.30$^{c}$ | 9.81 ± 0.47$^{b}$ | 13.06 ± 0.29$^{b}$ | 15.76 ± 0.30$^{a}$ | 9.31 ± 0.49$^{c}$ | 7.22 ± 0.25$^{d}$ | 7.12 ± 0.14$^{d}$ |
| Σn-6 | 32.64 ± 0.53$^{b}$ | 27.93 ± 1.26$^{c}$ | 43.45 ± 0.67$^{a}$ | 24.15 ± 0.93$^{d}$ | 25.32 ± 1.26$^{c,d}$ | 31.17 ± 1.08$^{b}$ | 28.55 ± 0.86$^{b,c}$ | 43.26 ± 2.09$^{a}$ | 31.76 ± 1.54$^{b}$ | 25.62 ± 0.65$^{c}$ |
| Σn-3 | 5.19 ± 0.41$^{b}$ | 5.69 ± 0.42$^{b}$ | 4.08 ± 0.15$^{b}$ | 26.25 ± 1.07$^{a}$ | 26.69 ± 0.74$^{a}$ | 6.50 ± 0.39$^{c}$ | 4.34 ± 0.21$^{d}$ | 5.82 ± 0.51$^{c,d}$ | 20.95 ± 0.85$^{b}$ | 26.11 ± 0.97$^{a}$ |
| n-6/n-3 | 6.29/1 | 4.91/1 | 10.65/1 | 0.92/1 | 0.95/1 | 4.80/1 | 6.58/1 | 7.43/1 | 1.52/1 | 0.98/1 |

[a]Expressed as percentage of total fatty acids and mean ± standard error of the mean ($n = 9$). Values with different superscripts (a, b, c, d, and e) are significantly different at $P < 0.05$ by Duncan's multirange test. Sprague-Dawley rats were fed each of the experimental diets for 4 wk in long-term and 4 d in short-term feeding. For abbreviations see Tables 1 and 2.

olism in time-course (3, 7, 20, and 50 d) experiments and reported that the levels of serum TC and phospholipid in the rats fed the PO diet were markedly lower than those fed the safflower oil diet after the seventh day. Thus, as in the fasting state, a 4-d feeding in experiment II is thought to be insufficient to make a significant decrease in TC levels between PO and CO diets.

Another interesting result was that CO feeding did not result in any significant decrease in plasma lipid levels. This is contrary to previous reports (9,22–24) that unsaturated fat reduced the plasma lipid levels over saturated fat but consistent with the result of Sanders *et al.* (18). Postprandially, plasma triglyceridemia was greater on the n-6 LA diet and lower on the long-chain n-3 diet, with the saturated diet being intermediate. The 3-h feeding protocol and matched feeding in our study enabled us to feed exactly the same amount of total fat content and other nutrients, such as protein and carbohydrate, so the results can be considered as the sole effect of different dietary fats, compared with other reports. Even though this does not fully explain the different results, it is still questionable that n-6 LA has a plasma lipid-lowering effect.

Although there was no significant difference in total hepatic lipid, hepatic TG and TC levels were low in the PO and FO groups. This shows that the concentrations of hepatic TG and TC were lower in the n-3 PUFA diet than in the n-6 LA diet. Considering that the low serum TG concentration is due to the decreased synthesis and secretion of hepatic very low density lipoprotein-TG (25), the reduction of hepatic TG levels as well as plasma TG levels observed in the PO and FO groups suggests the significance for application to human coronary heart disease.

Hepatic microsomes are predominantly smooth microsomes participating in lipid synthesis and intracellular membrane traffic (26). According to the report of Bernasconi *et al.* (27), the fatty acid composition of total hepatic microsomal lipids and that of phosphatidylcholine, the principal lipid of the microsomal membrane, showed similar results. In addition, they were correlated with the changes in desaturase activities (27,28). Total fatty acid composition in hepatic microsomal fraction was measured in our study as supporting data for the lipid level. As shown in Table 4, the significant differences in hepatic microsomal fatty acid content among groups reflected the dietary fatty acid composition. The major fatty acids in the BT group were palmitic, stearic, and oleic. Similarly, LA in the CO group, ALA in the PO group, and long- chain PUFA (EPA and DHA) in the FO group were detected in high proportion. In addition to the direct incorporation of dietary fatty acids into membrane lipids, there must be a considerable amount of metabolic conversion of fatty acids. In the CO group, a significantly high content of AA was detected, in contrast to the PO group in which the AA content was reduced with concomitant increases of EPA and DHA. It is likely that this result was due to the suppressed biosynthesis of AA from LA by the presence of ALA. The biosynthesis of AA from LA competes with that of EPA from ALA for $\Delta^6$-desaturase, with the relative rate of enzymatic elongation and desaturation in the order n-3 > n-6 > n-9 (29). The resulting high concentration of EPA and DHA in

the PO group might have controlled plasma and hepatic lipid levels. Both EPA and DHA were reported to have a marked hypotriglyceridemic effect, although DHA was more pronounced (30). Thus, the hypotriglyceridemic effect of PO compared with CO can be ascribed to the resulting high concentration of EPA and DHA.

In our study, microsomal DHA content was negatively correlated with plasma TG and TC levels ($r = -0.613$ for TG; $r = -0.458$ for TC, $P < 0.05$), but no significant correlation was observed between other n-3 fatty acids and plasma lipids. These results explain why FO rich in DHA was more effective in reducing plasma TG than PO. In addition, the contents of hepatic TG and TC were negatively correlated with the EPA and DHA contents in microsomal fraction ($r = -0.548$ in EPA, $r = -0.629$ in DHA, for hepatic TG; $r = -0.629$ in EPA, $r = -0.560$ in DHA, for hepatic TC), by which the significant decrease of hepatic lipids in the PO and FO groups can be explained.

It is generally accepted that dietary fat is a normal determinant of membrane fatty acid composition and a constant modulator of the biological process that may be regulated through membranes (31). With young rats, approximately 4–10 wk were required to approach a new steady state in most tissue lipids after a change in dietary lipid (14). Even though the change in fatty acid content occurs most rapidly in the liver, some studies have shown that dietary lipid brings changes of fatty acid profile in plasma and hepatic membrane after 10 d (11,32). Thus, it was assumed that biochemical and physiological changes in the short-term feeding trial would be caused by factors other than fatty acid composition of membrane. Contrary to our expectation, a 4-d feeding protocol in experiment II was sufficient to reflect the dietary fatty acids, even though the extent of change was not as great as in long-term experiment I. From this result, the change of microsomal fatty acid composition seems to be achieved in less time than expected, although a more sophisticated time-course study is needed.

Dietary PO as well as FO was effective to reduce postprandial lipemia and hepatic lipid level. This result suggests that n-3 ALA has considerable hypolipidemic effect compared with n-6 LA. The increase of EPA and DHA in hepatic membrane may be partly related to the hypolipidemic effect of n-3 PUFA.

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# Effect of Dexamethasone on the Fatty Acid Composition of Total Liver Microsomal Lipids and Phosphatidylcholine Molecular Species

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**ABSTRACT:** Dexamethasone depresses Δ6 and Δ5 and increases Δ9 desaturase and synthase activities. Therefore, we investigated the effect on the fatty acid composition of microsomal liver lipids and phosphatidylcholine (PtdCho) molecular species. After 15 d of treatment we found a notable decrease in arachidonic acid, a small decrease in stearic acid, and increases of linoleic, oleic, palmitoleic, and palmitic acids in liver microsomal total lipids and PtdCho. The study of the distribution of the PtdCho molecular species indicated that 18:0/20:4n-6, 16:0/20:4n-6, and 16:0/18:2n-6 predominated in the control animals. Dexamethasone, as expected because of its depressing effect on arachidonic acid synthesis and activation of oleic and palmitic acid synthesis, evoked a very significant decrease in 18:0/20:4n-6 PtdCho ($P<0.001$) and an important increase in 16:0/18:2n-6. The invariability of 16:0/20:4n-6 PtdCho could be related to the antagonistic effect of arachidonic and palmitic acid synthesis. PtdCho species containing oleic acid were not significant. The bulk fluidity and dynamic properties of the microsomal lipid bilayer measured by fluorometry using the probes 1,6-diphenyl-1,3,5-hexatriene and 4-trimethylammonium-phenyl-6-phenyl-1,3,5-hexatriene showed no significant modification, probably owing to a compensatory effect of the different molecular species, but changes of particular domains not detected by this technique are possible. However, the extremely sensitive Laurdan detected increased lipid packing in the less-fluid domains of the polar-nonpolar interphase of the bilayer, possibly evoked by the change of molecular species and cholesterol/phospholipid ratio. The most important effect found is the decrease of arachidonic acid pools in liver phospholipids as one of the corresponding causes of dexamethasone-dependent pharmacological effects.

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Polyunsaturated fatty acids derived from linoleic and α-linolenic acids play important roles in the biological functions of mammals, including human beings. The Δ6 desaturase (1) is the key enzyme that regulates polyunsaturated fatty acid biosynthesis at the level of 18-carbon fatty acids. A second step in this regulation at the level of 20-carbon fatty acids is produced by the Δ5 desaturase.

Since 1966, when we discovered that the Δ6 desaturase was depressed in experimental diabetes and could be recovered by insulin injection (2,3), we have enlarged the study regarding the effect of nearly all other hormones, not only upon the Δ6 but also upon the Δ5 desaturase. Several reviews have been published (4,5), and we have found that except for insulin, all hormones for example, glucagon, adrenaline, glucocorticoids (corticosterone, hydrocortisone, dexamethasone, triamcynolone), 11-deoxycorticosterone, aldosterone, estradiol, estriol, testosterone, and ACTH inhibit both enzymes. Progesterone, cortexolone, and pregnanediol were inactive.

When we compared the effects of these hormones *in vivo* upon the hepatic Δ9 desaturase activity, we found (2,3,6), as Gellhorn and Benjamin (7) showed that experimental diabetes depressed Δ9 desaturation of palmitic and stearic acids, and insulin injection recovered the activity. Unlike what happened to the Δ6 and Δ5 desaturases, glucocorticoids, 11-deoxycorticosterone, testosterone, estradiol, and L-triiodothyronine also enhanced Δ9 desaturase activity (Brenner, R.R., unpublished data).

In the case of glucocorticoids, our pioneering experiments done in 1979 (8) showed that *in vivo* hydrocortisone and the synthetic glucocorticoids triamcynolone and dexamethasone were depressors of Δ6 and Δ5 desaturation activity in liver microsomes and activators of Δ9 desaturase, although both synthetic products were more active than hydrocortisone. The strongest effect was found 24 h after the injection. The depressing effect of dexamethasone on [1-$^{14}$C]eicosa-8,11,14-trienoic acid conversion to arachidonic acid was also shown by direct incubation of the isolated rat hepatocytes or hepatoma tissue culture (HTC) cells with this synthetic glucocorticoid (9). Moreover, *in vivo* experiments demonstrated (10) that this modulation of Δ6 and Δ5 desaturation was produced by the biosynthesis of a cytosolic protein sensitive to trypsin that loosely binds to the microsomes. By incubating rat liver hepatocytes and HTC cells with labeled eicosa-

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; Δτ, differential polarized phase lifetime; ELSD evaporative light-scattering detector; GLC, gas–liquid chromatography; GP, generalized polarization; HPLC, high-performance liquid chromatography; HTC, hepatoma tissue culture; Laurdan-6-lauroyl-2,4-dimethyl aminonaphthalene; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; $r_\infty$, limiting anisotropy; $r_0$, fundamental anisotropy; $r_s$, steady-state fluorescence anisotropy; S, order parameter; τ lifetime; $\tau_P$, phase lifetime; $\tau_M$, modulation lifetime; $\tau_R$, rotational correlation time; TAT, tyrosine aminotransferase; TMA-DPH, 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene.

8,11,14-trienoic acid in the presence of various corticoids, it was shown that not only dexamethasone but also corticosterone and the mineralocorticoids 11-deoxycorticosterone and aldosterone depressed the biosynthesis of arachidonic acid (11). Similarly, both mineralocorticoids depressed Δ6 desaturation *in vivo* (12). However, the mechanisms of action of each one of these hormones were different, and only the effect of 11-deoxycorticosterone, like dexamethasone, was mediated through a soluble protein of approximately 18 kD present in the liver cytosolic fraction. This protein was induced by the occupancy of the glucocorticoid receptor (13). The inhibitory effect of aldosterone was mediated by a different mechanism. Like dexamethasone (14), 11-deoxycorticosterone induced a cytosolic protein that stimulated Δ9 desaturase activity in rat liver microsomes (15).

Since the above-mentioned corticoids alter the biosynthesis of monoenoic acids and polyunsaturated acids of the n-6 and n-3 families, it was important to recognize the corresponding changes evoked in the composition of tissue lipids. To explore these effects, we investigated the changes produced by a long-term (15 d) administration of dexamethasone on the fatty acid composition of total rat liver microsomal lipids and specifically on phosphatidylcholine (PtdCho), which is the principal component of this membrane and a good change detector in polyunsaturated fatty acids. Moreover, considering that the real effect is evoked in the distribution of the different molecular species of this phospholipid, we studied them as well. In addition, we checked the possible changes in the biophysical properties of the microsomal membrane lipid bilayers by fluorescence techniques.

## MATERIALS AND METHODS

*Animal treatment.* All studies performed with animals were carried out in accordance with accepted international standards. Male Wistar rats weighing between 180 and 200 g were separated into two groups. They were fed a commercial diet (Nutrimento, type 3; Escobar, Argentina). The relative percentages of the fatty acids in the diet were 17.0 palmitic, 0.2 palmitoleic, 5.9 stearic, 25.6 oleic, 1.7 vaccenic, 46.2 linoleic, and 3,4 α-linolenic. One group was used as control. The treated animals were injected daily intraperitoneally for 15 d with 0.5 mg/kg weight dexamethasone phosphate (Sidus, Buenos Aires, Argentina). Thereafter, the injected animals had a mean weight of 165 g, and those in the control group had a mean weight of 250 g. All the animals were killed by decapitation without anesthesia. The liver from each animal was rapidly excised and received in ice-cold homogenizing solution (1:3 wt/vol) which was composed of 0.25 M sucrose, 1 mM EDTA, and 10 mM phosphate buffer (pH 7.2). Microsomes were obtained by differential ultracentrifugation at 100,000 × *g* (Beckman Ultracentrifuge) as described elsewhere (16). They were kept frozen at −80°C. The protein concentration was measured according to the procedure of Lowry *et al.* (17).

To check the effect of the glucocorticoid injection, tyrosine aminotransferase (TAT) activity was determined in the 100,000 × *g* supernatants (cytosol fraction) from both control and dexamethasone-treated rats. All measurements were performed according to the method of Dradmonstone (18). The specific activity in noninduced rat liver cytosol ranged from 5.9 to 8.1 TAT units/mg protein and in treated animals from 32.4 to 44.7 TAT units/mg protein. Only those animals with more than 32.4 TAT units/mg protein were used in the present experiments.

*Lipid analysis.* Lipids were extracted from microsomes according to the procedure of Folch *et al.* (19). Total lipid content was measured by aliquot evaporation to constant weight. Cholesterol content was determined by the procedure of Huang *et al.* (20) and total phosphorus by the method of Gomori (21).

Phosphatidylcholine (PtdCho) and other phospholipid classes were separated from total lipids by high-performance liquid chromatography (HPLC) using an evaporative light-scattering detector (ELSD) (22). An Econosil silica column of 10 μm and 250 × 4.6 mm from Alltech Associates (Deerfield, IL) was used. Elution was performed at a flow rate of 1 mL/min by a gradient of hexane/isopropanol/dichloromethane (40:48:12 by vol) to hexane/isopropanol/dichloromethane/water (40:42:8:8 by vol) for 15 min followed by additional elution with the latter solvent for 30 min.

Nebulization in the ELSD was set at 90°C drift tube temperature and 2.20 L/min of nitrogen gas flow to the nebulizer. PtdCho peaks were collected manually from the column effluent using a flow splitter. The solvent was evaporated under $N_2$ and redissolved in methanol/triethylamine (2:1 vol/vol).

The fatty acid compositions of total lipid PtdCho as well as its molecular species were determined by capillary gas–liquid chromatography (GLC) in a Shimadzu Chromatograph, model GC-R9A. A 30 m × 0.25 mm internal diameter with 0.25 μm thickness Omega Wax 250 column (Supelco Inc., Bellefonte, PA) was used. Temperature was programmed for a linear increase of 3°C/min after 3 min at the starting temperature of 185°C and 19 min at the final temperature of 230°C. Helium was used as carrier gas. The chromatographic peaks were identified by comparison of the retention times with those of standards.

*PtdCho molecular species separation*. The separation of the molecular species was done using the method of Browers *et al.* (23). Resolution of molecular species was performed on two 5-μm endcapped Lichrosphere 100-RP18 columns in series (Merck, Darmstadt, Germany). Isocratic elution was applied using a solvent composed of methanol/acetonitrile/triethylamine (58:40:2 by vol) at a flux of 1 mL/min. The detection and quantification were done in an ELSD using $N_2$ as nebulizer at a flux of 1.8 L/min and temperature of 100°C (23). A sample of 1 mg of PtdCho was injected; 5 of 100 parts went to the detector and the remaining materials of the peaks were collected and identified by GLC analysis.

*Fluorescent probes*. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Sigma Chemical Co. (St. Louis, MO). 6-Lauroyl-2,4-dimethylaminonaphthalene (Laurdan) and 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene

(TMA-DPH) were purchased from Molecular Probes (Eugene, OR).

*Preparation and labeling of lipid vesicles*. Microsomal lipids (1 mg) in chloroform solution were added to a round-bottomed glass tube. The solvent was evaporated under an $N_2$ stream, and 1 mL of buffer A (50 mM sodium phosphate pH 7.4) was added. After 15 min at room temperature to allow hydration, samples were vigorously vortexed for 2 min. Then, the lipid suspensions were extruded 11 times through a 100-nm pore polycarbonate filter using a Liposo-Fast extruder (Avestin, Inc., Ottawa, Canada). For labeling, 0.25 mL of the extruded vesicles was mixed with 0.25 mL of a 4-μM suspension of the fluorescent probe (DPH, TMA-DPH, or Laurdan) in buffer A and vigorously vortexed. Then, samples were kept at room temperature for at least 30 min and diluted five times with buffer A before the fluorescence measurements were made. The final concentration of the samples was 0.1 mg/mL of lipids and 4 μM of the fluorescent probe.

*Fluorescence measurements.* All measurements were made in an SLM 4800 spectrofluorometer in 1 × 1 cm cuvettes. DPH or TMA-DPH steady-state fluorescence anisotropy ($r_s$), lifetime ($\tau$), and differential polarized phase lifetime ($\Delta\tau$) were measured using an excitation wavelength of 361 nm and observing the total emission at wavelengths >389 nm through a sharp cut-off filter (KV389) according to Lakowicz *et al.* (24,25) with some modification (26–28). For the $\tau$ and $\Delta\tau$ measurements, the exciting light was modulated sinusoidally in amplitude at 18 or 30 MHz with a Debye-Sears modulator and vertically polarized with a Glan-Thompson polarizer. For $\tau$, the emission was observed through a Glan-Thompson polarizer oriented 55° to the vertical to eliminate the effect of Brownian rotation (29). The phase shift and demodulation of the emitted light were measured relative to the reference standard 1,4-bis(5-phenyloxazol-2-yl)benzene in ethanol ($\tau$ = 1.35 ns) (30) and used to compute the phase ($\tau_P$) and modulation ($\tau_M$) lifetimes of the samples (31). $\Delta\tau$ was obtained from the phase shift between the parallel and perpendicular components of the emission observed with the emission polarizer vertically or horizontally oriented, respectively. Data were interpreted according to the model of hindered wobbling rotation (32). As described elsewhere (33,34), the values obtained for $r_s$, $\tau$, and $\Delta\tau$ were used to compute (i) the rotational correlation time ($\tau_R$), which is inversely related to the rotational rate and reflects the local viscous resistance to the probe rotation; and (ii) the limiting anisotropy ($r_\infty$), which is related to the order parameter $S$ ($S^2 = r_\infty/r_0$) and reflects the limitation imposed by the local environment to the extent or range of the probe wobbling.

Fluorescence spectra of Laurdan were taken with monochromator bandpasses of 8 nm in excitation and emission as previously described (34–36). All spectra were corrected for background contribution by subtracting the signal of unlabeled samples. Generalized polarization spectra were obtained by measuring the excitation intensity spectra using 440 nm ($I_{440}$) and 490 nm ($I_{490}$) for the emission and the emission intensity spectra at 340 nm ($I_{340}$) and 410 nm ($I_{410}$) excitation wavelength. Generalized polarization in the excitation (exGP) and emission (emGP) bands were obtained from $exGP = (I_{440} - I_{490})/(I_{440} + I_{490})$ and $emGP = (I_{410} - I_{340})/(I_{410} + I_{340})$, respectively. Laurdan emission lifetime was measured as for DPH and TMA-DPH by exciting at 360 nm and isolating the emission with a KV389 filter.

## RESULTS

*Effect of dexamethasone on the lipid and fatty acid composition of liver microsomes.* The effect of a daily injection of 0.5 mg/kg weight dexamethasone phosphate for 15 d on rat liver microsomal lipid composition is depicted in Table 1. First, it shows that the cholesterol/phospholipid ratio was somewhat increased by the glucocorticoid treatment. However, only very small changes were observed in the relative proportions of the different glycerophospholipids. There was a small decrease in the percentage of PtdCho, whereas the proportions of phosphatidylethanolamine (PtdEtn), phosphatidylinositol, and phosphatidylserine were slightly increased. By far, PtdCho was the predominant phospholipid of the liver microsomal membrane.

The fatty acid composition of rat liver microsomal lipids is shown in Table 2. The 15-d dexamethasone treatment evoked significant increases of palmitic, palmitoleic, oleic, linoleic, and eicosatrienoic n-6 acids and a decrease of stearic and arachidonic acids.

When we analyzed the general fatty acid composition of liver microsomal PtdCho, a similar effect was found as expected (Table 3). The percentages of palmitoleic, oleic, and linoleic acids were increased. Palmitic acid increased and stearic acid decreased, but without statistical significance, whereas arachidonic acid decreased significantly.

Therefore, these results correspond rather well to a decrease of Δ6 and Δ5 fatty acid desaturation activity that inhibits linoleic acid conversion to arachidonic acid and to an increase of the Δ9 desaturation activity that enhances the conversion of palmitic and stearic acids to palmitoleic and oleic acids, respectively, as already demonstrated (8–11,13,15).

*Effect of dexamethasone on microsomal PtdCho molecular species.* In fact, the fatty acid analysis of the microsomal PtdCho only gives an average composition of the lipid. The

**TABLE 1**
**Percentage Composition of Phospholipids, Cholesterol, and Phospholipid Classes in Liver Microsomal Lipids[a]**

| | Control | Dexamethasone-treated |
|---|---|---|
| Phospholipids | 61.5 ± 0.6 | 63.4 ± 0.2 |
| Cholesterol | 4.2 ± 0.2 | 5.0 ± 0.2 |
| Cholesterol/phospholipid | 0.068 ± 0.004 | 0.079 ± 0.003 |
| PtdCho | 80.5 ± 0.4 | 74.6 ± 0.7 |
| PtdEtn | 14.7 ± 0.4 | 17.6 ± 0.4 |
| PtdIns | 3.8 ± 0.5 | 5.1 ± 0.3 |
| PtdSer | 1.0 ± 0.3 | 2.7 ± 0.9 |

[a]Results are the mean ± standard error of five animals analyzed separately. PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine.

**TABLE 2**
**Fatty Acid Composition (mol%) of Total Microsomal Lipids[a]**

| Fatty acids | Control | Dexamethasone-treated | *P* |
|---|---|---|---|
| 16:0 | 22.42 ± 0.86 | 28.21 ± 0.98 | <0.01 |
| 16:1 | 0.37 ± 0.08 | 1.68 ± 0.28 | <0.01 |
| 18:0 | 24.62 ± 0.48 | 21.58 ± 0.70 | <0.01 |
| 18:1n-9 | 6.91 ± 0.19 | 8.89 ± 0.69 | <0.02 |
| 18:1n-7 | 2.67 ± 0.23 | 1.55 ± 0.03 | <0.01 |
| 18:2n-6 | 14.68 ± 0.17 | 15.71 ± 0.38 | <0.05 |
| 18:3n-6 | 0.56 ± 0.19 | 0.46 ± 0.06 | |
| 18:3n-3 | 0.82 ± 0.17 | 0.72 ± 0.20 | |
| 20:3n-9 | 0.51 ± 0.04 | 0.40 ± 0.09 | |
| 20:3n-6 | 0.58 ± 0.17 | 1.27 ± 0.13 | <0.02 |
| 20:4n-6 | 21.86 ± 0.71 | 15.57 ± 0.73 | <0.001 |
| 22:4n-6 | 0.38 ± 0.08 | 0.49 ± 0.06 | |
| 22:5n-6 | 0.27 ± 0.04 | 0.47 ± 0.03 | |
| 22:5n-3 | 0.85 ± 0.13 | 0.75 ± 0.06 | |
| 22:6n-3 | 2.50 ± 0.22 | 2.16 ± 0.10 | |

[a]Only principal acids were considered. Results are the average of five animals ± standard of the mean.

**TABLE 4**
**Percent Distribution by Weight of PtdCho Molecular Species in Liver Microsomes**

| Molecular species | Control | Dexamethasone-treated | *P* |
|---|---|---|---|
| 18:2/20:4 | 1.44 ± 0.30 | 1.18 ± 0.25 | |
| 18:2/18:2 | 1.59 ± 0.28 | 1.42 ± 0.07 | |
| 16:0/22:6 | 4.32 ± 0.24 | 4.14 ± 0.48 | |
| 18:1/20:4 | 3.69 ± 0.28 | 0.30 ± 0.30 | <0.001 |
| 16:0/20:4 | 21.83 ± 0.60 | 22.26 ± 0.56 | |
| 18:1/18:2 | 1.98± 0.16 | None detected | |
| 16:0/18:2 | 12.62 ± 0.53 | 22.10 ± 1.00 | <0.001 |
| 18:2/22:6 | 3.80 ± 0.66 | 4.68 ± 0.75 | |
| 18:0/20:4 | 36.22 ± 1.03 | 24.72 ± 1.14 | <0.001 |
| 16:0/18:1 | 3.68 ± 0.28 | 5.66 ± 0.52 | <0.01 |
| 18:0/18:2 | 8.83 ± 0.57 | 13.54 ± 0.64 | <0.001 |

[a]Molecular species were separated by high-performance liquid chromatography as described in the Materials and Methods section. Results are the average of five animals ± standard error of the mean. For abbreviation see Table 1.

real situation in the membrane is that PtdCho constitutes an entire family of different molecular species, each one having different pairs of fatty acids. The molecular species of rat liver microsomal PtdCho and the changes induced by dexamethasone treatment are given in Table 4.

Eleven major molecular species were separated, characterized, and quantitated by the method of Browers *et al.* (23) in both control and treated rats.

As reported in other experiments (34,36,37), the predominant liver microsomal PtdCho species in control rats was found to be 18:0/20:4n-6 followed by 16:0/20:4n-6 and 16:0/18:2n-6.

These three species, constituted by one saturated and another unsaturated acid, represent 70.6% of all molecular species of PtdCho in the microsomes. Therefore, they mainly determine the contribution of this phospholipid to the general biophysical properties of this lipid bilayer.

Dexamethasone treatment evoked changes in the proportion of some PtdCho molecular species (Table 4). The most noticeable modification was a sharp decrease of 18:0/20:4n-6 and an important increase of 16:0/18:2n-6 species. Other changes were a decrease of 18:1n-9/20:4n-6 and an increase of 18:0/18:2n-6 and 16:0/18:1n-9 species. However, the proportion of 16:0/20:4n-6 species was not modified. Despite this last important peak and the small 18:2n-6/20:4n-6 peak, which were not altered, the decrease of the other 20:4n-6 containing peak and the notable increase of 16:0/18:2n-6 and 18:0/18:2n-6 species confirm the changes of polyunsaturated fatty acid mean compositions found in PtdCho (Table 3). They also correspond well to a decrease of dietary linoleic acid conversion to arachidonic acid already shown in glucocorticoid-treated rats (8–11).

No significant changes were found in the 22:6n-3 containing species. The expected small increase of 18:1n-9 containing species was only found in the 16:0/18:1n-9 peak but not in the 18:1n-9/20:4n-6 and 18:1n-9/18:2n-6 peaks.

*Effect of dexamethasone on the order and dynamics of microsomal lipids*. The effect of dexamethasone administration on the biophysical properties of the microsomal lipids was studied in large unilamellar vesicles of these lipids using three different fluorescent probes. The rotational behavior of DPH and TMA-DPH was studied by using phase and modulation fluorometry (Table 5). These probes sense the order and dynamics of the lipid bilayer at different depths. The neutral DPH probe locates deeply into the bilayer, whereas the amphipathic TMA-DPH is anchored through its positive charge to the phospholipid polar groups and locates the fluorescent moiety more externally than DPH. Thus, TMA-DPH senses a more polar environment, which is indicated by its shorter lifetime in comparison with DPH. The lifetimes of these probes obtained by phase ($\tau_P$) and modulation measurements ($\tau_M$) at 18 MHz excitation frequency are shown in Table 5. No effect of dexamethasone administration was observed, indicating no change in the polarity in both sensed bilayer regions. Similar lifetime values were obtained by measurements at 30 MHz (data not shown). This fact and also the small differences be-

**TABLE 3**
**Fatty Acid Composition (mol%) of Rat Liver Microsomal PtdCho[a]**

| Fatty acids | Control | Dexamethasone-treated | *P* |
|---|---|---|---|
| 16:0 | 21.31 ± 0.49 | 23.54 ± 1.24 | |
| 16:1 | 0.36 ± 0.05 | 1.02 ± 0.14 | <0.01 |
| 18:0 | 23.43 ± 0.59 | 21.83 ± 0.58 | |
| 18:1n-9 | 4.48 ± 0.05 | 5.73 ± 0.65 | |
| 18:1n-7 | 3.11 ± 0.21 | 1.81 ± 0.21 | <0.01 |
| 18:2n-6 | 13.90 ± 0.33 | 16.95 ± 0.59 | <0.01 |
| 18:3n-6 | 0.78 ± 0.07 | 0.42 ± 0.13 | |
| 18:3n-3 | 2.06 ± 0.31 | 1.73 ± 0.19 | |
| 20:3n-9 | 0.54 ± 0.04 | 0.27 ± 0.01 | <0.0001 |
| 20:3n-6 | 0.94 ± 0.12 | 1.20 ± 0.13 | |
| 20:4n-6 | 24.74 ± 0.32 | 20.41 ± 1.01 | <0.01 |
| 22:4n-6 | 0.24 ± 0.03 | 0.40 ± 0.06 | |
| 22:5n-6 | 0.29 ± 0.03 | 0.49 ± 0.04 | |
| 22:5n-3 | 0.62 ± 0.03 | 0.67 ± 0.07 | |
| 22:6n-3 | 3.20 ± 0.19 | 3.53 ± 0.29 | |

[a]Results are the average of five animals ± standard error of the mean. For abbreviation see Table 1.

**TABLE 5**
**Effect of Treatment with Dexamethasone on the Fluorescence Lifetime and Rotational Parameters of TMA-DPH and DPH in Lipid Vesicles of Rat Liver Microsomes[a]**

| Parameter | | Control group[b] | Treated group[b] |
|---|---|---|---|
| TMA-DPH | $r_s$ | 0.217 ± 0.002 | 0.216 ± 0.002 |
| | $\tau_P$ (ns)[c] | 4.07 ± 0.08 | 4.12 ± 0.07 |
| | $\tau_M$ (ns)[c] | 4.58 ± 0.11 | 4.58 ± 0.10 |
| | $\tau_R$ (ns)[c] | 1.31 ± 0.02 | 1.34 ± 0.04 |
| | $r_\infty$[c] | 0.161 ± 0.002 | 0.159 ± 0.01 |
| DPH | $r_s$ | 0.107 ± 0.001 | 0.109 ± 0.002 |
| | $\tau_P$ (ns)[c] | 7.99 ± 0.08 | 8.09 ± 0.14 |
| | $\tau_M$ (ns)[c] | 8.57 ± 0.07 | 8.55 ± 0.11 |
| | $\tau_R$ (ns)[c] | 1.29 ± 0.08 | 1.26 ± 0.02 |
| | $r_\infty$[c] | 0.058 ± 0.002 | 0.061 ± 0.002 |

[a]TMA-DPH, 1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene; DPH, 1,6-diphenyl-1,3,5-hexatriene.
[b]The values given are the mean ± standard deviation of four animals for the control group and five animals for the treated group. Student's tests do not indicate a significant difference at the 5% probability level for any of the measured parameters.
[c]The indicated values for phase ($\tau_P$) and modulation ($\tau_M$) lifetimes, rotational correlation time ($\tau_R$) and limiting anisotropy ($r_\infty$) were obtained using 18 MHz excitation modulation frequency.

tween $\tau_P$ and $\tau_M$ indicate that there is no large heterogeneity in the probe distribution into different microdomains in the vesicle bilayer. The fact reasonably allows us to apply the hindered wobbling rotation model (32) assuming a unique rotamer population to calculate the rotational correlation time ($\tau_R$) and limiting anisotropy ($r_\infty$) parameters. The $\tau_M$ and $r_\infty$ parameters calculated from measurements at 18 MHz (shown in Table 5) indicate that dexamethasone treatment does not significantly affect the rotational behavior of these probes in the microsomal lipid vesicles.

The effect of dexamethasone administration on the lipid dynamics in vesicles of microsomal lipids was also studied by using the fluorescence properties of Laurdan. This probe locates its fluorescent moiety at the polar-nonpolar interphase of the lipid bilayer, and its emission spectra are highly sensitive to solvent relaxation (38). Small changes in the lipid packing can influence both the amount and mobility of water molecules in the bilayer. An increased water amount at the interfacial region of the bilayer would result in a shorter lifetime and a red-shifted emission of Laurdan fluorescence, whereas an increased mobility of water molecules would produce a red-shifted emission without affecting the fluorescence lifetime (38). Spectral shifts of Laurdan are generally expressed with the generalized polarization (GP) parameter (39). Figure 1 shows the influence of dexamethasone administration on the lipid composition of microsomal membranes and the effect on the GP values calculated from the Laurdan emission spectra at different excitation wavelengths at 25 and 37°C. The observed decrease in GP with increasing excitation wavelength is typical of liquid-crystalline bilayers (40). It is clear from the figure that dexamethasone treatment produces an increase in the GP values when lower excitation wavelengths are used. At lower excitation wavelengths, the less fluid lipid domains should be preferentially selected (40). It is important to note that there was no significant effect of the treatment on the Laurdan fluorescence lifetime because $\tau_R$ was 3.81 ± 0.06 and 3.81 ± 0.03 for control and treated groups, respectively; $\tau_M$ was 4.21 ± 0.03 and 4.20 ± 0.08 for control and treated groups, respectively, measured at 37°C and 18 MHz, indicating no change in the amount of water in the interfacial region. Thus, the observed increase in GP produced by dexamethasone administration can be attributed to an increased lipid packing, which results in reduced water mobility in the less fluid lipid domains preferentially selected at lower excitation wavelengths.

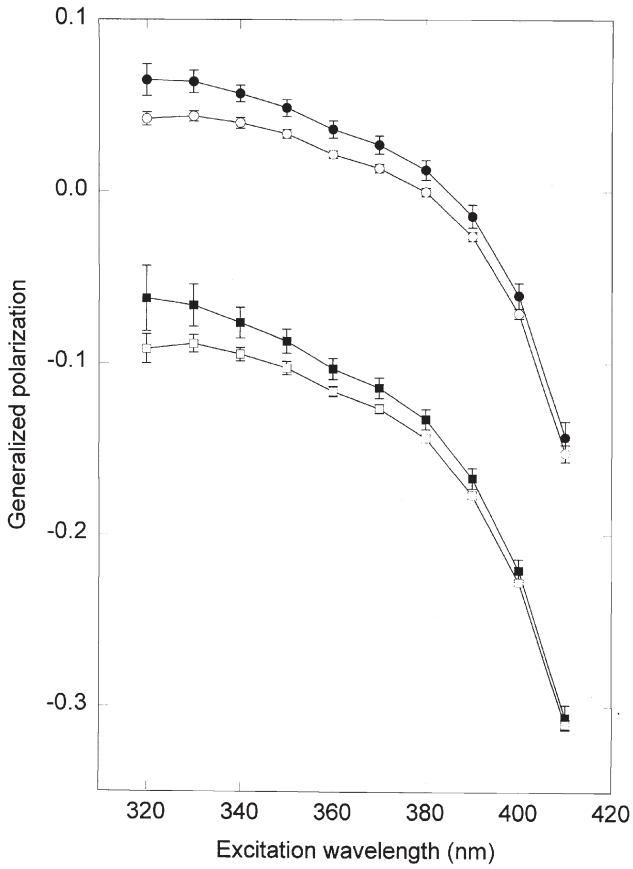


**FIG. 1.** Effect of dexamethasone administration on the Laurdan generalized polarization in microsomal lipid vesicles. Measurements were made on liver microsomal vesicles of control (○,□) and treated animals (●,■) at 25°C (○,●) and 37°C (□,■) as described in the Materials and Methods section. The values are the mean of four and five animals for the control and treated groups, respectively. The standard deviations are indicated by bars.

## DISCUSSION

It is now well accepted that glucocorticoids perform very important functions in glucose and fatty acid metabolism. As already indicated, these hormones depress liver microsomal fatty acid Δ6 and Δ5 desaturase activity and enhance Δ9 desaturase. These changes in the activity of the desaturases are apparently evoked through the biosynthesis of a cytosolic

protein (10,12,13). In the case of Δ9 desaturase, it is important to indicate that the enhancing effect of the hormone has been shown on both [1-$^{14}$C]palmitic and [1-$^{14}$C]stearic acid desaturation. This clarification is pertinent because of the two Δ9 desaturases detected in rodents (41); only one, $SCD_1$ (stearoyl-CoA desaturase 1), is present in liver (42,43), and it is able to desaturate both saturated acids. It could be differently regulated from $SCD_2$, also found in adipose tissue (44). This last isoform is also an exclusive component of other organs.

The biological changes evoked by the alteration of these fatty acid desaturase activities are, in general, the consequence of the alteration produced in the proportion of polyunsaturated fatty acids of n-6 and n-3 families, mainly arachidonic and docosahexaenoic acids, and the monounsaturated acids of n-9 family (oleic acid) in liver and in other organs.

To evaluate these changes evoked by dexamethasone, a very efficient synthetic glucocorticoid that modifies Δ6, Δ5, and Δ9 desaturase activity (8–10,15), we analyzed the fatty acid composition of liver microsomes after 15 d of treatment. This target membrane readily responds to alterations in the availability of polyunsaturated acids of n-6 and n-3 series. This scheme of evaluation is pertinent because the only source of these acids in our experimental conditions was the biosynthesis from the precursor acids, linoleic and α-linolenic. They are the only n-6 and n-3 acids provided by the diet.

As shown in Tables 2 and 3, significant increases in the proportion of linoleic acid and decreases of arachidonic acid of the n-6 family were found in both total lipids of liver microsomes and PtdCho, the main compound (≅80% of the membrane phospholipids) of this bilayer. These facts are in agreement with and apparently the consequence of the decrease of microsomal Δ6 and Δ5 desaturase activities evoked by dexamethasone treatment. In spite of that, it can be argued that the changes produced in the arachidonic and linoleic acid proportions might be due to a redistribution between microsomes and other organelles; the fact that dexamethasone also evokes similar changes in total lipids of cultures of Morris 7288c rat cells confirms our interpretation (45). Moreover, it has also been shown (14) that the corticoid-11-deoxycorticosterone, which similarly to dexamethasone depresses Δ6 and Δ5 desaturases, evoked similar decreases in arachidonic and increases of linoleic acid proportions in liver microsomes, total liver homogenate, cytosol, and plasma lipids.

No significant decrease was detected in the proportion of docosahexaenoic acid of the n-3 family even though its biosynthesis from α-linolenic acid is also mainly controlled by the same enzymes at the level of 18- and 20-carbon fatty acids. However, a difference is found in relation to arachidonic acid because its synthesis requires further steps of elongation to 22 and 24 carbons, a new desaturation by a poorly characterized Δ6 desaturase, and β-oxidation at the peroxisomes (31) that could modify or mask the effect on the mentioned desaturases. Another difference found in our experiments between the mentioned n-6 and n-3 polyunsaturated fatty acid biosynthesis in the animal is that the rats received a diet very rich in linoleic acid (46.2%) but poor in α-linolenic acid (3.4%). Also, less than 3% of 22:6n-3 is found in the total lipids of the membrane compared to more than 20% of 20:4n-6. As a consequence, the possibility is that arachidonic acid, in addition to being a major component of the membrane, is constantly deacylated and reacylated into PtdCho whereas docosahexaenoic acid is more likely to remain in the membrane. Therefore, it is possible that the effect found on the desaturases and transmitted to the arachidonic acid content would be more easily detected than in the n-3 polyunsaturated fatty acid.

However, we must consider that when we studied the effect of experimental diabetes (29), in which the activity of both liver Δ6 and Δ5 desaturases is decreased, we found that, although the amount of arachidonic acid detected in liver microsomal lipids dropped, a significant increase in docosahexaenoic acid was recorded. This last result has not been properly explained yet, but we suggested (29) it could be due to a specific effect on the decreased oxidation of docosahexaenoic acid. It may indicate that the amount of 22:6n-3 acid in the liver microsomes is regulated by an additional mechanism besides that of 20:4n-6 acid.

A remarkable conclusion from these composition studies is that, at least in rat liver microsomal membranes, 15-d glucocorticoid treatment decreases quantitatively the proportion of the biologically very important arachidonic acid of the n-6 family, but it does not alter the amount of the also very important docosahexaenoic acid of the n-3 series.

The increase evoked by dexamethasone in liver microsomal Δ9 desaturase (15) correlates well with the increase of palmitoleic and oleic acid percentages found in microsomal total lipids and PtdCho fatty acids (Tables 2 and 3). However, whereas the decrease of stearic acid found in total lipids (Table 2) also agrees with that effect, a significant change of this acid was not found in PtdCho fatty acids. As well, the increase in the percentage of palmitic acid in both total lipids and PtdCho would not correlate with an increase of Δ9 desaturation activity. However, this result is not an important objection because the relative amount of palmitoleic acid compared to palmitic is extremely low in these lipids, and any possible effect of increased Δ9 desaturation on the palmitic acid amount would pass undetected. Moreover, an even more important explanation for this result is that the increase of palmitic acid in total microsomal lipids and PtdCho is undoubtedly due to the already demonstrated dexamethasone stimulation of fatty acid synthase activity (46,47). This is apparently evoked through an increase of the synthase mRNA transcription and by posttranscriptional events, too (48).

The increase of synthase mRNA evoked by dexamethasone may be also considered, at least in part, as a consequence of the decrease of Δ6 desaturase activity produced by the same glucocorticoid. The decrease of the Δ6 desaturase activity lowers the synthesis and hepatic content of arachidonic acid that, as it is known, suppresses the synthase mRNA transcription (49). The authors (49) also showed that this effect is

not produced by linoleic acid if it is not previously desaturated by the Δ6 desaturase.

*PtdCho molecular species.* The important change evoked by dexamethasone *in vivo* on the molecular species of PtdCho, which are basic structural components of rat liver endoplasmic reticulum bilayer membranes, is clearly shown in Table 4.

As already found in other experiments (34,36,37), the highly predominant PtdCho species in the rat liver microsomes were 18:0/20:4n-6, 16:0/20:4n-6, and 16:0/18:2n-6. They were the principal determinants of the contribution of this phospholipid to the general biophysical properties and some of the chemical properties of the bilayer. Similar predominant molecular species were also shown in rat liver microsomal PtdEtn (37). In PtdIns, 18:0/20:4n-6 and 16:0/20:4n-6 were nearly exclusive components (37).

The decrease in the proportion of arachidonic acid and the increase of linoleic acid shown in PtdCho (Table 3) and evoked by dexamethasone are the consequence of the significant decrease of the predominant 18:0/20:4n-6 and small 18:1n-9/20:4n-6 PtdCho molecular species, and the increase of the predominant 16:0/18:2n-6 and also important 18:0/18:2n-6 PtdCho molecular species. However, other minor species containing 18:2n-6 or 20:4n-6 acids as 18:2n-6/20:4n-6 and 18:1n-9/18:2n-6 are little changed. But what is really remarkable is that the very important 16:0/20:4n-6 PtdCho species is not lowered compared to 18:0/20:4n-6. This undoubtedly indicates that the decrease of arachidonic acid biosynthesis evoked by the inactivation of Δ6 and Δ5 desaturases by dexamethasone determines less availability of this acid for modeling the fatty acids at the 2-position of PtdCho molecules, but the other fatty acids at position 1, and other factors as mechanisms of phospholipid synthesis and specificity of enzymes, also determine the final molecular compositions. The effect of glucocorticoids on phospholipase $A_2$ activity may be an additional factor contributing to the remodeling of PtdCho molecular species.

In the specific case of 16:0/20:4n-6 and 18:0/20:4n-6 species changes, the difference might be due, at least in part, to the different ways 16:0 and 18:0 are affected by dexa-methasone. In the case of 16:0 it has been repeatedly shown that fatty acid synthase is activated by glucocorticoids (46,47).

As expected from the fatty acid composition of microsomal lipids displayed in Tables 2 and 3, no significant modification in the amounts of both 16:0/22:6n-3 and 18:0/22:6n-3 species was evoked by dexamethasone. Although these species are minor components of PtdCho, the reason for no modification is not clear, as previously discussed.

Oleic acid-containing species are not abundant in the liver microsomes of rats fed a linoleic acid rich-diet, and only 16:0/18:1n-9 was nearly doubled by dexamethasone treatment, the consequence of the increase of Δ9 desaturase activity. Indeed, they are unimportant species for the determination of PtdCho contribution to the dynamic properties of the lipid bilayer.

*Effect on dynamic properties of microsomal lipid bilayer.* The changes in the lipidic composition and PtdCho molecular species of rat liver microsomes and the small increase of the cholesterol/phospholipid ratio evoked by dexamethasone administration showed no significant effect on the rotational behavior of DPH and TMA-DPH in vesicles made with these microsomal lipids (Table 5) in spite of testing different depths of the bilayer. The lack of effect on the fluorescent lifetimes of these probes as well as those of Laurdan indicate no change in the environment polarity of these probes at their different locations. However, as indicated by the increased Laurdan GP at lower excitation wavelengths, dexamethasone produces an increased lipid packing in the preferentially selected less fluid domains, resulting in a decreased mobility of water molecules present in the interfacial region of the bilayer in these domains.

It is not surprising that the increased lipid packing as detected by Laurdan does not result in any detectable change in the rotational mobility of DPH and TMA-DPH. We previously showed that higher sensitivity of Laurdan detected small changes in the lipid packing that were not detected by DPH or TMA-DPH, for instance those produced by streptozotocin diabetes (36) or polyunsaturated fatty acid deficiency (35) in rat liver microsomes. The high sensitivity of Laurdan was attributed to the fact that it senses the rotational mobility of a very small molecule, such as water (35). Small changes in the lipid packing that do not appreciably alter the rotational behavior of relatively large molecules like DPH or TMA-DPH can change the motion rate of smaller molecules such as water. Moreover, DPH and TMA-DPH would sense the motion in the different lipid domains, which, on average, were apparently not greatly changed by dexamethasone administration. On the contrary, Laurdan detects an increased lipid packing after dexamethasone treatment only in the less fluid lipid domains, and this might be related to the change of the molecular species of PtdCho and/or to the small increase of the cholesterol/phospholipid ratio. This effect of dexamethasone in the less fluid lipid domains contrasts with the effects produced in the more fluid domains (i.e., at longer wavelengths) by streptozotocin diabetes (36) (which decreases GP) and polyunsaturated fatty acid deficiency (35) (which increases GP). The relationship between compositional changes and these effects on particular lipid domains is not yet easily interpretable.

The main effect evoked by the glucocorticoid in liver is a decrease of arachidonic acid in the membrane phospholipids that alters the proportion of the molecular species of PtdCho, decreasing the 18:0/20:4n-6 species. However, since the proportion of saturated/unsaturated acid molecules is roughly maintained in spite of a small increase in the cholesterol/phospholipid ratio, no important change is evoked in the bulk fluidity of microsomal bilayer as tested by fluorometric techniques. Only a small packing increase is found in the less fluid lipid domains. However, fluorometric techniques used cannot detect changes in specific particular lipid domains that are very probably produced.

The decrease of arachidonic acid stores in liver evoked by dexamethasone would result in lower availability of this acid for its main biological functions. An inadequate availability

of arachidonic acid is associated with impaired nerve transmission, reduced eicosanoid synthesis, and impaired fetal growth. It exerts vital functioning effects in key steps of cell signaling and the expression of a wide array of genes, including those encoding proteins involved in lipid metabolism, thermogenesis, and cell differentiation.

Moreover, the decrease of arachidonic acid biosynthesis and its pools and the depression of phospholipase $A_2$ activity (50) evoked by dexamethasone are cooperative factors that prevent generation of arachidonic-acid derived prostaglandins and other eicosanoids and produce the well-known anti-inflammatory effect of glucocorticoids.

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# Analysis of Molecular Species of Peroxide Adducts of Triacylglycerols Following Treatment of Corn Oil with *tert*-Butyl Hydroperoxide

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**ABSTRACT:** We recently demonstrated that exposure of synthetic mono- and diunsaturated triacylglycerols to *tert*-butylhydroperoxide (TBHP) leads to formation of stable adducts of the oxidizing agent and the unsaturated esters (Sjövall, O., Kuksis, A., and Kallio, H., Reversed Phase High-Performance Liquid Chromatographic Separation of *tert*-Butyl Hydroperoxide Oxidation Products of Unsaturated Triacylglycerols, *J. Chromatogr. A 905*, 119–132, 2001). In the present study we isolated and identified the TBHP adducts of corn oil triacylglycerols. The much wider range of molecular species available in the corn oil permitted us to demonstrate that the yield of the adducts varies with the degree of unsaturation of the triacylglycerol. The highest yields were obtained for the linoleate (20% of linoleoyl-containing residual triacylglycerols) and the lowest ones for the oleate (5% of oleoyl-containing residual triacylglycerols) triacylglycerols, whereas the saturated triacylglycerols did not give TBHP adducts in readily detectable amounts. Normal-phase thin-layer chromatography along with reversed-phase high-performance liquid chromatography/mass spectrometry (LC/MS) with electrospray ionization was used to isolate and separate the major molecular species of polyunsaturated triacylglycerols and corresponding TBHP adducts. As an extreme example, the dilinoleoylmonooleoylglycerol was identified as the mono-, di-, tri-, tetra-, and penta-TBHP adduct. LC/MS with electrospray ionization at elevated capillary exit voltage (pseudo tandem mass spectrometry) was used to confirm structures of the $[M - RCOOH]^+$ ions and the absence of TBHP adducts of $[M - RCOOH]^+$. It is concluded that stable adduct formation is an unavoidable complication of preparation of oxotriacylglycerols by oxidation with concentrated TBHP solutions and care must be taken to resolve the adducts from the desired oxidation product.

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*tert*-Butyl hydroperoxide (TBHP) is a popular lipid-oxidizing agent (1) and has been extensively utilized for the preparation of hydroperoxides and epoxides of unsaturated fatty acid methyl esters (2,3), triacylglycerols (4,5), cholesteryl esters (6,7), and glycerophospholipids (8,9). We have recently detected significant peroxide bridging during preparation of hydroperoxide reference compounds, especially when more concentrated solutions of the hydroperoxide are employed (10). We observed that the oxotriacylglycerols included both chain desaturation and chain cleavage products, many of which formed peroxide bridges with TBHP. Since this reagent is commonly used for the hydroperoxidation of natural lipid mixtures for subsequent biochemical and physiological studies, the formation and recovery of the TBHP adducts during a simple lipid extraction of the reaction mixture is of interest, as is the identification of the adducts.

In the present report we describe the isolation and identification of the TBHP adducts from the residual triacylglycerol mixture recovered from rapid peroxidation of corn oil. Reversed-phase liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) analysis of the molecular species established that the yield of the adducts varies with the degree of unsaturation of the triacylglycerol. The linoleates yielded the most and the oleates the least, whereas saturates usually failed to give any TBHP adducts.

## MATERIALS AND METHODS

*Materials*. TBHP as 70% solution in water and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma Chemical Co. (St. Louis, MO). The solvents were of chromatography grade or of reagent grade and were dried over anhydrous sodium sulfate.

Synthetic hydroperoxides, epoxides, hydroxides, and core aldehydes of the common palmitoyl, oleoyl, and linoleoyl triacylglycerols were prepared in the laboratory as described in previous studies (4,5). The corn oil (Mazola, Englewood Cliffs, NJ) was obtained from a local grocer. The corn oil triacylglycerols were isolated by thin-layer chromatography (TLC) as previously described (5).

*Oxidation*. The oxidation of corn oil was performed by adding 1 mL of 35 to 70% TBHP in water to 10 mg of purified triacylglycerols in the presence of 10 μM $FeSO_4$ and 100 μL of 0.2% taurocholic acid (5). The reaction mixture was incubated on a mechanical agitator in darkness for 90 min at

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Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:0, arachidic acid; CapEx 300, capillary exit voltage of 300 V; DNPH, 2,4-dinitrophenylhydrazine; ELSD, evaporative light-scattering detector; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC, liquid chromatography; M, molecular ion; MS, mass spectrometry; Rt, retention time; TBHP, *tert*-butyl hydroperoxide; TCN, theoretical carbon number; TLC, thin-layer chromatography.

37°C. The reaction was stopped by adding to the mixture 5 mL of chloroform/methanol (2:1, vol/vol), 100 μL of 2% in water, and 10 μL of 2% butylated hydroxy toluene in methanol. The extracts were washed three times with water (3 × 1 mL). The solvent was evaporated under nitrogen at 38°C and the lipid residue was saved for TLC, high-performance liquid chromatography/evaporative light-scattering detection (HPLC/ELSD), and LC/ESI/MS.

*Preparation of DNPH derivatives*. The DNPH derivatives of triacylglycerol core aldehydes were synthesized by adding freshly prepared DNPH in 1 N HCl (0.5 mg/mL) to an aliquot of dry sample (5). The mixture was shaken vigorously and kept in the dark at room temperature for 4 h and overnight at 4°C. The lipids were extracted by 5 mL of chloroform/methanol (2:1, vol/vol), the chloroform phase was blown down under nitrogen, and the residue was taken up in an appropriate solvent for chromatography and MS as described next.

*TLC.* Normal-phase TLC was used to purify triacylglycerols and to resolve the oxotriacylglycerols and their DNPH derivatives. Silica gel H (Merck & Co., West Point, PA) plates were prepared in the laboratory and heptane/isopropyl ether/acetic acid (60:40:4, by vol) solution was used as a mobile phase (5). The complex mixture resolved into a total of nine yellow bands and a band for residual triacylglycerols (TLC Band 10), which were present mostly as the TBHP adducts (see below). The DNPH derivatives of the core aldehydes were seen as yellow bands on the chromatoplates (in daylight). The compounds were recovered from the silica gel scrapings by extraction with chloroform/methanol 2:1 (vol/vol). Extracts were washed with distilled water, dried with anhydrous $Na_2SO_4$, and saved for subsequent HPLC/ELSD and LC/ESI/MS analysis.

*HPLC/ELSD and LC/ESI/MS.* Procedures for HPLC and LC/ESI/MS were as described previously (5,11). Triacylglycerols and oxidation products were resolved by reversed-phase HPLC on a Supelcosil LC-18 column (Bellefonte, PA; 250 × 4.6 mm i.d.), using a linear gradient of 20–80% 2-propanol in methanol (0.85 mL/min) in 30 min. A Hewlett-Packard model 1050 liquid chromatograph was coupled to a Varex ELSD II light-scattering detector (Varex, MD) using nitrogen as nebulization gas and an evaporation temperature of 85°C. LC/ESI/MS was performed using a Hewlett-Packard model 1090 liquid chromatograph interfaced with a nebulizer-assisted ESI source connected to a Hewlett-Packard model 5989A quadrupole mass spectrometer (10). The HPLC conditions were the same as described above except that ammonia/isopropanol (1%) was added postcolumn at a flow rate of 0.15 mL/min in order to enhance ionization (10).

*Peak identification*. The chromatographic peaks of the oxotriacylglycerols were identified on the basis of reversed-phase HPLC retention times of standards and estimated elution factors for functional groups and their positional distribution as previously described (5). The identification of major peaks was confirmed by single-ion mass chromatograms extracted by computer from the total positive ion current spectra.

*Quantification.* The yields of the major oxidation products were quantified by HPLC/ELSD. The results were compared to those obtained with LC/ESI/MS. The ammonia, sodium, and potassium adducts were summed to obtain a total estimate for the mass of species in each mass spectrum. The TBHP adducts were estimated separately as they were clearly resolved from other species during TLC and the reversed-phase HPLC/ELSD and LC/ESI/MS.

## RESULTS

*LC/ESI/MS profiles of total corn oil and total oxidized corn oil.* Figure 1 compares the total ion current profiles of natural corn oil and corn oil exposed to TBHP for 90 min as obtained by reversed-phase LC/ESI/MS. Corresponding peaks are assigned the same numbers in the elution profiles and the peak identities are listed in Tables 1–3. The native oil gives seven major peaks ranging in theoretical carbon number from 42 to 54, along with seven minor ones mostly of lower carbon number. Owing to extensive peak splitting, the oxidized oil gives a somewhat larger number of peaks, which, however, are eluted over the same range of retention times. The peaks

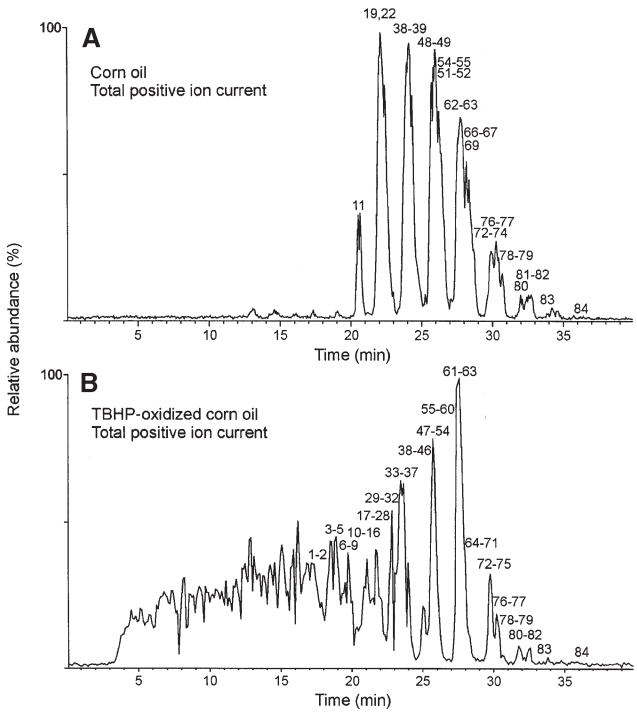


**FIG. 1.** Total liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) ion current profiles of native (A) and oxidized (B) corn oil. Oxidized corn oil obtained by exposure to *tert*-butyl hydroperoxide (TBHP) for 90 min. Peak identities are given in Tables 1–3. LC/ESI/MS conditions are as follows: Supelco (Bellefonte, PA) LC-18 column (250 × 4.6 mm i.d.) installed in a Hewlett-Packard Model 1090 liquid chromatograph interfaced with a nebulizer-assisted ESI source connected to a Hewlett-Packard Model 5989A quadrupole mass spectrometer (10). The high-performance liquid chromatography column was developed with a linear gradient of 20–80% 2-propanol in methanol (0.85 mL/min) in 30 min. Ammonia (1%) in isopropanol was added postcolumn at a flow rate of 0.15 mL/min in order to enhance ionization.

**TABLE 1**
**Residual TAG from TBHP-Oxidized Corn Oil Triacylglycerols (TLC Band 10) as Obtained by Reversed-Phase LC/ESI/MS (uncorrected ion abundances)[a]**

| Peak no.[b] | Rt | TCN | ACN/DB | Mass[c] | Abundance[d] | TLC band[e] | Molecular structure[f] |
|---|---|---|---|---|---|---|---|
| 11 | 20.48 | 41.15 | 54:7 | 894 | 8053 | 10 | 18:2/18:3/18:2 |
| 19 | 21.79 | 41.87 | 52:5 | 870 | 3125 | 10 | 18:2/18:2/16:1 |
| 22 | 22.12 | 42.92 | 54:6 | 896 | 62159 | 10 | 18:2/18:2/18:2 |
| 38 | 24.01 | 43.87 | 54:5 | 898 | 82975 | 10 | 18:2/18:2/18:1 |
| 39 | 24.13 | 44.38 | 52:4 | 872 | 69823 | 10 | 16:0/18:2/18:2 |
| 48 | 25.64 | 45.48 | 54:4 | 900 | 86623 | 10 | 18:1/18:2/18:1 |
| 49 | 25.95 | 45.71 | 52:3 | 874 | 96319 | 10 | 16:0/18:2/18:1 |
| 51 | 26.17 | 45.26 | 50:2 | 848 | 64479 | 10 | 14:0/18:1/18:1 |
| 52 | 26.18 | 46.12 | 56:5 | 926 | 4275 | 10 | 18:2/18:2/20:1 |
| 54 | 26.36 | 46.38 | 54:4 | 900 | 71711 | 10 | 18:0/18:2/18:2 |
| 55 | 26.49 | 46.00 | 46:0 | 796 | 1799 | 10 | 14:0/16:0/16:0 |
| 62 | 27.37 | 47.45 | 54:3 | 902 | 136767 | 10 | 18:1/18:1/18:1 |
| 63 | 27.72 | 47.26 | 52:2 | 876 | 71231 | 10 | 16:0/18:1/18:1 |
| 66 | 28.13 | 47.71 | 54:3 | 902 | 69311 | 10 | 18:0/18:2/18:1 |
| 67 | 28.39 | 48.22 | 52:2 | 876 | 89535 | 10 | 16:0/18:2/18:0 |
| 69 | 28.53 | 48.38 | 56:4 | 928 | 17503 | 10 | 20:0/18:2/18:2 |
| 72 | 29.45 | 48.62 | 56:3 | 930 | 61487 | 10 | 18:1/18:1/20:1 |
| 73 | 29.48 | 48.00 | 48:0 | 824 | 15351 | 10 | 16:0/16:0/16:0 |
| 74 | 29.69 | 49.26 | 54:2 | 904 | 113919 | 10 | 18:0/18:1/18:1 |
| 76 | 30.20 | 49.49 | 52:1 | 878 | 130463 | 10 | 16:0/18:1/18:0 |
| 77 | 30.29 | 49.71 | 56:3 | 930 | 29063 | 10 | 20:0/18:2/18:1 |
| 78 | 30.52 | 50.22 | 54:2 | 904 | 53503 | 10 | 18:0/18:2/18:0 |
| 79 | 30.71 | 50.38 | 58:4 | 956 | 3530 | 10 | 22:0/18:2/18:2 |
| 80 | 31.80 | 50.85 | 56:2 | 932 | 127231 | 10 | 18:0/18:1/20:1 |
| 81 | 32.18 | 51.71 | 58:3 | 958 | 13283 | 10 | 22:0/18:2/18:1 |
| 82 | 32.32 | 51.49 | 54:1 | 906 | 104159 | 10 | 18:0/18:1/18:0 |
| 83 | 33.73 | 53.26 | 58:2 | 960 | 61839 | 10 | 22:0/18:1/18:1 |
| 84 | 36.08 | 55.49 | 58:1 | 962 | 45887 | 10 | 18:0/18:1/22:0 |

[a]Abbreviations: LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; Rt, retention time; TCN, theoretical carbon number; ACN, acyl carbon number; DB, double bond number; TBHP, *tert*-butylhydroperoxide; TLC, thin-layer chromatography; TAG, triacylglycerol.
[b]Numbers of the compounds refer to peaks in Figures 1 and 4.
[c]Nominal mass of $[M + NH_4]^+$.
[d]Abundance of an ion in the first-mentioned TLC band.
[e]The most abundant band mentioned first. Minor amounts of residual TAG were detected also in other bands.
[f]The most likely structure is given, although the positional order of fatty acids may not be as presented.

eluted over the range of 3 to 15 min are due to oxidation products of molecular weights below those of triacylglycerols and were not further examined at this time. Although many of the peaks overlap in retention time, they differ greatly in composition of molecular species because of the exposure to TBHP. Figure 2 compares the total positive ion current mass spectra averaged over the elution time of the native and oxidized corn oil. The major difference in the spectra is the great proportion of the *m/z* 963, 989, and higher (e.g., *m/z* 1019–1075) masses in the mass spectrum of the oxidized oil.

*Isolation of residual triacylglycerols.* Figure 3 shows the TLC separation of the corn oil triacylglycerol oxidation products following derivatization with DNPH. A total of nine yellow bands were obtained along with a colorless 10th band migrating ahead of the others. Since the yellow color was due to dinitrophenylhydrazone formation, it was obvious that most, if not all, of the oxidized triacylglycerol bands contained at least one triacylglycerol with a carbonyl function. On the other hand, TLC Band 10 was not expected to contain carbonyl esters, the bulk of the material presumably representing the unoxidized (residual) triacylglycerols. An LC/ESI/MS examination of TLC Band 10, however, revealed that it contained ion masses far exceeding those anticipated for the simple oxidation products of corn oil triacylglycerols.

Table 4 compares the fatty acid composition of the original corn oil and of TLC Band 10 of the oxidized oil. There was extensive destruction of linoleic acid and, to a lesser extent, oleic acid during the oxidation, as indicated by a fourfold decrease in the proportion of the linoleate and a twofold increase in the proportion of the oleate. The proportion of palmitate doubled, and that of the stearate tripled. The minor saturated long-chain fatty acids proportionally increased.

Figure 4 shows the total positive ion current profile of TLC Band 10 as obtained by reversed-phase LC/ESI/MS, along with the full mass spectrum (*m/z* 700 to 1400) averaged over the entire elution profile. The total ion current profile of native corn oil overlaps partly with that of the total oxidized corn oil, suggesting the presence of residual triacylglycerols,

**TABLE 2**
**Mono-TBHP Adducts of TAG from TBHP-Oxidized Corn Oil TAG (TLC Band 10) as Obtained by Reversed-Phase LC/ESI/MS (Uncorrected Ion Abundances)[a]**

| Peak no.[b] | Rt | TCN | ACN/DB | Mass[c] | Abundance[d] | TLC band[e] | Molecular structure[f] |
|---|---|---|---|---|---|---|---|
| 6 | 19.28 | 40.87 | 54:6 | 984 | 23199 | 10(8) | 18:2/18:2/18:2 TBHP |
| 9 | 19.94 | 40.98 | 54:6 | 984 | 49839 | 10(8) | 18:2/18:2/18:2 TBHP |
| 10 | 20.22 | 39.93 | 52:5 | 958 | 8303 | 10(9,8) | 18:2/18:2/16:1 TBHP |
| 13 | 20.95 | 43.43 | 54:4 | 988 | 28951 | 10(9,8) | 18:1/18:2/18:1 TBHP |
| 14 | 21.05 | 41.82 | 54:5 | 872 | 96479 | 10(9,8) | 18:2/18:2/18:1 TBHP |
| 15 | 21.12 | 42.23 | 52:4 | 960 | 53343 | 10(9,8) | 16:0/18:2/18:2 TBHP |
| 17 | 21.80 | 42.44 | 52:4 | 960 | 61839 | 10(9,8) | 16:0/18:2/18:2 TBHP |
| 18 | 21.60 | 43.54 | 54:4 | 988 | 27375 | 10(9,8) | 18:2/18:1/18:1 TBHP |
| 20 | 21.75 | 41.93 | 54:5 | 986 | 116799 | 10(9,8) | 18:2/18:2/18:1 TBHP |
| 28 | 22.63 | 44.33 | 54:4 | 988 | 102783 | 10(9,8) | 18:0/18:2/18:2 TBHP |
| 29 | 22.88 | 45.15 | 54:3 | 990 | 28799 | 10(9) | 18:1/18:1/18:1 TBHP |
| 30 | 22.93 | 42.96 | 50:2 | 936 | 13983 | 10(8) | 14:0/18:1/18:1 TBHP |
| 31 | 22.93 | 43.66 | 52:3 | 962 | 77439 | 10(9,8) | 16:0/18:2/18:1 TBHP |
| 32 | 22.95 | 44.96 | 52:2 | 964 | 20063 | 10(9) | 18:0/18:2/18:1 TBHP |
| 33 | 23.33 | 44.44 | 54:4 | 988 | 108863 | 10(9,8) | 18:0/18:2/18:2 TBHP |
| 34 | 23.46 | 45.29 | 54:3 | 990 | 24631 | 10(9) | 18:1/18:1/18:1 TBHP |
| 35 | 23.60 | 43.77 | 52:3 | 962 | 71519 | 10(9,8) | 18:2/18:1/16:0 TBHP |
| 36 | 23.69 | 45.10 | 52:2 | 964 | 18143 | 10 | 18:0/18:1/18:1 TBHP |
| 37 | 23.80 | 43.10 | 50:2 | 936 | 20215 | 10(8) | 14:0/18:1/18:1 TBHP |
| 44 | 24.90 | 45.02 | 56:4 | 1016 | 8623 | 10 | 18:1/18:2/20:1 TBHP |
| 45 | 25.03 | 45.66 | 54:3 | 990 | 139455 | 10(8) | 18:0/18:2/18:1 TBHP |
| 46 | 25.14 | 46.17 | 52:2 | 964 | 68063 | 10(8) | 16:0/18:2/18:0 TBHP |
| 47 | 25.48 | 46.33 | 56:4 | 1016 | 15127 | 10 | 20:0/18:2/18:2 TBHP |
| 50 | 25.97 | 46.28 | 52:2 | 964 | 16623 | 10(8) | 16:0/18:0/18:2 TBHP |
| 53 | 26.33 | 46.44 | 56:4 | 1016 | 13839 | 10 | 20:0/18:2/18:2 TBHP |
| 60 | 27.07 | 46.46 | 56:3 | 1018 | 24063 | 10 | 18:1/18:1/20:1 TBHP |
| 61 | 27.12 | 47.10 | 54:2 | 992 | 24079 | 10 | 18:0/18:1/18:1 TBHP |
| 64 | 28.05 | 47.66 | 56:3 | 1018 | 17103 | 10 | 18:1/18:2/20:0 TBHP |
| 65 | 28.08 | 48.17 | 54:2 | 992 | 14607 | 10 | 18:0/18:2/18:0 TBHP |
| 68 | 28.40 | 48.44 | 58:4 | 1044 | 3593 | 10 | 22:0/18:2/18:2 TBHP |
| 71 | 29.37 | 48.55 | 56:2 | 1020 | 20407 | 10 | 18:0/18:1/20:1 TBHP |
| 75 | 30.11 | 48.69 | 56:2 | 1020 | 7511 | 10 | 18:0/18:1/20:1 TBHP |

[a]For abbreviations see Table 1.
[b]Numbers of the compounds refer to peaks in Figures 1 and 4.
[c]Nominal mass of $[M + NH_4]^+$.
[d]Abundance of an ion in the first-mentioned TLC Band.
[e]The most abundant band mentioned first.
[f]The most likely structure is given, although the *sn*-positional order of fatty acids may not be as presented. Underlined double bond shows the *sn*-position of TBHP group for calculation of TCN value.

although the splitting of the peaks is much deeper. The peak numbers are assigned as explained above and their identities are given in Tables 1–3. The mass spectra of the individual HPLC peaks revealed that the masses of the ions corresponded to the mono- and di-TBHP adducts of the residual corn oil triacylglycerols as well as to nonoxygenated triacylglycerols, which, however, had undergone desaturation of fatty chains and conjugation of double bonds. The individual HPLC peaks were identified on the basis of the molecular and fragment ions recorded under each of the peaks and from the relative retention times of standard normal and oxygenated triacylglycerols.

*Identification of residual triacylglycerols.* Figure 5 gives the single-ion chromatograms for the six major residual triacylglycerol masses. In order of decreasing retention time they are *m/z* 933 (18:0/18:1/20:1), 931 (a, 18:1/18:1/20:1; b, 20:0/18:2/18:1), 929 (20:0/18:2/18:2), 877 (a, 16:0/18:1/18:1; b, 16:0/18:2/18:0), 875 (16:0/18:2/18:1), and 873 (16:0/18:2/18:2). Several of these ion peaks are double peaks or possess advance or tailing shoulders. These can be readily attributed to homologs that possess the same total carbon and double bond number but differ in the distribution of the double bonds. Thus, the presence of two double bonds in the same fatty chain shortens the retention time more than the presence of two double bonds in separate fatty chains. Similar single-ion mass chromatograms were obtained for other homologous series of residual triacylglycerols as shown in Table 1. The structures proposed on the basis of relative retention times of standard triacylglycerols were confirmed by the finding of the corresponding $[M - RCOOH]^+$ ions as minor peaks in the total ion spectrum of each peak or as major fragment ions following reanalysis of the sample at a higher

**TABLE 3**
**Di-TBHP Adducts of TAG from TBHP-Oxidized Corn Oil TAG (TLC Band 10) as Obtained by Reversed-Phase LC/ESI/MS (Uncorrected Ion Abundances)[a]**

| Peak no.[b] | Rt | TCN | CAN/DB | Mass[c] | Abundance[d] | TLC band[e] | Molecular structure[f] |
|---|---|---|---|---|---|---|---|
| 1 | 17.31 | 40.25 | 54:6 | 1072 | 54479 | 10(9,8) | 18:2/18:2/18:2 di-TBHP |
| 2 | 17.96 | 40.26 | 54:6 | 1072 | 54255 | 10(9,8) | 18:2/18:2/18:2 di-TBHP |
| 3 | 18.34 | 41.20 | 54:5 | 1074 | 57807 | 10(9) | 18:2/18:2/18:1 di-TBHP |
| 4 | 19.04 | 41.21 | 54:5 | 1074 | 111143 | 10(9) | 18:2/18:2/18:1 di-TBHP |
| 5 | 19.10 | 41.71 | 52:4 | 1048 | 78087 | 10 | 16:0/18:2/18:2 di-TBHP |
| 7 | 19.69 | 41.21 | 54:5 | 1074 | 112866 | 10(9,8) | 18:2/18:2/18:1 di-TBHP |
| 8 | 19.71 | 41.72 | 52:4 | 1048 | 70235 | 10(9,8) | 16:0/18:2/18:2 di-TBHP |
| 12 | 20.50 | 42.61 | 54:4 | 1076 | 40012 | 10(9,8) | 18:1/18:2/18:1 di-TBHP |
| 16 | 21.41 | 42.81 | 54:4 | 1076 | 36784 | 10 | 18:1/18:2/18:1 di-TBHP |
| 21 | 22.06 | 42.81 | 54:4 | 1076 | 46456 | 10(9) | 18:1/18:2/18:1 di-TBHP |
| 23 | 22.30 | 43.71 | 54:4 | 1076 | 51487 | 10 | 18:0/18:2/18:2 di-TBHP |
| 24 | 22.34 | 43.04 | 52:3 | 1050 | 64175 | 10(8) | 16:0/18:2/18:1 di-TBHP |
| 25 | 22.34 | 42.39 | 50:2 | 1024 | 14359 | 10 | 14:0/18:1/18:1 di-TBHP |
| 26 | 22.36 | 44.39 | 52:2 | 1052 | 20599 | 10 | 16:0/18:1/18:1 di-TBHP |
| 27 | 22.60 | 44.56 | 56:4 | 1104 | 2676 | 10 | 18:1/18:2/20:1 di-TBHP |
| 40 | 24.16 | 45.75 | 56:3 | 1106 | 1106 | 10 | 18:1/18:1/20:1 di-TBHP |
| 41 | 24.27 | 45.71 | 56:4 | 1104 | 6003 | 10 | 20:0/18:2/18:2 di-TBHP |
| 42 | 24.34 | 44.58 | 54:3 | 1078 | 29719 | 10(8) | 18:1/18:1/18:1 di-TBHP |
| 43 | 24.41 | 45.55 | 52:2 | 1052 | 13327 | 10 | 16:0/18:2/18:0 di-TBHP |
| 56 | 26.59 | 47.04 | 56:3 | 1106 | 5537 | 10 | 18:1/18:2/20:0 di-TBHP |
| 57 | 26.60 | 47.98 | 56:2 | 1108 | 830 | 10 | 18:0/18:1/20:1 di-TBHP |
| 58 | 26.62 | 47.71 | 58:4 | 1132 | 1341 | 10 | 22:0/18:2/18:2 di-TBHP |
| 59 | 27.00 | 47.72 | 58:4 | 1132 | 941 | 10 | 22:0/18:2/18:2 di-TBHP |
| 70 | 28.61 | 48.39 | 56:2 | 1108 | 1981 | 10 | 20:0/18:1/18:1 di-TBHP |

[a]For abbreviations see Table 1.
[b]Numbers of the compounds refer to peaks in Figures 1 and 4.
[c]Nominal mass of $[M + NH_4]^+$.
[d]Abundance of an ion in the first-mentioned LC Band.
[e]The most abundant band mentioned first.
[f]The most likely structure is given, although the *sn*-positional order of fatty acids may not be as presented. Underlined double bonds show the *sn*-positions of TBHP groups for calculation of TCN value.

positive exit voltage [capillary exit voltage of 300 V, (CapEx 300)] (data not shown).

*Identification of mono-TBHP adducts*. Figure 6 gives the single-ion chromatograms for the mono-TBHP adducts of the six major residual triacylglycerol masses shown in Figure 5. In order of decreasing retention time they are: *m/z* 1021 (a, 18:0/18:1/20:1 + TBHP; b, 18:0/18:1/20:1 + TBHP), 1019 (a, 18:1/18:1/20:1 + TBHP; b, 18:1/18:2/20:0 + TBHP), 1017 (a, 18:1/18:2/20:1 + TBHP; b, 20:0/18:2/18:2 + TBHP; c, 20:0/18:2/18:2 + TBHP), 965 (a, 16:0/18:1/18:0 + TBHP; b, 16:0/18:1/18:1 + TBHP; c, 16:0/18:2/18:0 + TBHP; and d, 16:0/18:0/18:2 + TBHP), 963 (a, 16:0/18:2/18:1 + TBHP; b, 18:2/18:1/16:0 + TBHP), and 961 (a, 16:0/18:2/18:2 + TBHP; b, 16:0/18:2/18:2 + TBHP). Several of these ion peaks are double peaks or possess advance or tailing shoulders. These can be readily attributed to homologs that possess the same total carbon and double bond number but differ in the distribution of the double bonds. The location of the TBHP groups is indicated by underscoring the appropriate fatty chain. Thus, the presence of two double bonds in the same fatty chain shortens the retention time more than the presence of two double bonds in separate fatty chains. The mono-TBHP adducts migrate slightly ahead of the parent triacylglycerols. Similar single-ion mass chromatograms were obtained for other homologous series of residual triacylglycerols as shown in Table 2.

Table 2 indicates that some of the adducts were also found in TLC Bands 9 and 8. The structures advanced on the basis of the molecular masses of presumed TBHP adducts of the triacylglycerols were confirmed by finding the corresponding $[M - RCOOH - TBHP]^+$ ions of the parent triacylglycerols as minor peaks in the total ion spectrum of each peak or as major fragment ions following reanalysis of the sample at a higher positive exit voltage (CapEx 300) (data not shown). Fragment ions carrying the adducted TBHP moiety, however, were not found. In several instances, the minor diacylglycerol-like fragment ions possessed one double bond more than anticipated from the masses of the major residual triacylglycerols. This suggested that a double bond had been introduced during the adduct formation.

*Identification of di-TBHP adducts.* Figure 7 gives the single ion chromatograms for the di-TBHP adducts of the six major residual triacylglycerol masses. In order of decreasing retention time they are: *m/z* 1109 (a, 18:0/18:1/20:1 + 2×TBHP; b,

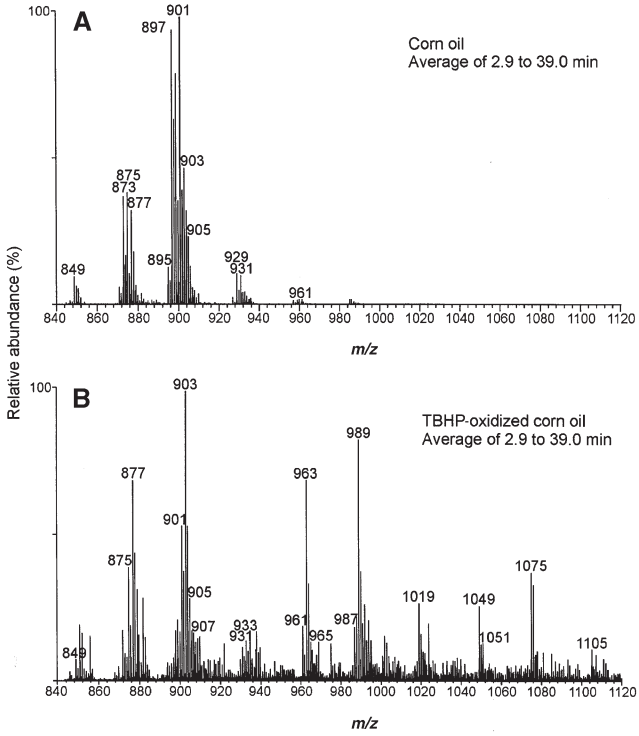


**FIG. 2.** Comparison of total positive ion current mass spectra averaged over the elution times of native (A) and oxidized (B) corn oil. Ions are identified in Tables 1–3. LC/ESI/MS conditions are as given in Figure 1. For abbreviations see Figure 1.

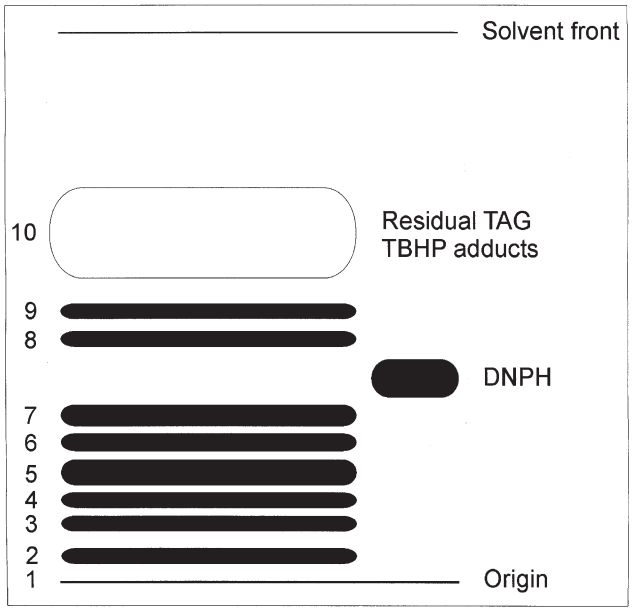


**FIG. 3.** Thin-layer chromatography (TLC) separation of the oxidation products of corn oil triacylglycerols (TAG) following derivatization with dinitrophenylhydrazine (DNPH). Silica gel H (Merck & Co.) plates (20 × 20 cm) were prepared in the laboratory and developed with heptane/isopropyl ether/acetic acid (60:40:4, by vol). DNPH derivatives of TAG containing core aldehydes appeared as yellow bands; other bands were stained with 2,7-dichlorofluorescein. Aldehyde-containing bands (Bands 2–9) are identified in Reference 10. For other abbreviation see Figure 1.

20:0/18:1/18:1 + 2×TBHP), 1107 (a, 18:1/18:1/20:1 + 2×TBHP; b, 20:0/18:2/18:1 + 2×TBHP), 1105 (a, 18:1/18:2/20:1 + 2×TBHP; b, 20:0/18:2/18:2 + 2×TBHP), 1053 (a, 16:0/18:1/18:1 + 2×TBHP; b, 16:0/18:2/18:0 + 2×TBHP), 1051 (16:0/18:2/18:1 + 2×TBHP), and 1049 (a, 16:0/18:2/18:2 + 2 × TBHP; b, 16:0/18:2/18:2 + 2×TBHP). Several of these ion peaks are double peaks or possess advance or tailing shoulders. These can be readily attributed to homologs, which possess the same total carbon and double bond number but differ in the distribution of the double bonds, as explained previously. In some instances, both TBHP groups were assigned to the same fatty chain. Similar single-ion mass chromatograms were obtained for other homologous series of residual triacylglycerols as shown in Table 3.

Table 3 indicates that some of the adducts were also found in TLC Bands 9 and 8. The structures proposed on the basis of the molecular masses of presumed TBHP adducts of the triacylglycerols were confirmed by finding the corresponding $[M - RCOOH]^+$ ions of the parent triacylglycerols as minor peaks in the total ion spectrum of each peak or as major fragment ions following reanalysis of the sample at a higher positive exit voltage (CapEx 300) (data not shown). Fragment ions carrying the adducted TBHP moiety, however, were not found. In several instances, the minor diacylglycerol-like fragment ions appeared to possess one double bond more than anticipated for the mass of the $[M - RCOOH - 2 \times TBHP]^+$ generated from the major corn oil triacylglycerols. This indicated that an extra double bond had been introduced during the TBHP adduct formation.

Figure 8 compares the retention times of one of the major residual triacylglycerols (18:2/18:2/18:1) as the mono-, di- , tri-, tetra-, and penta-TBHP adducts as obtained by single-ion mass chromatograms. In order of increasing retention time they are: *m/z* 1340 (18:2/18:2/18:1 + 5×TBHP), 1251 (a, 18:2/18:2/18:1 + 4×TBHP; b, 18:2/18:2/18:1 + 4×TBHP), 1163 (a, 18:2/18:2/18:1 + 3×TBHP; b, 18:2/18:2/18:1 +

**TABLE 4**
**Fatty Acid Composition of TLC-Purified Corn Oil and Residual TAG (mol%)[a]**

| Fatty acid | Corn oil[b] | Residual TAG[c] |
|---|---|---|
| 16:0 | 10.96 ± 1.0 | 18.90 ± 1 |
| 16:1n-7 | 0.09 ± 0.2 | 0.71 ± 0.5 |
| 18:0 | 1.85 ± 0.1 | 5.95 ± 1 |
| 18:1n-9 | 28.83 ± 1.5 | 58.47 ± 3 |
| 18:2n-6 | 56.59 ± 2.5 | 14.13 ± 1 |
| 18:3n-3 | 0.70 ± 0.1 | |
| 20:0 | 0.46 ± 0.2 | 1.05 ± 0.5 |
| 20:1n-9 | 0.40 ± 0.2 | 0.54 ± 0.5 |
| 22:0 | 0.11 ± 0.2 | 0.24 ± 0.5 |

[a]Gas–liquid chromatography analysis on a polar capillary column (4,5). For abbreviations see Table 1.
[b]Mean ± standard deviation.
[c]Mean ± range/2.

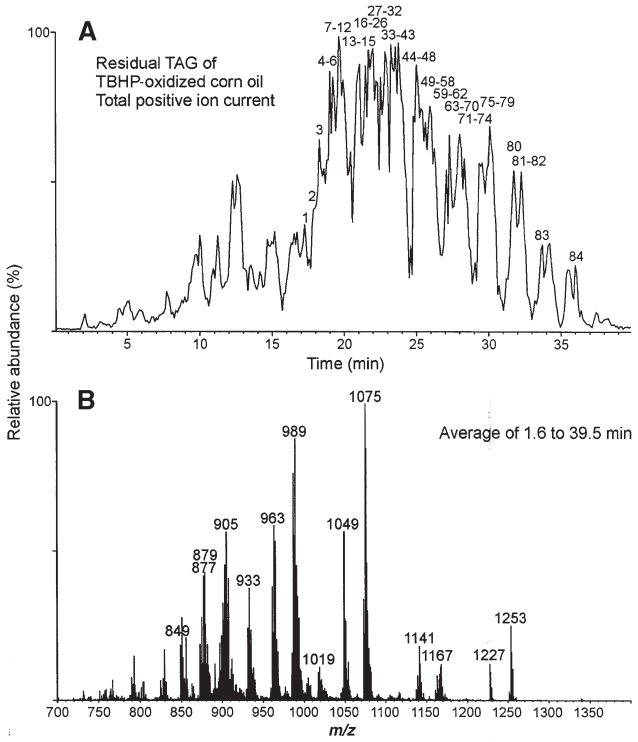


**FIG. 4.** Total positive ion current profile of TLC Band 10 as obtained by reversed-phase LC/ESI/MS (A) along with the full mass spectrum (*m/z* 700 to 1400) averaged over the entire elution profile (B). Chromatographic peaks and ions are identified in Tables 1–3. LC/ESI/MS conditions and abbreviations are given in Figure 1.

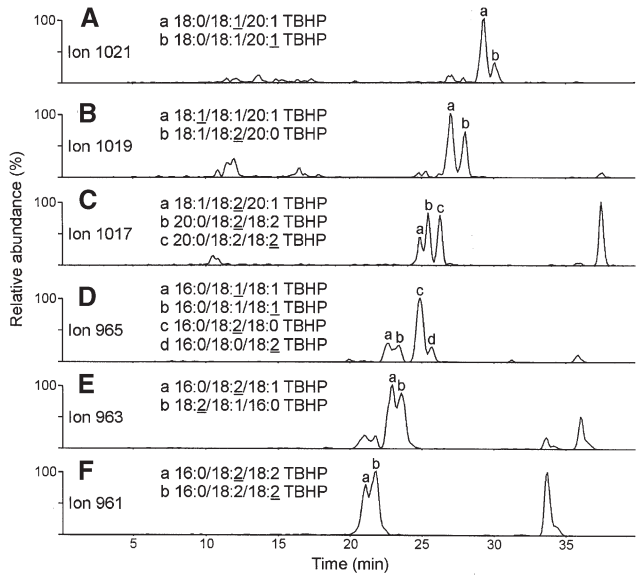


**FIG. 6.** Single-ion mass chromatograms for the mono-TBHP adducts of the six major residual TAG shown in Figure 5. Ion identifications are given in the figure. Chromatographic peaks and ions are identified in Tables 1–3. LC/ESI/MS and conditions abbreviations are given in Figure 1. Underlined double bond shows the *sn*-position of TBHP group for calculation of the theoretical carbon number.

3×TBHP), 1075 (a, 18:2/18:2/18:1 + 2×TBHP; b, 18:2/18:2/18:1 + 2×TBHP; c, 18:2/18:2/18:1 + 2×TBHP), 987 (a, 18:2/18:2/18:1 + TBHP; b, 18:2/18:2/18:1 + TBHP), and 899 (18:2/18:2/18:1). It should be noted that some of the fatty chains carry two TBHP groups as indicated by doubly underscoring the appropriate fatty chains in Figure 8. The locations of the TBHP groups were assigned from the knowledge of the contributions made by specific positional location of component fatty acids, double bonds, and other functional groups to

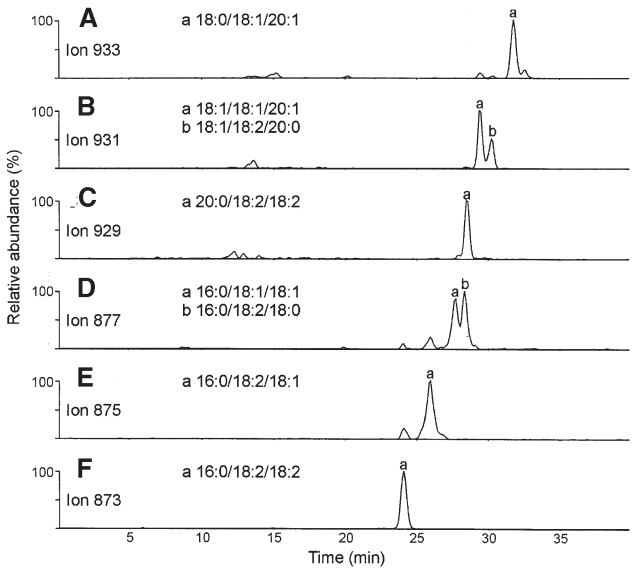


**FIG. 5.** Single-ion mass chromatograms for the six major residual TAG masses. Ion identifications are given in the figure. Chromatographic peaks and ions are identified in Tables 1–3. LC/ESI/MS conditions and abbreviations are given in Figure 1.

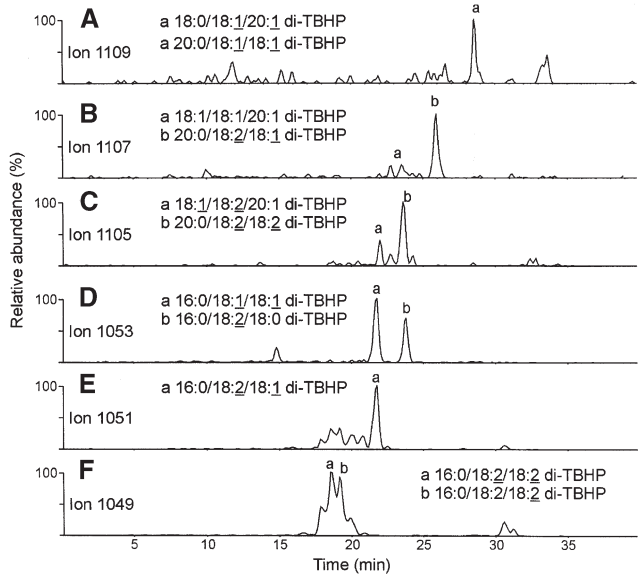


**FIG. 7.** Single ion mass chromatograms for the di-TBHP adducts of the six major residual TAG shown in Figure 5. Ion identifications are given in the figure. Chromatographic peaks and ions are identified in Tables 1–3. LC/ESI/MS conditions and abbreviations are given in Figure 1.

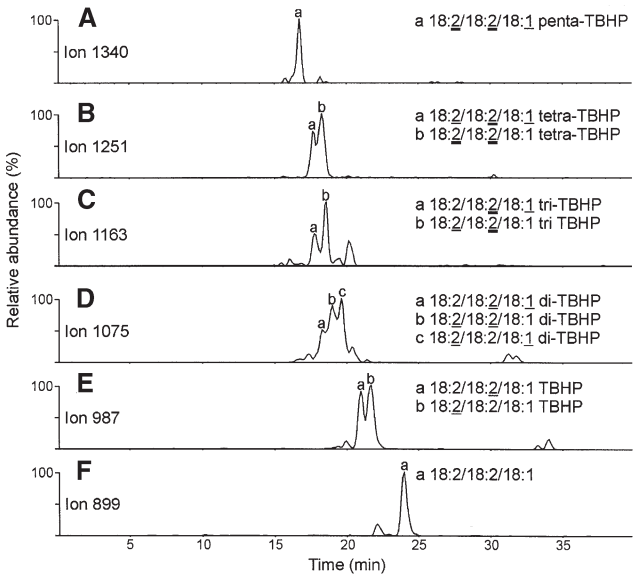


**FIG. 8.** Comparison of retention times of one of the major residual TAG (18:2/18:2/18:1) as the mono-, di-, tri-, tetra-, and penta-TBHP adducts as obtained by single-ion monitoring. Ion identifications are given in the figure. Chromatographic peaks and ions are identified in Tables 1–3. LC/ESI/MS conditions and abbreviations are given in Figure 1.

the overall chromatographic retention time of each triacylglycerol derivative. Each additional TBHP group decreases the retention time with respect to the parent triacylglycerol by an amount that is related to the location of the fatty chain involved in the adduct formation. This is indicated by the split and/or multiple peaks in the single-ion mass chromatograms. A complete alignment of the peaks, however, is not possible because the various derivatives represent different homologous series despite similar incremental increases in the total number of the TBHP groups.

*Quantification.* Table 5 gives quantitative estimates for the residual triacylglycerols and the mono-, di-, and tri-TBHP adducts isolated. The results are expressed as percentage of total positive ion current. TLC Band 10 was estimated to represent over 10% of the total ion current of the oxidized corn oil in the present experiments.

**TABLE 5**
**Yield of Residual TAG and Mono- and Di-TBHP Adducts of Corn Oil TAG[a]**

| | Mol% of TLC band 10 | Mol% of total ion current |
|---|---|---|
| Residual TAG | 80.1 | 10–15 |
| Mono-TBHP | 11.7 | 1.5–2 |
| Di-TBHP | 8.7 | 1–1.5 |
| Tri-TBHP | Trace | Trace |
| Tetra-TBHP | Trace | Trace |
| Penta-TBHP | Trace | Trace |

[a]Average ± range/2. Estimated from selected ion intensities by allowing an additional 50% for isotope effect. For abbreviations see Table 1.

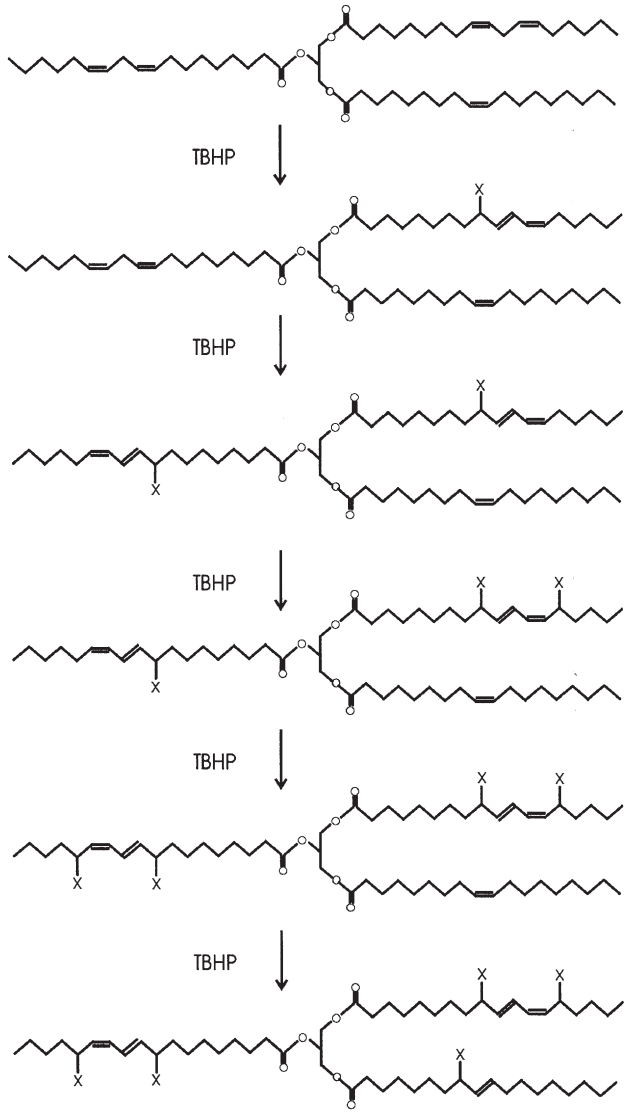


**FIG. 9.** Hypothetical location of TBHP groups in an oxidized corn oil TAG (18:2/18:2/18:1) based on Figure 8. The locations of the TBHP groups are marked with ×. Only one of several bis-TBHP isomers is shown (9,14-bis-TBHP).

## DISCUSSION

The present study demonstrates the formation of significant amounts of TBHP adducts of polyunsaturated corn oil triacylglycerols under experimental conditions commonly employed to generate model oxolipids (4,5,9) to serve as standards for the isolation and identification of autoxidation products of natural fats and oils. The TBHP adducts apparently are of two types, one type resulting from the addition of TBHP functions [X = $(CH_3)_3COO$] to individual fatty chains, the other from addition of two TBHP functions per chain (Fig. 9). The formation of the TBHP adducts can be attributed to free radical termination or annihilation reactions (12) where the *tert*-butyl alkoxy radical reacts with a radical of the fatty chain formed by abstraction of the bis(allylic) hydrogen atom.

Alternatively, TBHP adduct could be formed by reacting a hydroperoxide radical with a *tert*-butyl radical.

The presence of the $Fe^{2+}$ ions is believed to catalyze the formation of the *tert*-butyl alkoxy radical responsible for initiating removal of a bis(allylic) hydrogen atom from the polyunsaturated fatty acids found esterified to the glycerol backbone. The addition of molecular oxygen to the free radical site on the fatty acid would then form an initial hydroperoxide radical, which either abstracted a hydrogen atom to yield a hydroperoxide or became reduced to the hydroxy fatty acyl-substituted triacylglycerol. More complex reactions, however, are also possible as demonstrated previously with oleic (2,3) and linoleic (13) acid methyl esters and polyolefins (14). The dehydration of the hydroperoxides during TBHP oxidation appears to be similar to that reported during autoxidation (15) and would result in introduction of unsaturation, which would become subject to further oxidation. The present study confirms and extends our earlier work on the high molecular weight products of TBHP oxidation of standard mono- and diunsaturated triacylglycerols (10).

The study shows that the TBHP adducts of corn oil triacylglycerols can be readily isolated by normal-phase TLC where they migrate ahead of the other oxotriacylglycerols. The TBHP adducts of polyunsaturated corn oil triacylglycerols overlap with the residual triacylglycerols, which are enriched in the more saturated species of triacylglycerols. The TBHP adducts of some of the more unsaturated triacylglycerols, however, were found to migrate with the less polar oxotriacylglycerols recovered in TLC Bands 8 and 9. The TBHP adducts were stable to strong acid as evidenced by their isolation following preparation of the dinitrophenylhydrazones in 1 N HCl. The adducts decomposed on storage in organic solvents.

The triacylglycerols were identified in the positive ion mode as the ammonia adducts of the molecular ions and the $[M - RCOOH]^+$ ions, which were detected as minor ions during routine ESI and as major ions during the more intense ionization brought about by increased capillary exit voltage (16), although this may lead to possible dehydration of the hydroperoxides (17). The triacylglycerols were identified on the basis of the masses of the molecular ions and the corresponding fragment ions obtained by increasing the capillary exit voltage. There were no adducts found for the $[M - COOH]^+$ ions for any of the triacylglycerols examined in this way. Apparently, the TBHP adducts were readily decomposed at elevated capillary exit voltage. However, in several instances the $[M - RCOOH]^+$ ions with one of two additional double bonds could be recognized.

The proportion of adducted triacylglycerol in relation to the total residual corn oil triacylglycerol was similar to that previously observed during peroxidation of synthetic triacylglycerols with TBHP (10).

In preliminary studies (5) we noted that TBHP oxidation at laboratory scale can yield milligram quantities of oxoacylglycerols, but by-product formation constitutes an undesirable complication. The present work identifies the by-products as mainly the adducts of TBHP. The TBHP adducts are readily resolved from the hydroperoxides and epoxides of simple triacylglycerols by normal-phase TLC and reversed-phase HPLC, but the oxidation products of more complex triacylglycerols overlap extensively with the more complex oxidation products. Because the peroxide bridge formation constitutes a part of the reaction mechanism, it may not be entirely avoided; however, milder reaction conditions minimize the bridge formation.

## ACKNOWLEDGMENTS

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# Sterol Biosynthesis by the Arbuscular Mycorrhizal Fungus *Glomus intraradices*

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**ABSTRACT:** Ri- T-DNA-transformed carrot roots were used for investigating sterol metabolism by the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* under three distinct experimental conditions: (i) a symbiotic stage (fungus still attached to the host roots); (ii) a detached stage (fungus physically separated from the roots); and (iii) a germinating stage (germinating spores). In all three stages, *G. intraradices* was found to contain a mixture of 24-alkylated sterols, with 24-methyl and 24-ethyl cholesterol as the main compounds, but no ergosterol, the predominant sterol in most fungi. Feeding experiments with [1-$^{14}$C]sodium acetate were performed to check the ability of the fungus to synthesize sterols. Whatever the experimental conditions, *G. intraradices* was able to actively take up exogenous acetate and to incorporate it into sterols and their precursors. Our data provide first evidence for *de novo* sterol synthesis by an AM fungus.

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Arbuscular mycorrhizal (AM) fungi of the order Glomales (Zygomycota) form a mutualistic symbiosis with the roots of most land plants. These fungi are obligate biotrophs and colonize the root cortex in order to obtain carbon from the host plant. They also develop a network of external hyphae, which play a pivotal role in the acquisition by the plant of mineral nutrients from the soil (1). The complex association of the two organisms makes the study of the fungal partner difficult. The mechanism or mechanisms by which fungal growth is regulated remain unclear. In the absence of the host plant, the growth of AM fungi is limited to a relatively short period of time (2). During this period, the production of a mycelium by germinating spores triggers gluconeogenesis (3) as well as hydrolysis of storage lipids (i.e., triacylglycerols) (4,5). After establishment of the symbiosis, the fungus becomes able to metabolize glucose in carbohydrates (glycogen and trehalose) and lipids (6,7). These data highlight the versatility of lipid metabolism, which is closely related to the physiological stage of the fungus.

The recent development of monoxenic culture systems (8–11) offers unique advantages for investigating fungal lipid metabolism. This approach involves the use of *in vitro* cultures of transformed plant roots colonized by the AM fungus. In particular, the use of divided petri plates, in which the spores and the mycelium are physically separated from the mycorrhizal roots, allows the selective addition of labeled substrates to either compartment and the straightforward isolation of each of these tissues (10).

The present work is an attempt to investigate sterol metabolism by *Glomus intraradices*. Little attention has been paid to this class of lipids in AM fungi, which have been reported to belong to a primitive taxon (12). Whereas ergosterol is by far the predominant sterol in most fungi (13), AM fungi contain cholesterol as well as 24-methyl and 24-ethyl cholesterol, but no ergosterol (14–17). Higher plants also have 24-alkylated sterols, mainly represented by sitosterol, stigmasterol, and 24-methyl cholesterol (18). Thus, the question of whether these biotrophic fungi are able to synthesize their own sterols or have to take them up from the plant partner remains open.

To investigate the ability of the AM fungus *G. intraradices* to synthesize sterols, labeling experiments using [1-$^{14}$C]sodium acetate as a precursor were performed. Monoxenic cultures of Ri- T-DNA-transformed carrot roots colonized by *G. intraradices* were maintained in two-compartment petri plates for 3 or 4 mon. The radioactive precursor was added only to the fungal compartment. Three distinct experimental stages of the fungus have been taken into account: (i) a symbiotic stage, corresponding to the fungus still attached to the host plant roots; (ii) a detached stage, consisting of the fungus physically separated from the host roots; and (iii) a germinating stage (germinating spores). Our data provide first evidence for a *de novo* sterol synthesis by the AM fungus *G. intraradices* in the three stages. A preliminary communication of the present work was previously presented (19).

## MATERIALS AND METHODS

*Chemicals*. [1-$^{14}$C]Sodium acetate (60 mCi mmol$^{-1}$) was purchased from Amersham (Buckinghamshire, United Kingdom). Mevinolin was from Sigma (St. Louis, MO). Before use, the lactone was converted to the open-acid form according to the procedure described by Kita *et al.* (20).

*Fungal growth conditions and labeling experiments*. Ri T-DNA-transformed carrot (*Daucus carota* L.) roots colonized

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Abbreviations: AM, arbuscular mycorrhizal; ES, esterified sterols; FS, free sterols; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; TLC, thin- layer chromatography.

by *Glomus intraradices* Schenck & Smith (DAOM 197198) were grown in two-compartment petri plates (10) containing a medium M (9), in the dark at 27°C. The two compartments were separated by a watertight plastic wall. The mycorrhizal roots were confined in one compartment, but the fungus was allowed to grow over the central separation and into the other compartment. The cultures were examined weekly, and roots were cut when needed. After 4 mon, the fungus completely filled the distal compartment and sporulated extensively. These monoxenic culture systems were usually maintained for 4 mon.

The ability of the fungus to synthesize sterols was investigated using [1-$^{14}$C]sodium acetate as a precursor under three distinct experimental conditions: (i) a symbiotic stage (fungus still attached to the host roots), (ii) a detached stage (fungus detached from roots), and (iii) a germinating stage (germinating spores). For each experimental condition, 11 petri plates were used. For symbiotic and detached stages, 10 μCi of [1-$^{14}$C]sodium acetate was added to each plate, in the fungal compartment only. In the case of the detached stage, carrot roots were removed just before addition of acetate. For the germinating stage, fungal material was collected from the fungal compartment by blending the solidified medium in sodium citrate solution (10 mM) (21). Spores were collected by filtration on a 53-μm sieve, rinsed with sterile water, and applied onto two plates with 55 μCi of sodium acetate per plate. Then, spores were allowed to germinate on M medium (in 2% $CO_2$, in the dark) (9). In all cases, after addition of sodium acetate, the plates were kept at 27°C in the dark for 12, 24, 48, 72, and 96 h, respectively. After incubation, the culture media were solubilized with sodium citrate (21), and the fungal material was recovered by filtration and frozen at –80°C. These experiments were done in triplicate.

*Incubation with mevinolin*. The ability of *G. intraradices* to synthesize sterols in the symbiotic stage was also investigated in the presence of mevinolin, a potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. For this experiment, fungal material from 4-mon-old cultures, applied onto three petri plates, was used. Mevinolin was added to each plate at a concentration of 20 μM 6 h before radioactive acetate (10 μCi). Fungal material was collected after 48 h, and sterols and their precursors were isolated as described below.

*Sterol analysis.* Sterol analyses were performed on the fungal material collected at 0, 12, 24, 48, 72, and 96 h after addition of [1-$^{14}$C]sodium acetate. After being freeze-dried, fungal material was ground and extracted by refluxing three times with dichloromethane/methanol (2:1, vol/vol) for 3 h. Extracts were combined and dried under reduced pressure. Free sterols (FS) and sterols in their esterified forms (ES) were separated on silica gel thin-layer chromatography (TLC) plates (60F254; Merck, Darmstadt, Germany) with hexane/diethyl ether/glacial acetic acid (78:20:4, by vol) as the solvent. The radioactivity was detected on TLC plates by using a linear radioactivity analyzer (Berthold LB 2820-1, Wilbach, Germany). Bands corresponding to squalene and ES ($R_f$ 0.97), 4,4-dimethylsterols ($R_f$ 0.40), and 4α-methyl- and 4-demethylsterols ($R_f$ 0.35) were scraped off and eluted with dichloromethane. ES were saponified with 6% (wt/vol) KOH in methanol at 90°C for 1 h. Sterols and 4,4-dimethyl- and 4α-methylsterols from free and esterified forms were acetylated (22). Acetate derivatives were then purified and analyzed with the use of a PerkinElmer Autosystem gas chromatograph (GC) equipped with a flame-ionization detector (Norwalk, CT) and a DB-5 (J&W Scientific, Folsom, CA) capillary column (30 m × 0.25 mm i.d.), with hydrogen as the carrier gas (2 mL min$^{-1}$). The temperature program included a fast rise from 60 to 270°C at 30°C/min and then a rise from 270 to 310°C at 2°C/min. Cholesterol (not acetylated) was used as an internal standard. Sterol acetates were identified by gas chromatography–mass spectrometry (GC–MS Varian, Walnutcreek, CA) (23,24). Major 4-demethylsterol acetates were separated from each other by reversed-phase high-performance liquid chromatography (HPLC; Waters, Milford, MA) using a $C_{18}$ ODS Ultrasphere column (4.6 × 250 mm). The eluent was methanol/$H_2O$ (99.7:0.3, vol/vol), at a flow rate of 1 mL min$^{-1}$. An evaporative light-scattering detector (Eurosep Instruments DDL 31, Cergy Pontoise, France; 700 mV, 55°C) was used. Each compound was identified by GC–MS and quantified by GC. Elution times for 24-methyl and 24-ethyl cholesterol acetates were 36 and 42 min, respectively. After addition of carriers, acetates of 4,4-dimethylsterols were separated on TLC plates impregnated with 10% $AgNO_3$ and diluted using cyclohexane/toluene (6:4, vol/vol) (3 runs) as solvent system. Radioactivity was determined by liquid scintillation counting.

## RESULTS

*Sterol composition of* G. intraradices. We first determined the sterol composition of *G. intraradices* in the three experimental conditions corresponding to symbiotic, detached, and germinating stages. Sterol analyses were performed as described in the Materials and Methods section on the fungal material collected after 0, 12, 24, 48, 72, and 96 h. As only low amounts of fungal material were recovered, reliable quantitative data for sterol contents could not be obtained. Because no change in the relative sterol composition of the various samples was observed between 0 and 96 h, results are given as mean values (Table 1). Whatever the experimental conditions, sterols occurred in their free forms (FS) (60%) and as esterified conjugates (ES). Predominant fungal sterols were found to be 24-methyl cholesterol and 24-ethyl cholesterol. The other identified compounds were cholesterol, 24-ethylcholesta-5,22-dien-3β-ol, 24-ethylidene cholesterol, and ergosta-7,24(24)$^1$-dien-3β-ol. No ergosterol was detected. Traces (less than 0.5% of total sterols) of 4,4-dimethylsterols (lanosterol and 24-methylene lanosterol) were also found. Both FS and ES contained the same sterols, but a higher proportion of 24-methyl cholesterol was observed in ES. Table 2 gives the sterol composition of Ri- T-DNA-transformed carrot roots colonized or not colonized by *G. intraradices*. In the absence of the fungus, sterols of carrot roots were identified

**TABLE 1**
**Free and Esterified 4-Demethylsterol Composition of *Glomus intraradices* in Symbiotic, Detached, and Germinating Stages[a]**

| | Symbiotic stage | | Detached stage | | Germinating stage | |
|---|---|---|---|---|---|---|
| Sterol | FS[b] | ES[c] | FS | ES | FS | ES |
| Cholesterol | 2 | 1 | 2 | 1 | 1 | 2 |
| 24-Methyl cholesterol | 58 | 67 | 60 | 72 | 57 | 72 |
| 24-Ethylcholesta-5,22-dien-3β-ol | 3 | 2 | 2 | 1 | 5 | 2 |
| 24-Ethyl cholesterol | 35 | 25 | 32 | 20 | 34 | 21 |
| 24-Ethylidene cholesterol | 3 | 2 | 2 | 3 | 2 | 2 |
| Ergosta-7,24(24)[1]-dien-3β-ol | 1 | 2 | 1 | 3 | 1 | 2 |

[a]Data are presented as percentages of 4-demethylsterol content.
[b]FS, free sterols.
[c]ES, esterified sterols.

as stigmasterol, sitosterol, 24-methyl cholesterol, and isofucosterol, i.e., the typical plant $\Delta^5$-sterols. Carrot roots also contained an unusual sterol, which exhibited a GC relative retention time (RRT) of 1.32, very close to that of sitosterol (RRT 1.34). Based on the MS for the acetate derivative [*m/z* 394 ($M^+$ – AcOH) (100), 379 (22), 352 (3), 310 (7), 296 (19), 281 (25), 255 (14), 253 (33), 228 (13), 213 (20), 211 (14)], this sterol was identified as 24-epiclerosterol, the 24α epimer of 24-ethyl-24(25)-methylene cholesterol, in agreement with data reported by Akihisa *et al.* (25). We also detected other compounds such as cycloartenol, 24-methylene cycloartanol, obtusifoliol, and cycloeucalenol, which correspond to usual precursors of the plant sterol pathway. After colonization by *G. intraradices*, carrot roots were found to contain a higher sterol content than control roots, but the same compounds were detected. However, the symbiosis appears to trigger a significant increase in the relative percentage of 24-methyl cholesterol and/or 24-methylene cholesterol, which were not separated under our GC conditions. Traces of lanosterol and 24-methylene lanosterol were also detected, probably originating from the fungus. Because of low sterol contents, the stereochemistry at C-24 of fungal sterols could not be determined. In higher plants, most 24-ethylsterols have a 24α configuration whereas 24-methylsterols are mixtures of both 24α and 24β epimers. Ergosterol and fungal sterols usually have a 24β configuration (26). Under our GC experimental conditions, epimers at C-24 were not separated. Thus, it was not possible to discriminate between plant and fungal 24-alkylated sterols. However, the presence of traces of lanosterol and 24-methylene lanosterol suggested the existence of a fungal sterol biosynthetic pathway.

**TABLE 2**
**4-Demethylsterol Composition of Carrot Roots Colonized and Noncolonized by *Glomus intraradices*[a]**

| Sterol | C[b] | M[c] |
|---|---|---|
| Cholesterol | — | Tr[d] |
| 24-Methyl cholesterol | 11 | 21[e] |
| Stigmasterol | 75 | 67 |
| 24-Epiclerosterol | 1 | 2 |
| Sitosterol | 12 | 9 |
| Isofucosterol | 1 | 1 |
| Total sterols (mg/g dry wt ± SD) | 1.7 ± 0.1 | 2.3± 0.2 |

[a]Data are presented as percentages of 4-demethylsterol content and are means of three experiments.
[b]C, control carrot roots.
[c]M, mycorrhizal carrot roots.
[d]Tr = trace, amounts < 0.5%.
[e]24-Methyl cholesterol + 24-methylene cholesterol not separated by gas chromatography.

*Labeling experiments with [1-$^{14}$C]acetate.* In order to investigate the ability of the fungus *G. intraradices* to synthesize sterols, feeding experiments with [1-$^{14}$C]acetate were conducted as described in the Materials and Methods section. The three experimental situations (i.e., symbiotic stage, detached stage, and germinating stage) were taken into account, with incubation periods ranging from 12 to 96 h. In all three stages, exogenous acetate was found to be actively taken up by the fungus with very similar kinetics. Indeed, only 15–35% of the initial radioactivity remained in the external medium after 12 h, and almost complete absorption of the precursor was observed after 96 h.

Sterols in their free and esterified forms were separated from each other, quantified, and their radioactivity determined as described in the Materials and Methods section. Figure 1A shows the distribution of the radioactivity incorporated into FS and ES of the fungus in the three situations. In all cases, most of the acetate radioactivity was recovered in FS, mainly after 72 and 96 h, giving clear evidence that the fungus was able to synthesize sterols whatever the experimental stage. However, some differences between the three stages were apparent. Whereas the fungus in the symbiotic stage exhibited the most active ability to synthesize sterols, a lower capacity was observed for the fungus in the detached stage, with a maximum at 48 h instead of 96 h. Germinating spores were found to display an intermediate ability for sterol synthesis. In all the stages, labeled ES were presumably formed but were not detectable before 48 h.

It was interesting to check whether the ability of the fungus to synthesize sterols could be affected by an inhibitor. We chose to use mevinolin, a competitive inhibitor of 3-hydroxymethyl-3-glutaryl coenzyme A reductase (27), an enzyme located early in the sterol biosynthetic pathway. The effect of

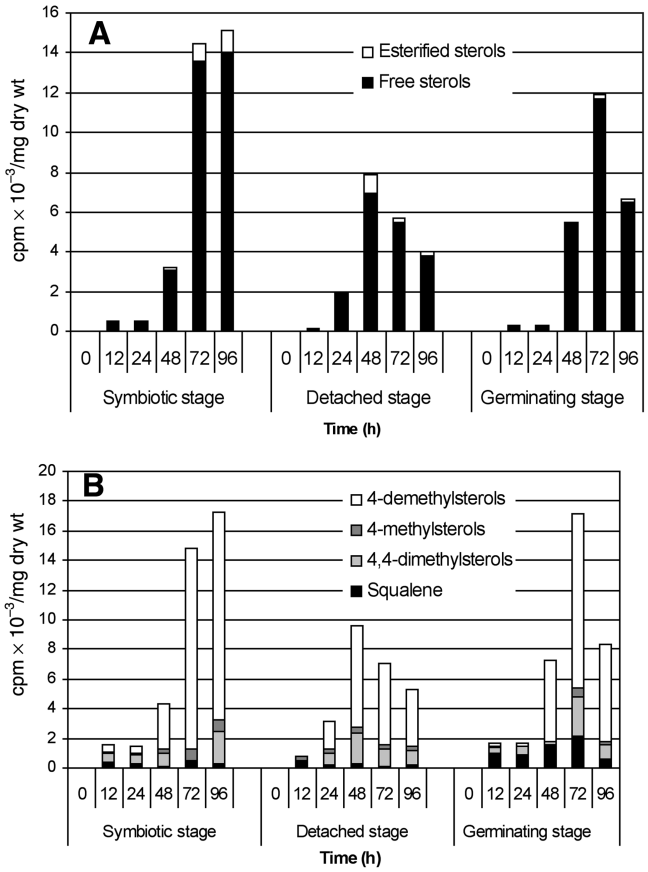


**FIG. 1.** (A) Incorporation of radioactivity from [1-$^{14}$C]acetate into free and esterified sterols of *Glomus intraradices* in symbiotic, detached, and germinating stages after periods of time ranging from 0 to 96 h. (B) Distribution of radioactivity from [1-$^{14}$C]acetate in free sterols and their precursors of *G. intraradices* in symbiotic, detached, and germinating stages after periods of time ranging from 0 to 96 h.

mevinolin on sterol synthesis by *G. intraradices* in the symbiotic stage was tested. Radioactive acetate was given to the fungus after 6 h of contact with the inhibitor, and the fungal material was recovered after 48 h. A strong decrease (85%) in the amount of radioactivity incorporated into sterols was observed (data not shown), indicating that mevinolin was able to inhibit the fungal sterol synthesis. This result also indicates that *G. intraradices* synthesized sterols *via* the classical acetate/mevalonate pathway.

*Distribution of radioactivity among the different classes of free sterols.* Figure 1B gives the distribution of radioactivity among the different classes of FS: 4-demethylsterols and their precursors, (i.e., squalene, 4,4-dimethylsterols, and 4α-methylsterols). The height of each bar in the histograms corresponds to the total radioactivity incorporated into FS. All the classes of sterols were labeled, but most of the acetate radioactivity was recovered in 4-demethylsterols, the end products of the pathway. A relatively higher proportion of squalene was found in germinating spores, suggesting a less active sterol metabolism.

The 4,4-dimethyl- and 4-demethylsterol fractions were analyzed in more detail. The early biosynthetic precursors, 4,4-dimethylsterols, of FS fractions isolated from the fungus in the symbiotic stage were separated from each other as acetate derivatives by argentation TLC in the presence of carriers as described in the Materials and Methods section. Only lanosterol and 24-methylene lanosterol were found to incorporate radioactivity, with the labeling of these compounds increasing as a function of the time of contact with the precursor (Fig. 2). Lanosterol was more labeled than 24-methylene lanosterol, in agreement with the precursor-to-product relationship between the two compounds. As significant amounts of α-amyrine were previously found in spores of AM fungi (17), we checked for the occurrence of radioactivity in the band corresponding to the position of α-amyrine on argentation TLC plates. Whatever the labeling period of time, no radioactive α-amyrine was detected, indicating that although this compound was present in the fungus, it might originate from the plant partner.

The end products, 4-demethylsterols, were separated from each other by reversed-phase HPLC and quantified by GC as reported in the Materials and Methods section. The major compounds, 24-methyl cholesterol and 24-ethyl cholesterol, were clearly found to incorporate radioactivity from acetate in all the experimental situations (Fig. 3).

*Distribution of radioactivity among sterol classes of ES.* Sterols (4-demethylsterols) and their precursors (4,4-dimethylsterols) were recovered from ES and analyzed for their radioactivity. As shown in Figure 4, both classes of compounds were found to contain significant amounts of radioactivity in the case of the fungus in the symbiotic and asymbiotic stages. In contrast, germinating spores exhibited only a low ability to synthesize ES.

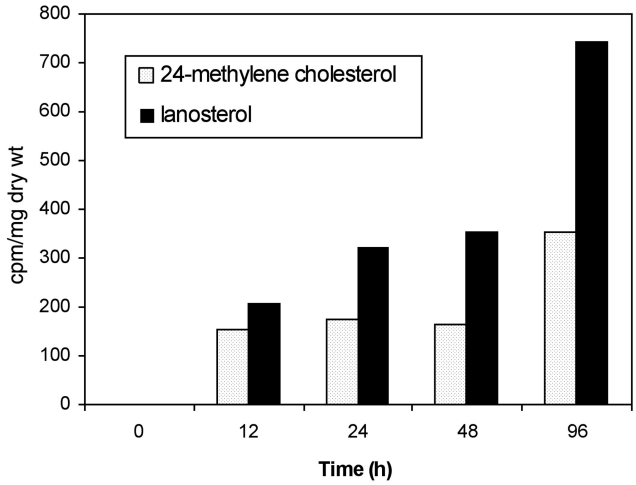


**FIG. 2.** Incorporation of radioactivity from [1-$^{14}$C]acetate into major free 4,4-dimethylsterols of *Glomus intraradices* in the symbiotic stage after periods of time ranging from 0 to 96 h.

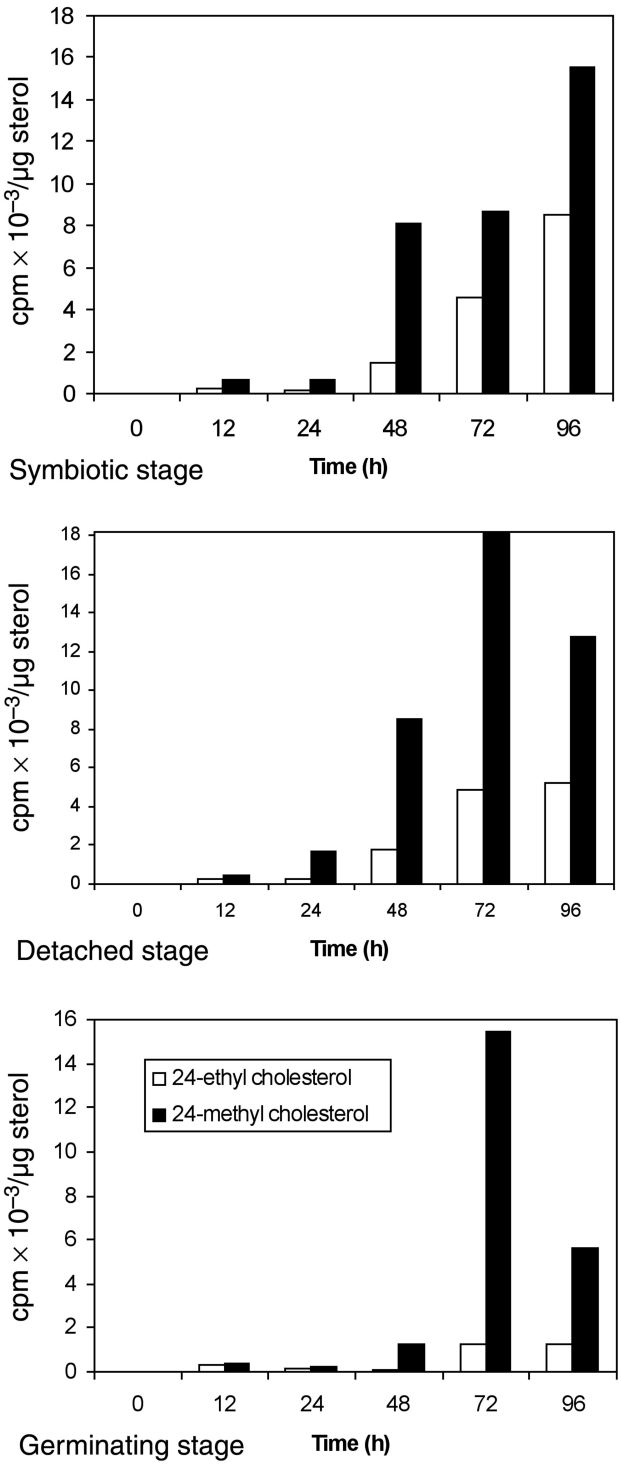


**FIG. 3.** Incorporation of radioactivity from [1-$^{14}$C]acetate into free 24-methyl and 24-ethyl cholesterol of *Glomus intraradices* in symbiotic, detached, and germinating stages after periods of time ranging from 0 to 96 h.

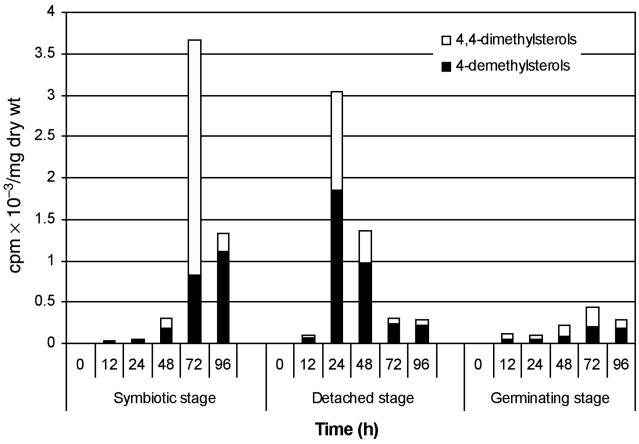


**FIG. 4.** Distribution of radioactivity from [1-$^{14}$C]acetate in 4,4-dimethyl- and 4-demethylsterols from esterified conjugates of *Glomus intraradices* in symbiotic, detached, and germinating stages after periods of time ranging from 0 to 96 h.

## DISCUSSION

The use of monoxenic cultures of transformed carrot roots colonized by the AM fungus *G. intraradices* allowed us to investigate the sterol metabolism of this fungus at three different stages. In contrast to most fungi, *G. intraradices* was found to contain no ergosterol but a mixture of 24-alkylated sterols, represented mainly by 24-methyl and 24-ethyl cholesterol. Other compounds such as cholesterol, 24-ethylidene cholesterol, 24-ethylcholesta-5,22-dien-3β-ol, and ergosta-7,24(24)$^1$-dien-3β-ol were also detected in very low amounts. All these compounds were present as both free sterols and esterified conjugates. The sterol composition did not appear to depend on the fungal experimental situations. The occurrence of 24-ethylsterols in fungi is relatively unusual. These sterols were identified in primitive organisms Chytridiomycota and Hyphochytridiomycota (13). However, 24-ethylsterols were also found in spores of AM fungi, which are classified as Zygomycota (14–17), and also in other typical Zygomycetes such as *Phycomyces blaskesleeanus* (Mucorales) (28,29) and *Umbelopsis nana* (Mucorales) (12). Finally, 24-ethylsterols were also found in "higher fungi." They were detected in pathogenic fungi such as rust fungi (Uredinales, Basidiomycota) (30,31,13) and *Blumeria graminis* f. sp. *tritici* (Erysiphales, Ascomycota) (32), the agent of wheat powdery mildew and also in *Pneumocystis carinii* (Pneumocystidales, Ascomycota), a mammal opportunistic fungus (33). Triterpenoids with 24-alkyl groups are present in mycelia of *Pisolithus tinctorius* (Gasteromycetes, Basidiomycota), an ectomycorrhizal fungus (34).

Because of the low amounts of the fungal sterols, the stereochemistry at C-24 of these sterols could not be determined. However, a 24β configuration is expected (26). As shown in Table 2, control carrot roots were found to contain the usual plant $\Delta^5$-sterols represented by stigmasterol, the predominant sterol, 24-methyl cholesterol, and sitosterol. In higher plants, 24-ethylsterols usually have a 24α configuration whereas 24-methylsterols are a mixture of both epimers (24α, i.e., campesterol, and 24β, i.e., 22-dihydrobrassicasterol) (18). Carrot roots colonized by *G. intraradices* exhibited a sterol

composition similar to that of control roots. However, an increase in the relative percentage of 24-methyl cholesterol was found in mycorrhizal roots. A careful examination of this fraction by GC–MS revealed the presence of 24-methylene cholesterol, which could not be detected in control roots or in spores of *G. intraradices*. A similar observation was previously reported for mycorrhizal roots from other plants (35,36). Another effect of symbiosis was an increase in the total sterol content (in mg $g^{-1}$ dry wt) of carrot mycorrhizal roots. Because epimers at C-24 were not separated under our GC conditions, it was not possible to discriminate between plant and fungal sterols in mycorrhizal roots.

In order to check whether the fungus was able to synthesize its own sterols, labeling experiments were performed using [1-$^{14}$C]sodium acetate as a precursor. The behavior of the fungus was examined in the symbiotic, detached, and germinating stages. In all these stages, the fungus was found to actively take up exogenous acetate and to incorporate it into sterols. Thus, clear evidence for *de novo* synthesis of sterols by *G. intraradices* was obtained for the first time. Radioactivity was mainly recovered in free sterols. The symbiotic stage appeared to be the most active one. Radioactivity was associated with the two main fungal sterols—24-methyl cholesterol and 24-ethyl cholesterol—but also with the early biosynthetic intermediates, lanosterol and 24-methylene lanosterol.

Sterol biosynthesis by the fungus in the symbiotic stage was almost completely inhibited by 20 μM mevinolin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. This result indicates that in *G. intraradices* acetate is metabolized into sterols by the classical mevalonate pathway as in the filamentous fungus *Aschersonia aleyrodis* and the yeast *Rhodotorula glutinis*. (37).

Our results show that we could detect a less active sterol metabolism of free sterols and a low synthesis of esterified sterols in germinating spores of *G. intraradices*. Nevertheless, the growth of the germinating tubes needs a strong synthesis of membranes. This leads to the hypothesis that sterols required for this membrane synthesis may be provided by hydrolysis of the many sterol esters that are present in *G. intraradices*. These esters are considered to be a storage form for sterols in fungi (38). Such a sterol synthesis by *G. intraradices* even in the absence of symbiosis clearly indicates that the inability of the fungus to complete its life cycle cannot be explained by a lack of sterols and the consequent need to take them up from the plant partner.

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# A New Trihydroxy Fatty Acid from the Ascomycete, Chinese Truffle *Tuber indicum*

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**ABSTRACT:** From the chloroform/methanol extract of the fruiting bodies of the ascomycete Chinese truffle *Tuber indicum* Cooke et Massee, a new trihydroxylated monounsaturated fatty acid (**1**) has been isolated. The structure of this new linoleic acid–derived metabolite was established as 9,10,11-trihydroxy-(12*Z*)-12-octadecenoic acid by means of spectroscopic and chemical methods. The fatty acid composition of the chloroform-soluble fraction of this fungus was analyzed by gas chromatography–mass spectrometry. The content of the predominant unsaturated fatty acids (oleic and linoleic acids) is as high as 68%. The use of dimethyl disulfide adduct was effective in the determination of the position of the double bond, and the glycol oxidation fission reaction with sodium metaperiodate supported on silica gel was helpful in establishing the location of the trihydroxylic groups in the new fatty acid.

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Truffles, also known as "black diamonds," are subterranean edible fungi of the family Tuberaceae (order Tuberales) that grow in symbiosis with certain trees. There are more than 60 different kinds of truffles around the world (1), most of which grow in various parts of Europe, particularly in France. They are thought to be a "miracle of nature" and have been since ancient times the ultimate in gastronomy because of their superior nutritional attributes. In addition to their use as a costly food, truffles have been used in making liqueurs, for scenting tobacco, and in certain perfumes.

Recent studies have proven that some truffles contain steroids as major components (2,3) as well as volatile organic compounds for mushroom aroma (4–6). The white truffle (*Tuber magnatum* Pico) and the black truffle (*T. melanosporum* Vitt) are highly appreciated for their unique aroma, which is characteristically sulfurous. The predominant sulfur compounds in white truffle aroma are dimethyl sulfide and bis(methylthio)-methane and dimethyl sulfide in black truffle aroma (4). Interestingly, the ability of pigs to detect truffles underground has been linked to the presence of trace amounts of odorous steroidal pheromone in both black and white truffles (5).

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Abbreviations: CC, column chromatography; DMDS, dimethyl disulfide; EI–MS, electron impact–mass spectrometry; FAB–MS, fast atom bombardment–mass spectrometry; GC–MS, gas chromatography–mass spectrometry; HPODE, hydroperoxyoctadecadienoic acid; IR, infrared; LA, linoleic acid; LOX, lipoxygenase; MS, mass spectrometry; NMR, nuclear magnetic resonance; OA, oleic acid.

The lipid content of mushrooms has been the object of many investigations in relation to studies in subjects as varied as metabolism, nutrition, and medicine. In the past decades, it has been proven that fatty acids with 16 and 18 carbons in higher fungi are most abundant and that linoleic and/or oleic acids as principal unsaturated fatty acids (6–8) occur in complex bonding forms. Several higher mushrooms are also found to contain polyhydroxylated $C_{18}$ fatty acids in the free acid forms (9–11).

About 25 species of the genus *Tuber* are found in China. Chinese truffles, *T. indicum* Cooke et Massee, are distributed mainly in the provinces of Yunnan and Sichuan. This truffle strongly resembles the black truffle. As part of our search for naturally occurring bioactive secondary metabolites of higher fungi in the Yunnan Province, we reported a rare polyhydroxylated ergosterol glycoside in the preceding paper (2). In continuation of our investigation on chemical constituents of *T. indicum*, a new fatty acid (**1**) was isolated from the fruiting bodies of this fungus, along with known compounds adenosine, uracil, 5-hydroxypyrrolidin-2-one, D-allitol, ergosterol, and nonanedioic acid (azelaic acid). The present report deals with the structural elucidation of this new metabolite, and the fatty acid composition of its chloroform-soluble fraction is briefly described.

## EXPERIMENTAL PROCEDURES

*Chromatographic and instrumental methods.* Melting points were obtained on an XRC-1 apparatus (Sichuan University, Sichuan, People's Republic of China). Optical rotations were taken on a Horiba SEPA-300 automatic polarimeter (Horiba, Tokyo, Japan). The nuclear magnetic resonance (NMR) one- and two-dimensional NMR spectra were acquired on Bruker AM-400 and Bruker DRX-500 instruments (Karlsruhe, Germany); tetramethylsilane was used as an internal standard and coupling constants were represented in hertz. Mass spectrometry (MS) spectra were measured with a VG Autospec-3000 mass spectrometer (VG, Manchester, England). Infrared (IR) spectra were obtained in KBr pellets on a Bio-Rad (Richmond, CA) FTS-135 infrared spectrophotometer. Gas chromatography (GC)–MS was performed with a Finnigan 4510 GC–MS spectrometer (San Jose, CA) employing the electron impact (EI) mode (ionizing potential 70eV) and a capillary column (30 m × 0.25 mm) packed with 5% phenyl/95% methylsilicone on HP-5 (Hewlett-Packard, Palo Alto, CA). Hydrogen was

used as carrier gas and other conditions were as follows: hydrogen gas flow (30 mL/min), air flow rate (300 mL/min), hydrogen gas carrier flow rate (1.0 mL/min), and column temperature (160–240°C, rate of temperature increase: 5°C/min).

*Materials.* Column chromatography (CC) was performed over silica gel (200–300 mesh; Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China). Reversed-phase chromatography was carried out on LiChroprep$^R$ RP-8 (40–63 μm) (Merck, Darmstadt, Germany). Thin-layer chromatographic analysis was carried out on plates precoated with silica gel $F_{254}$ (Qingdao Marine Chemical Ltd.), and detection was achieved by spraying with 10% $H_2SO_4$ followed by heating. All solvents were distilled before use.

*Fungal samples.* The dried fruiting bodies of *T. indicum* were purchased in Yunnan Province in April 2000 and identified by Profs. P.G. Liu and X.H. Wang (Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, People's Republic of China). A voucher specimen was deposited at the Herbarium of Kunming Institute of Botany.

*Extraction and isolation.* The dried and powdered fruiting bodies (4.7 kg) were extracted successively three times with $CHCl_3$ and four times with $CHCl_3$/MeOH (1:1, vol/vol) at room temperature. These extracts were concentrated to dryness *in vacuo*, respectively, to give both $CHCl_3$ (154 g) and $CHCl_3$/MeOH extracts (122 g). The $CHCl_3$/MeOH (1:1, vol/vol) extracts were chromatographed over silica gel using $CHCl_3$ by using increasing concentrations of MeOH in $CHCl_3$ as eluent. The fractions (3.5 g) eluted with $CHCl_3$/MeOH (95:5, vol/vol) were subjected to silica gel CC using $CHCl_3$/MeOH (9:1, vol/vol) to provide a residue (139 mg), which was rechromatographed on silica gel with cyclohexane/acetone (7:3, vol/vol) to furnish pure compound **1** (18.7 mg).

*Fatty acid analysis.* Two milliliters $Et_2O$/*n*-hexane (2:1, vol/vol), 2 mL MeOH, 2 mL 0.8 mol/L KOH-MeOH, and 19 mL $H_2O$ were added to the 110 mg $CHCl_3$ extract containing fatty acids in a 25-mL volumetric flask; after shaking for 5 min, the supernatant obtained was subjected to GC–MS.

*9,10,11-Trihydroxy-(12Z)-12-octadecenoic acid (**1**).* White amorphous crystals ($CHCl_3$); m.p. 73–75°C; $[\alpha]_D^{22}$ = +10.0 (*c* 0.008, $CHCl_3$); IR (KBr) *v* 3368 (OH), 2928, 2852 (aliphatic C-H), 1713 (acidic C=O), 1464, 1399, 1242, 1075, 1017 (C-O), 940, and 723 $[(CH_2)_n]cm^{-1}$; EI–MS (70 eV) (relative intensity %) *m/z* 330 $[M]^+$ (0.5), 302 (1.0), 285 (1.5), 275 (1.0), 273 (1), 271 (1.2), 203 (7.0), 187 (4.5), 185 (41), 173 (7.5), 168 (50), 155 (29), 143 (2.5), 139 (12), 127 (19.5), 121 (5), 110 (14), 109 (35), 98 (24), 97 (29.5), 83 (68), 71 (29), 69 (58.2), 57 (77.5), and 55 (100); and $^1H$ and $^{13}C$ NMR data are given in Table 1. High-resolution fast atom bombardment (FAB)–MS (negative ion mode*s*) at *m/z* 329.2264 $[M-1]^-$, calcd. for $C_{18}H_{33}O_5$, 329.2328; FAB–MS (positive ion mode) *m/z* 331 $[M+1]^+$ (10), 313 $[M+1-H_2O]^+$ (23), 173 (100), 109 (25.5), 81(29.5).

*Acetylation of **1**.* Compound **1** (8.0 mg) was treated with $Ac_2O$/pyridine (1:1) for 36 h at room temperature to yield a crude product, to which 3 mL of water was added and then extracted with EtOAc (3 × 3 mL). The resulting residue was chromatographed on silica gel with *n*-hexane/EtOAc (10:1–8:2 vol/vol) to afford 8.9 mg of its triacetate derivative (**1a**) as a colorless oil. $^1H$ NMR 400 MHz ($CDCl_3$) δ ppm **1a**: 5.70 (1H, *dt*, *J* = 4.2, 6.7, 10.7 Hz), 5.64 (1H, *dd*, *J* = 11.0, 7.4 Hz), 5.29 (1H, *br d*, *J* = 9.7 Hz), 5.17 (1H, *t*, *J* = 5.0, 6.2 Hz), 4.95 (1H, *dd*, *J* = 2.9, 9.8 Hz), 2.20 (2H, *t*, *J* = 7.6 Hz), 1.98 (3H, *s*, $COCH_3$), 1.99 (3H, *s*, $COCH_3$), 2.04 (3H, *s*, $COCH_3$), 1.58 (4H, *m*), 1.22–1.27 ($[CH_2]_n$, *br s*), 0.86 (3H, *t*, *J* = 6.6 Hz, terminal methyl); EI–MS (70 eV) (relative intensity %) *m/z* 456 $[M]^+$ (1), 439 $[M-OH]^+$ (8), 411 (0.5), 410 (0.5), 399 (1), 397 $[M-AcO]^+$ (25), 336 $[M-2\times AcOH]^+$ (41), 294 $[M-2\times AcOH-Ac+1]^+$ (72), 287 (8), 277 (40), 276 $[M-3\times AcOH]^+$ (57), 248 (21), 241 (8), 233 (5), 227 (80), 219 (8.5), 215 (4), 182 (86), 169 (72), 168 (80), 155 (53), 143 (3), 140 (84), 139 (48), 127 (86), 122 (15), 110 (18), 109 (59),

**TABLE 1**
**$^1H$ (400 MHz) and $^{13}C$ (100 MHz) Nuclear Magnetic Resonance (NMR) Data of Compound 1 in Pyridine-$d_5$[a]**

| Atom. no. | $^{13}C$ (multiplicity) | | $^1H$ (multiplicity, *J* in Hz) | $^1H$-$^1H$ COSY |
|---|---|---|---|---|
| 1 | 175.9 | C | | |
| 2 | 34.9 | $CH_2$ | 2.47 (*t, J* = 7.5 Hz) | H-3 |
| 3 | 25.7 | $CH_2$ | 1.74 (*m*) | H-2, H-4 |
| 4–6 | 22.7–31.7 | $3CH_2$ | 1.19–1.38 (*br m*) | |
| 7 | 34.3 | $CH_2$ | 2.25 (*m*) | H-6, H-8 |
| 8 | 34.3 | $CH_2$ | 1.91 (*m*) | H-7, H-9 |
| 9 | 72.6 | CH | 4.26 (*m*) | H-8, H-10 |
| 10 | 78.6 | CH | 3.93 (*dd, J* = 6.7, 3.4 Hz) | H-9, H-11 |
| 11 | 67.8 | CH | 5.30 (*dd, J* = 9.1, 3.4 Hz) | H-10, H-12 |
| 12 | 132.6 | CH | 6.16 (*dd, J* = 11.0, 9.2 Hz) | H-11, H-13 |
| 13 | 131.5 | CH | 5.60 (*dt, J* = 11.0, 7.4 Hz) | H-12, H-14 |
| 14 | 28.2 | $CH_2$ | 2.10 (*m*) | H-13 |
| 15–17 | 22.7–31.7 | $3CH_2$ | 1.19–1.38 (*br m*) | |
| 18 | 14.1 | $CH_3$ | 0.80 (*t, J* = 7.0 Hz) | H-17 |

[a]Assignments were made by distortionless enhancement by polarization transfer and heteronuclear multiple quantum coherence analysis; COSY, correlation spectroscopy.

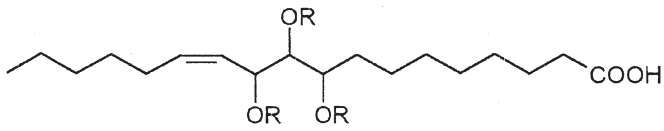
98 (43), 97 (39), 95 (55), 83 (75), 69 (60), 57 (75), 55 (100); high-resolution EI–MS *m/z* 456.2693 $[M]^+$ (calcd. for $C_{24}H_{40}O_8$, 456.2723).

*Sodium periodate oxidation of **1***. Sodium periodate (0.26 g in 1.2 mL water) was heated, with stirring, to 75°C over a period of 25 min. Silica (1.0 g) was added to the stirred solution. The mixture was then cooled and shaken vigorously for 20 min to give a coarse powder. $CH_2Cl_2$ (6 mL) was added and then, after stirring, a solution of **1** (6.4 mg) in 6 mL of $CH_2Cl_2$/MeOH (1:1, vol/vol). The reaction mixture was stirred at room temperature for 90 min, then filtered and washed with $CH_2Cl_2$. The combined supernatant was concentrated *in vacuo* to afford a mixture of aldehydes, which was subjected to EI–MS and $^1$H NMR. EI–MS (70 eV) *m/z* 126 $[M]^+$ for 2-*cis*-octenal **2**, 172 $[M]^+$ for 9-oxo-nonanoic acid **3**; $^1$H NMR 500 MHz ($CDCl_3$) δ ppm **2**: 9.51 (1H, *d*, *J* = 7.8 Hz, 1-H), 6.21 (1H, *dd*, *J* = 11.0, 7.8 Hz, 2-H), 6.85 (1H, *dd*, *J* = 11.0, 7.0 Hz, 3-H), 2.09 (2H, *dt*, *J* = 7.2, 7.8 Hz, 4-H), 0.88 (3H, *t*, *J* = 7.0 Hz, 8-H); and **3**: 9.76 (1H, *t*, *J* = 1.8 Hz, 9-H), 2.42 (2H, *t*, *J* = 7.3 Hz, 8-H), 2.34 (2H, *t*, *J* = 7.5 Hz, 2-H). $^1$H NMR data were in agreement with those of authentic samples.

*Dimethyl disulfide (DMDS) derivative **4** of compound **1a***. To the solution of **1a** (3.1 mg) dissolved in DMDS (0.2 mL), DMDS (0.2 mL) and iodine (1 mg) were added. The mixture obtained was kept at 60°C for 44 h in a small-volume sealed vial. The reaction was quenched with aqueous $Na_2S_2O_3$ (5%), and the reaction mixture was extracted with *n*-hexane (0.6 mL). The extract was concentrated, and the residue was purified by silica gel CC using *n*-hexane/EtOAc (8:2, vol/vol) to give the DMDS adduct **4**. EI–MS (relative intensity %) (70 eV) *m/z* 550 $[M]^+$ (5), 533 (1), 506 (1.5), 452 (2), 419 (4), 404 (4), 390 (9), 355 (4), 340 (8), 323 (16), 309 (31), 239 (10), 226 (19), 183 (14), 167 (22), 154 (37), 131 (56), 111 (28), 97 (49), 83 (47.5), 69 (58), 57 (68.5), 55 (100); high resolution EI–MS *m/z* 131.0898 $[M]^+$ (calcd. for $C_7H_{15}S$, 131.0894).

## RESULTS AND DISCUSSION

Compound **1** was obtained as white amorphous crystals ($CHCl_3$), m.p. 73–75°C. The molecular formula of **1** was determined to be $C_{18}H_{34}O_5$ by high-resolution negative ion FAB–MS (*m/z* 329.2264 $[M - 1]^-$, calcd. 329.2328 for $C_{18}H_{33}O_5$) and $^{13}$C NMR spectra. Upon treatment with $Ac_2O$/pyridine, **1** was acetylated to furnish its acetate **1a** (Scheme 1), which showed a molecular ion at *m/z* 456, corresponding to the molecular composition of $C_{24}H_{40}O_8$ as determined by high-resolution EI–MS at *m/z* 456.2693 (calcd. for $C_{24}H_{40}O_8$, 456.2723). The IR spectrum of **1** revealed the absorption bands of hydroxyls at 3368 and 1017 $cm^{-1}$, and a carboxylic carbonyl at 1713 $cm^{-1}$. Also, the bands appearing at 2928, 2852, 1464, 1420, and 723 $cm^{-1}$ revealed its paraffinic nature (12). Compound **1** was considered to be a straight-chain compound due to a terminal methyl group at δ 14.1 ppm (13) in its $^{13}$C NMR spectrum. The $^1$H NMR spectrum of **1** showed the presence of one terminal methyl at δ

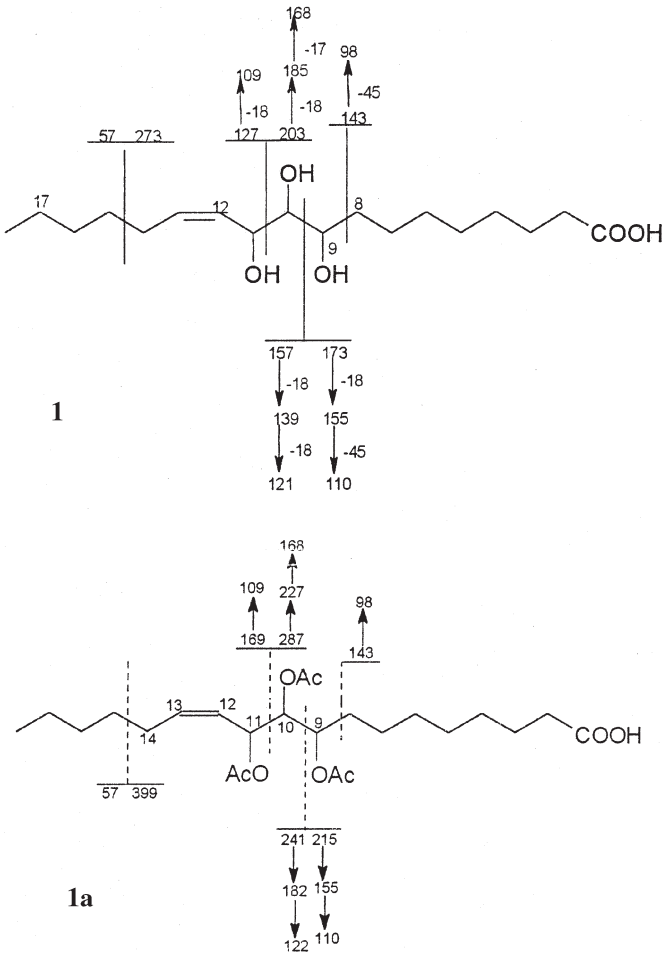



**1** R = H
**1a** R = Ac

**SCHEME 1**

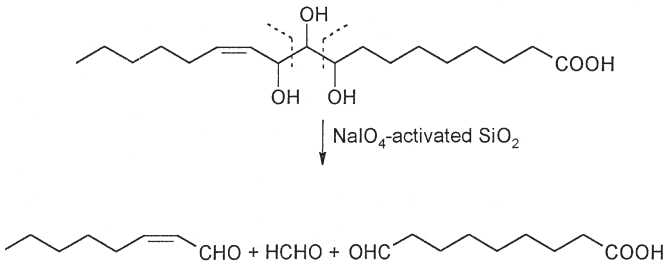
0.80 (3H, *t*), and methylenes at δ 1.19–1.38 (*br m*). The $^{13}$C NMR (distortionless enhancement by polarization transfer) spectrum of **1** further furnished a quaternary carbon, 5 methines, 11 methylenes, and 1 methyl (Table 1), in which one carboxylic carbon (*C*OOH) at δ 175.9 was given. These data revealed that **1** was an unbranched fatty acid.

The $^{13}$C NMR spectral signals at δ ppm 67.8 (CH), 72.6 (CH), and 78.6 (CH) and the $^1$H NMR resonances at δ ppm 5.30 (1H, *dd*, *J* = 9.1, 3.4 Hz), 4.26 (1H, *dt*, *J* = 9.1, 2.6 Hz), and 3.93 (1H, *dd*, *J* = 6.7, 3.4 Hz) infer the existence of three hydroxy groups in the molecule. This conclusion was further confirmed by the presence of the nine-proton (3H, each) singlets of three ester methyl groups at δ 1.98, 1.99, and 2.04 in the $^1$H NMR spectrum of **1a** as well as by a typical ion peak at *m/z* 276 due to the loss of three molecules of AcOH from the molecular ion of **1a**, respectively. A set of diagnostic fragment ions of *m/z* 173→155 and 157→139→121, 203→185→168, and 127→109 in the EI–MS of **1** (Scheme 2) showed these hydroxyls to be located at C-9, C-10, and C-11. This assignment was in turn supported by a series of characteristic mass spectral fragment ions at *m/z* 215→155 and 241→182→122, 287→227→168, and 169→109 in the EI–MS of **1a** (Scheme 2). To further prove the positions of trihydroxylic groups, two short-chain aldehydes, **2** and **3,** along with a formaldehyde (Scheme 3) were prepared by the $NaIO_4$-activated silica gel oxidation of **1**. The aldehydes thus obtained were analyzed by $^1$H NMR and EI–MS without further separation. The conjugated aldehyde **2** with an adjacent double bond gave three typical low-field protons at δ ppm 9.51 (1H, *d*, *J* = 7.8 Hz, H-1), 6.21 (1H, *dd*, *J* = 11.0, 7.8 Hz, H-2), and 6.85 (1H, *dd*, *J* = 11.0, 7.0 Hz, H-3), which was in agreement with the spectrum of an authentic sample. The assignment of H-2 appearing at δ 6.12 ppm is based on the conjugated effect in **2**. Because the olefinic protons of **2** had the coupling constant of $J_{2,3}$ = 11.0 Hz, the double bond was deduced to be *cis*, and the position of this double bond turned out to be at C-2/C-3 due to the coupling between the olefinic proton (H-2) and the aldehydic proton (H-1) by $J_{1,2}$ = 7.8 Hz. Additionally, the aldehyde protons of **3** were recognized as a triplet at δ ppm 9.76 (*t*, *J* = 1.8 Hz, H-9). The absence of a methyl group and the presence of two methylenes as triplets at δ 2.34 and 2.42, respectively, indicate that the carboxyl group was present in **3**. These data show that the oxidation products had key structures **2** and **3**. The precursor of **3**, namely, 9-oxononanoic acid, was found to be 9-hydroperoxy-10,12-octadecadienoic acid (9-HPODE) (14).




**SCHEME 2**

Furthermore, the $^1$H NMR signals at δ 5.60 (1H, *dt*, *J* = 11.0, 7.4 Hz) and 6.16 (1H, *dd*, *J* = 11.0, 9.1 Hz) and $^{13}$C NMR signals at δ 131.5 (CH) and 132.6 (CH) indicated the presence of a disubstituted double bond in **1**. The location and configuration of the double bond were determined as follows. The corresponding fragment ions at *m/z* 57, 127, and 203 due to the formation of $[C_4H_9]^+$, $[CH_3(CH_2)_4CH{=}CHCH(OH)]^+$, and $[CH(OH)CH(OH)(CH_2)_7COOH]^+$ species arising from allylic cleavages between C-14 and C-15 and between C-10 and C-11 supported the location of the olefinic linkage between C-12 and C-13 in the molecule, as evidenced from allylic cleavages of **1a** at *m/z* 57 $[C_4H_9]^+$,169 $[CH_3(CH_2)_4CH{=}CHCH(OAc)]^+$, and 287 $[CH(OAc)CH(OAc)(CH_2)_7COOH]^+$ fragment ions.



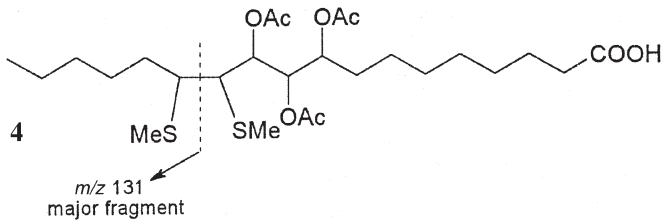

**SCHEME 3**

Moreover, the high-resolution EI–MS spectrum of the DMDS derivative **4** of **1a** showed a remarkable fragment-ion peak at *m/z* 131.0898, corresponding to the chemical composition of $C_7H_{15}S$ (calcd. 131.0894) due to cleavage of the bond between the carbons bearing a methylthio group (Scheme 4). This result also supports the location of the double bond between C-12 and C-13 in **1**.

It is known, on the other hand, that the geometry of the double bond in a long-chain alkene can be determined from the $^{13}$C NMR chemical shift of the methylene carbon next to the olefinic carbon, namely, the carbon signal observed between δ 27–28 ppm in *cis* type and between δ 32–33 ppm in *trans* type (15). The *cis* stereochemistry of this double bond was deduced from the chemical shift of C-14 (δ = 28.2 ppm). This *cis* configuration was also supported by the large vicinal coupling constant ($J_{12,13}$ = 11.0 Hz) displayed between H-12 and H-13. From the $^1$H-$^1$H correlation spectroscopy spectrum, the correlations between H-8 at δ 1.91 (*m*) and H-9 at δ 4.26 (*m*), H-9 and H-10 at δ 3.93 (*dd*), H-10 and H-11 at δ 5.30 (*dd*), H-11 and H-12 at δ 6.16 (*dd*), H-12 and H-13 at δ 5.60 (*dt*), and H-13 and H-14 at δ 2.10 (*m*) were observed in **1**. It strongly confirmed that **1** contained the partial structure -$CH_2$-HC=CH-CH(OH)-CH(OH)-CH(OH)-$CH_2$- (C-8 to C-14). Consequently, the preceding evidence led to the establishment of the structure of **1** as 9,10,11-trihydroxy-(12*Z*)-12-octadecenoic acid (Scheme 1).

The major fatty acids from a $CHCl_3$-soluble extract of *T. indicum* were linoleic acid (LA) (61.6%), stearic acid (16.0%), palmitic acid (13.7%), and oleic acid (OA) (6.66%). The high level of unsaturated fatty acids (68%) may contribute in part to explaining the nutritional quality of this mushroom.

Lipid peroxidation processes induced by lipoxygenase (LOX; EC 1.13.11.12), a nonheme iron-containing dioxygenase enzyme, are reported to occur in plant systems; they were observed, for instance, in *Rudbeckia fulgida* (16), in wheat flour suspensions (17), and in rice plants (18). The oxidation of the two unsaturated acids, LA and linolenic acid, is then catalyzed by LOX to lead, *via* an antarafacial process, to the hydroperoxide 9-HPODE or 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), and to mixtures of 9- and 13-HPODE depending on LOX type and appropriate conditions (14). Subsequently, HPODE are metabolized in the presence of various enzymes to mono-, epoxy-, and trihydroxy fatty acids and even a cascade of catabolic products. According to the pathway of metabolism, it is possible that the previously unknown metabolite reported in the present study is biosyn-

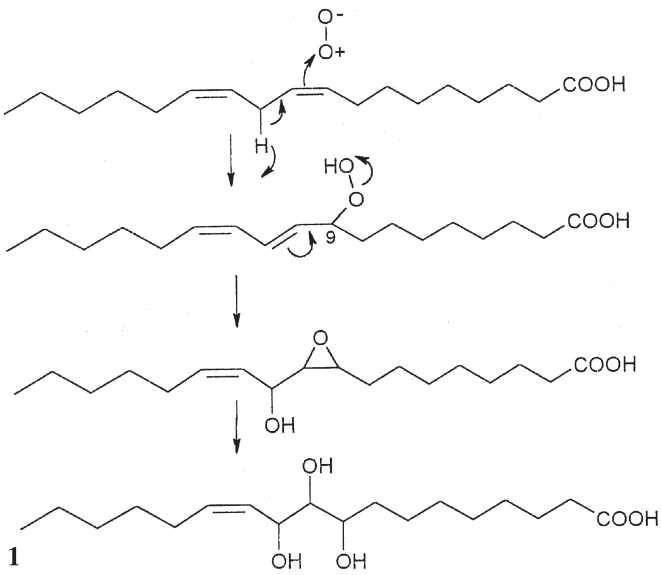



**SCHEME 4**

thetically derived from LA *via* the intermediate 9-HPODE (Scheme 5) in *T. indicum*. Hermann and Erwin (19) found that 9,10,11-trihydroxy-12-*trans*-octadecenoic acid from LA, a geometrical isomer of the title compound **1**, was present in the bee and may be considered one of the components responsible for stale bee flavors. There is no doubt that the first discovery of **1** could be required for an understanding of the role of *T. indicum* in mushroom metabolism.

The unsaturated fatty acids possess a broad spectrum of biological properties in both animals and plants. In particular, LA-derived hydroxylated unsaturated C-18 fatty acids have attracted considerable attention. Some of these compounds show biological activity, e.g., 9,10-dihydroxy-8-oxo-12*Z*-octadecaenoic acid exhibits cytotoxicity against HeLa cells and an inhibitory effect on tea pollen growth (9); 9*S*,12*S*,13*S*-trihydroxyoctadeca-10*E*-enoic acid is active against rice blast fungus (18); 9,10,13-trihydroxy-*trans*-11-octadecaenoic acid has prostaglandin E-like activity (20); and 13*S*-hydroxy-9,11-octadecadienoic acid (*S*-coriolic acid) shows nematocidal action (11). A fatty acid mixture consisting of linoleic, oleic, and palmitic acids as main components showed nematocidal activity, and the most active compound was LA with 50% inhibition lethal dose ($LD_{50}$) values between 5 and 10 μg/mL (11). LA and OA displayed antibacterial activity, the former inhibiting the growth of all the Gram-positive bacterial species with a minimum inhibitory concentration between 0.01 and 1.0 mg/mL, whereas the latter is active against three of the five Gram-positive bacteria at a minimum inhibitory concentration of 1.0 mg/mL. A synergistic effect between the two fatty acids was observed against *Staphylococcus aureus* and *Micrococcus kristinae* (21). The fungal metabolites that may be formed in the fruiting bodies of macrofungi constitute their chemical defense system against parasites as well as various predators such as bacteria, fungi, animals, and insects (22). This newly isolated fatty acid, in combination with LA and OA, might be presumed to act as phytoalexins existing in hypogenous fungi. Further investigation will be performed.

LA inhibits the activities of mammalian DNA polymerases; the addition of ergosterol peroxide to a polymerase reaction mixture led to selective enhancement of the inhibitory effect of LA on DNA polymerase β (23). Fortuitously, we have previously reported finding this sterol in this mushroom (2). The occurrence of LA and the sterol further offers the possibility of gaining insight into the biological functions of truffles.




**SCHEME 5**

## ACKNOWLEDGMENTS

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# Identification of Novel Nonmethylene-Interrupted Fatty Acids, 7*E*,13*E*-20:2, 7*E*,13*E*,17*Z*-20:3, 9*E*,15*E*,19*Z*-22:3, and 4*Z*,9*E*,15*E*,19*Z*-22:4, in Ophiuroidea (Brittle Star) Lipids

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**ABSTRACT:** Fatty acids of Ophiuroidea (brittle star) *Ophiura sarsi* have been investigated by gas–liquid chromatography (GLC). About 2–13% of four unidentified fatty acids were found in total fatty acids from a sample caught at a depth of 1,100 m. Structural analyses were undertaken after partial hydrogenation of their concentrates with hydrazine hydrate and subsequent isolation of the monoenoate products by argentation thin-layer chromatography. The structures of the unidentified fatty acids were determined as 7*E*,13*E*-eicosadienoic (20:2), 7*E*,13*E*,17*Z*-eicosatrienoic (20:3), 9*E*,15*E*,19*Z*-docosatrienoic (22:3), and 4*Z*,9*E*,15*E*,19*Z*-docosatetraenoic (22:4) acids by gas chromatography–mass spectrometry of dimethyl disulfide adducts and GLC of the monoenoates on a polar column. These fatty acids belong to a family of nonmethylene-interrupted (NMI) polyunsaturated fatty acids frequently observed in marine invertebrates and conifer seeds. As far as the authors know, however, these NMI fatty acid types with mixed geometry of ethylenic bonds have not been reported previously.

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Some species of marine invertebrates contain remarkable levels of distinctive fatty acids. In Ophiuroidea (brittle star), 6,9,12,15,18,21-tetracosahexaenoic acid (24:6n-3) has been observed at concentrations of 3–15% in total fatty acids from all of the samples examined (1–3). This fatty acid has anti-inflammatory and antiallergic properties similar to those of 22:6n-3 (4). Because Ophiuroidea are widely distributed with a high density in the upper bathyal zone around Japan, they are useful as a source of 24:6n-3 (2). However, there have been only a few studies on other fatty acid components. The present research on an additional sample of Ophiuroidea *Ophiura sarsi* revealed the occurrence of four unidentified fatty acids (2.2–12.7% of total fatty acids) along with the following major fatty acids: 14:0 (3.8%), 16:0 (6.6%), 16:1n-7 (3.3%), 18:0 (3.1%), 18:1n-9 (13.1%), 18:1n-7 (2.5%), 20:1n-11 + 20:1n-13 (4.3%), 20:5n-3 (4.5%), and 24:6n-3 (5.9%). This paper reports the structural assignments of the unidentified components as nonmethylene-interrupted (NMI) polyunsaturated fatty acids 7*E*,13*E*-20:2, 7*E*,13*E*,17*Z*-20:3, 9*E*,15*E*, 19*Z*-22:3, and 4*Z*,9*E*,15*E*,19*Z*-22:4.

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Abbreviations: DMDS, dimethyl disulfide; ECL, equivalent chain length; GC–MS, gas chromatography–mass spectrometry; GLC, gas–liquid chromatography; NMI fatty acids, nonmethylene-interrupted fatty acids; TLC, thin-layer chromatography.

## MATERIALS AND METHODS

Ophiuroidea *Ophiura sarsi* were caught at a depth of 1,100 m in Toyama Bay, the Sea of Japan (37°15′N, 137°35′E) on March 18, 1999, and kept frozen at –30°C for a month until analyzed. Total lipids were extracted from whole bodies by the method of Bligh and Dyer (5). The extracted lipids were converted to fatty acid methyl esters by direct transesterification with 7% $BF_3$/methanol for 1 h at 100°C in a screw-capped test tube under nitrogen. Methyl esters were purified by thin-layer chromatography (TLC) on a Kieselgel 60G plate (Merck, Darmstadt, Germany) with toluene for development (6).

The methyl esters were fractionated according to the degree of unsaturation by argentation TLC (Ag-TLC) on 10% (w/w) silver nitrate-impregnated layers of Kieselgel 60G with hexane/ethyl acetate (95:5, vol/vol) and double developments. The methyl esters were further fractionated according to their carbon number by reversed-phase TLC on a Whatman KC18F plate (Whatman International Ltd., Maidstone, England) with acetonitrile and double developments.

Hydrogenation of fatty acid methyl esters was carried out over palladium black in hexane.

Partial reduction of unsaturated fatty acids was carried out by the method previously described (7,8). A mixture of 1 mg of fatty acids and 10% (vol/vol) hydrazine hydrate in methanol (1 mL) was stirred in the presence of air at 50°C for 5–8 h. The products extracted with diethyl ether were converted to methyl esters with 7% $BF_3$/methanol. The resulting monounsaturated fatty acids were separated from other products by Ag-TLC on 30% (w/w) silver nitrate-impregnated Kieselgel 60G plates with hexane/benzene (50:50, vol/vol) and double developments.

Dimethyl disulfide (DMDS) adducts of the monounsaturated fatty acids were prepared by the procedure of Shibahara

*et al.* (9–11). Methyl esters were reacted with DMDS (1 mL) in the presence of $I_2$ (13 mg) as the catalyst for 1 h at 35°C.

Fatty acid methyl esters were analyzed by gas–liquid chromatography (GLC) with a Shimadzu GC-17A instrument (Shimadzu Seisakusho Co., Kyoto, Japan) equipped with a flame-ionization detector and a Supelcowax 10 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco Inc., Bellefonte, PA). The column temperature was either isothermal at 200°C or programmed from 180 to 240°C (1°C/min). The injector and detector temperatures were 250 and 260°C, respectively. The carrier gas was $H_2$ (95 kPa). Peak area percentages were obtained with a Shimadzu C-R6A integrator.

Monounsaturated fatty acids isolated from the hydrazine reduction products were analyzed by GLC with the same instrument equipped with a polar "CP-Sil 88 for FAME" capillary column (50 m × 0.25 mm i.d., 0.20 μm film thickness; Chrompack, Middelburg, The Netherlands) for separation of geometrical isomers. The column temperature was 200°C. The injector and detector temperatures were 250 and 260°C, respectively. The carrier gas was $H_2$ (125 kPa).

Gas chromatography–mass spectrometry (GC–MS) analyses of fatty acid methyl esters were carried out with a GCQ system (Thermo Quest Co., Tokyo, Japan) equipped with an SPB-1 (dimethylsiloxane) capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness; Supelco Inc.). Chemical ionization with methane reagent gas was used. The column temperature was programmed as follows: isothermal at 120°C for 1 min, increased from 120 to 220°C (25°C/min) and held for 5 min, increased from 220 to 230°C (0.5°C/min), and increased from 230 to 270°C (25°C/min) and held for 10 min. The injector temperature was 270°C. All spectra were obtained at an ionization energy of 70 eV and at a source temperature of 200°C.

GC–MS analyses of the DMDS adducts were carried out with the GCQ system and the SPB-1 capillary column described above. Electron ionization was used. The column temperature was programmed to increase from 120 to 275°C (25°C/min) after holding the initial temperature of 120°C for 2 min.

Fourier transform infrared spectra were measured in a $CCl_4$ solution with a JASCO FT-IR 5300 spectrometer (JASCO Co., Tokyo, Japan).

## RESULTS AND DISCUSSION

GLC analysis of the fatty acid methyl esters from the Ophiuroidea showed four large peaks of A (2.2% of total fatty acids), B (12.7%), C (5.6%), and D (3.0%) (Fig. 1). Their equivalent chain lengths (ECL) were 20.51 (A), 21.13 (B), 23.08 (C), and 23.28 (D) on Supelcowax 10 at 200°C. With 10% (w/w) Ag-TLC, the peak component of A was concentrated to 5.2% in a fraction along with ordinary monoenoic fatty acids; B and C were concentrated to 51.3 and 22.2%, respectively, in a fraction along with dienoic and trienoic fatty acids; D was concentrated to 19.3% in a fraction along with pentaenoic (primarily 20:5n-3) and tetraenoic (primarily 22:4n-3) fatty acids. Reversed-phase TLC of these Ag-TLC fractions gave further concentrates of A (8.6% of total fatty

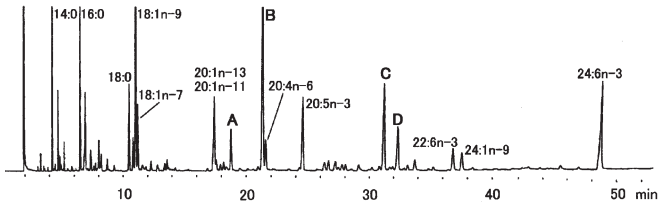


**FIG. 1.** Gas chromatogram of fatty acid methyl esters from the total lipids of Ophiuroidea (Supelcowax 10; Supelco, Bellefonte, PA; 180 to 240°C at 1°C/min). Peaks A–D: fatty acids identified as 7*E*,13*E*-20:2 (A), 7*E*,13*E*,17*Z*-20:3 (B), 9*E*,15*E*,19*Z*-22:3 (C), and 4*Z*,9*E*,15*E*,19*Z*-22:4 (D) in the present study.

acids), B (87.8%), C (75.6%), and D (79.6%). The concentrate of A included 18:1 acids (87.6%) and 19:1 acids (0.8%); concentrate of B included 18:2 acids (5.9%) and 20:2 or 20:3 acids (2.8%); concentrate of C included 20:2 acids (13.7%) and 22:2 or 22:3 acids (9.2%); and concentrate of D included 22:4n-3 acids (12.4%). Total hydrogenation of the concentrates yielded *n*-20:0 from A and B, and *n*-22:0 from C and D, indicating that these four components are fatty acids having straight carbon chains. Figure 2 shows the mass spectrum of fatty acid A. Chemical ionization mass spectrometry of the fatty acid methyl esters gave $[M - H]^-$ and $[M - H - 32]^-$ ions. Two prominent ions at *m/z* 321 and 289 indicated that fatty acid A is eicosadienoic acid (20:2). Prominent peaks observed at *m/z* 319 and 287 for B, at *m/z* 347 and 315 for C, and at *m/z* 345 and 313 for D indicated eicosatrienoic (20:3), docosatrienoic (22:3), and docosatetraenoic (22:4) acids, respectively.

Each concentrate of A–D was partially reduced by hydrazine to produce monoenoic fatty acids. The concentrates of A and B gave two and three isomers of 20:1, respectively, and C and D three and four isomers of 22:1, respectively. All of the monoenoic fatty acid isomers were separately recovered by 30% (w/w) Ag-TLC. DMDS adduct of each isomer was analyzed by GC–MS using an SPB-1 column. Figure 3 shows the mass spectra of the DMDS adducts of 20:1 isomers originated from fatty acid B (20:3). All of the spectra gave a molecular ion at *m/z* 418 and a series of characteristic key fragment ions showing the double bond position in the 20:1 isomers. In Figure 3A, the fragment ions at *m/z* 189 and 229 corresponded to cleavage between the methylthio-substituted

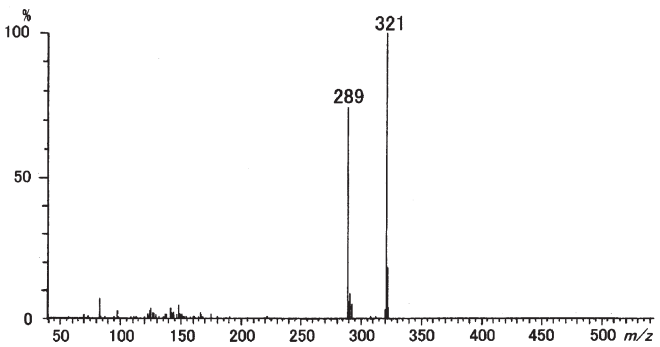


**FIG. 2.** Chemical ionization mass spectrum of methyl ester of fatty acid A, 7*E*,13*E*-20:2.

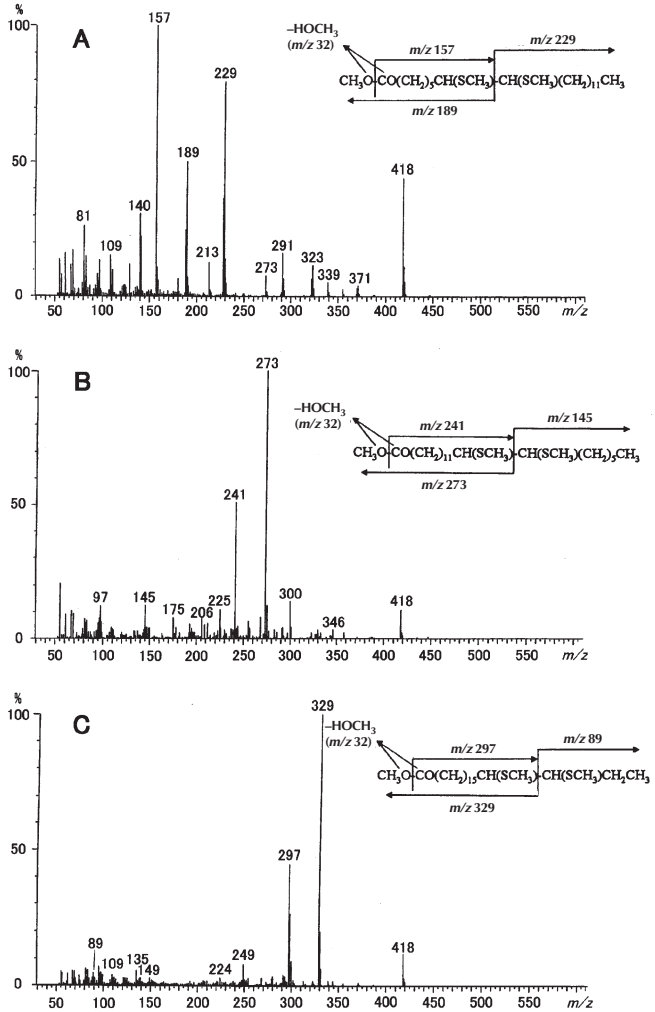


**FIG. 3.** Mass spectra of dimethyl disulfide adducts of 20:1 isomers formed by hydrazine reduction of fatty acid B, 7*E*,13*E*,17*Z*-20:3.

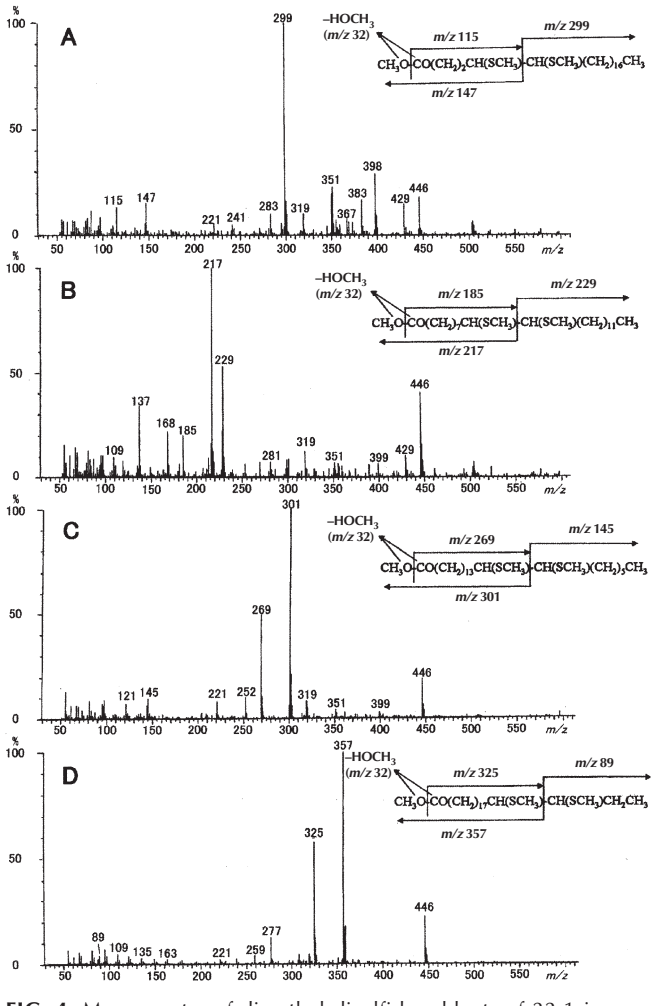


**FIG. 4.** Mass spectra of dimethyl disulfide adducts of 22:1 isomers formed by hydrazine reduction of fatty acid D, 4*Z*,9*E*,15*E*,19*Z*-22:4.

carbons of C-7 and C-8, and the ion at *m/z* 157 was due to loss of methanol (*m/z* 32) from the ion at *m/z* 189. A set of three fragment ions indicated the structure of 7-20:1. In the same manner, structures of 13-20:1 and 17-20:1 were indicated on the basis of key fragment ions at *m/z* 273, 145, and 241 (Fig. 3B), and at *m/z* 329, 89, and 297 (Fig. 3C), respectively. The DMDS adducts of 20:1 isomers originated from fatty acid A (20:2) showed mass spectra similar to those of Figures 3A and 3B. Figure 4 shows the mass spectra of the DMDS adducts of 22:1 isomers originated from fatty acid D (22:4). The structures of isomers 4-22:1, 9-22:1, 15-22:1, and 19-22:1 were indicated on the basis of sets of key fragment ions at 147, 299, and 115 (Fig. 4A); at 217, 229, and 185 (Fig. 4B); at 301, 145, and 269 (Fig. 4C); and at 357, 89, and 325 (Fig. 4D), respectively. The mass spectra of the DMDS adducts of 22:1 isomers originated from fatty acid C (22:3) were similar to those of Figures 4B–D. Hydrazine reduces olefinic bonds without positional and geometrical isomerization of the remaining olefinic bonds (8,12). Consequently, these results indicate that fatty acids A, B, C, and D are 7,13-20:2, 7,13,17-20:3, 9,15,19-22:3, and 4,9,15,19-22:4, respectively.

The infrared spectra of the concentrates of A–D showed a strong absorption near 970 $cm^{-1}$ indicating one or more *trans*-olefinic bonds. The positions of the *trans*-olefinic bonds were determined by GLC of the hydrazine-reduced monoenoate products on a polar capillary column CP-Sil 88 (13–15). Tables 1 and 2 compare the ECL values of the monoenoates with those of *cis*- and *trans*-monounsaturated fatty acid standards obtained from flathead flounder fatty acids (16), hydrazine-reduced products of 20:5n-3 and 22:6n-3, and their geometrical $NO_2$-isomerized products (17,18). ECL of 7-20:1 and 13-20:1 originated from fatty acids A and B were 20.39 and 20.51, whereas those of corresponding *cis*-isomers were 20.54 and 20.73, respectively. Early elution of 7-20:1 and 13-20:1, originated from fatty acids A and B, on this liquid phase indicated that both contained *trans*-olefinic bonds at positions C7 and C13 (i.e., 7*E*- and 13*E*-olefinic bonds) of the original. Table 2 shows corresponding 9*E*- and 15*E*-olefinic bonds in fatty acids C and D. In contrast, ECL values of 17-20:1, 4-22:1, and 19-22:1 originated from fatty acids B, C, and D were close to those of corresponding *cis*-isomers and higher than those of *trans*-isomers, indicating 17*Z*-, 4*Z*-, and 19*Z*-olefinic bonds.

**TABLE 1**
**Equivalent Chain Lengths of 20:1 Isomers, Produced from 7,13-20:2 (A) and 7,13,17-20:3 (B) of Ophiuroidea Fatty Acids, on a CP-Sil 88 Capillary Column**

| | Standard isomers | | Hydrazine-reduced products | |
|---|---|---|---|---|
| | *cis* (*Z*) | *trans* (*E*) | From 7,13-20:2 | From 7,13,17-20:3 |
| 7-20:1 | 20.54[a] | — | 20.39 | 20.39 |
| 13-20:1 | 20.73[b] | — | 20.51 | 20.51 |
| 17-20:1 | 21.01[c] | 20.66[d] | — | 21.02 |

[a]Calculated from the analysis of 20:1 originated from flathead flounder fatty acids (14).
[b]Calculated from the analysis of commercially available authentic standard.
[c]Calculated from the analysis of partially hydrazine-reduced products of 20:5n-3.
[d]Calculated from the analysis of $NO_2$-isomerized products of *cis*-isomer.

In this study, fatty acids A–D were identified as 7*E*,13*E*-20:2 (A), 7*E*,13*E*,17*Z*-20:3 (B), 9*E*,15*E*,19*Z*-22:3 (C), and 4*Z*,9*E*, 15*E*,19*Z*-22:4 (D). These fatty acids belong to the family of NMI polyunsaturated fatty acids. NMI fatty acids and particularly C20 and C22 dienoic acids have been found in lipids of many marine invertebrates (19,20), e.g., chiton (21), gastropods (21–23), mussels (22), oysters (22,24), quahaug (22,25), sea urchin (26), starfish (22), sand shrimps (23), and crab (27). Takagi *et al.* (1) detailed fatty acid compositions of two species of Ophiuroidea, *Ophioplocus japonicus* and *Asteronyx loveni,* caught at the coast of Usujiri near Hakodate, Japan and at a depth of 200 m in the Okhotsk Sea, respectively. Typical NMI dienoic fatty acids, 5,11-20:2 and 5,13-20:2, and their chain extension products, 7,13-22:2 and 7,15-22:2, were found in total lipids of *O. japonicus* at concentrations of 1.67, 0.63, 0.18, and 0.21% of total fatty acids, respectively. In *A. loveni*, 5,11-20:2 and 5,13-20:2 were found at concentrations of 0.49 and 0.04%, respectively. However, there was no information on the occurrence of 7,13-20:2, 7,13,17-20:3, 9,15,19-22:3, and 4,9,15,19-22:4 in their report.

Ackman (28) listed $C_{16}$–$C_{30}$ NMI polyunsaturated fatty acids of miscellaneous aquatic organisms including seaweeds, sponges, and marine invertebrates. Gunstone (29) also listed $C_{18}$–$C_{22}$ NMI fatty acids of plants (particularly conifer seeds) and marine origin. When the lists were combined, $C_{20}$ and $C_{22}$ NMI fatty acids listed were 5,11-20:2, 5,13-20:2, 7,11-20:2, 7,13-20:2, 5,11,14-20:3, 7,11,14-20:3, 5,11,14,17-20:4, 5,11-22:2, 5,13-22:2, 7,13-22:2, 7,15-22:2, 7,17-22:2, 9,13-22:2, 9,15-22:2, and 7,13,16-22:3. Of these fatty acids, 7,13-20:2 corresponds to fatty acid A identified in the present study, although configurations of the olefinic bonds were not determined. This fatty acid was found in white shrimp *Penaeus setiferus* together with 7,11-20:2, 7,13-21:2, 7,13-22:2, 7,15-22:2, 9,13-22:2, 9,15-22:2, and 7,17-22:2 (30). To the best of the authors' knowledge, 7*E*,13*E*,17*Z*-20:3, 9*E*,15*E*,19*Z*-22:3, and 4*Z*,9*E*,15*E*,19*Z*-22:4 identified in the present study have not been reported in previous papers. The first-mentioned trienoic acids seem to be derived from 7*E*,13*E*-20:2 by insertion of an additional *cis*-olefinic bond at the 17 position and subsequent chain extension. The tetraenoic acid is probably formed from 9*E*,15*E*,19*Z*-22:3, although direct insertion of a *cis*-olefinic bond at the 4 position is obscure. Biosynthesis of 22:6n-3 proceeds by way of 7,10,13,16,19-22:5 → 9,12,15,18,21-24:5 → 6,9,12,15,18,21-24:6→ 4,7,10,13,16,19-22:6 (31).

**TABLE 2**
**Equivalent Chain Lengths of 22:1 Isomers, Produced from 9,15,19-22:3 (C) and 4,9,15,19-22:4 (D) of Ophiuroidea Fatty Acids, on a CP-Sil 88 Capillary Column**

| | Standard isomers | | Hydrazine-reduced products | |
|---|---|---|---|---|
| | *cis* (*Z*) | *trans* (*E*) | From 9,15, 19-22:3 | From 4,9,15, 19-22:4 |
| 4-22:1 | 22.40[a] | 22.24[b] | — | 22.40 |
| 9-22:1 | 22.49[c] | — | 22.34 | 22.33 |
| 15-22:1 | 22.67[c] | — | 22.48 | 22.48 |
| 19-22:1 | 22.96[a] | 22.62[b] | 22.95 | 22.97 |

[a]Calculated from the analysis of partially hydrazine-reduced products of 22:6n-3.
[b]Calculated from the analysis of $NO_2$-isomerized products of *cis*-isomer.
[c]Calculated from the analysis of 22:1 originated from flathead flounder fatty acids (14).

**TABLE 3**
**Fatty Acid Composition of the Ophiuroidea Lipids**

| Fatty acid | Wt% | Fatty acid | Wt% |
|---|---|---|---|
| 12:0 | 0.12 | 20:1n-11 + 20:1n-13 | 4.30 |
| iso-13:0 | 0.30 | 20:1n-9 | 0.51 |
| 13:0 | 0.08 | 20:1n-7 | 0.34 |
| iso-14:0 | 0.07 | 5,11-20:2 | 0.47 |
| anteiso-14:0 | 0.01 | 5,13-20:2 + 20:1n-5[a] | 0.30 |
| 14:0 | 3.81 | 7*E*,13*E*-20:2 (A)[b] | 2.22 |
| 14:1 | 0.23 | 20:2n-6 | 0.16 |
| iso-15:0 | 1.60 | 5,11,14-20:3 | 0.17 |
| anteiso-15:0 | 0.32 | 20:3n-6 | 0.09 |
| 15:0 | 0.65 | 21:0[a] | 0.25 |
| 15:1 | 0.12 | 7*E*,13*E*,17*Z*-20:3 (B)[b] | 12.66 |
| iso-16:0 | 0.16 | 20:4n-6 + 21:1[a] | 1.75 |
| anteiso-16:0 | 0.03 | 20:3n-3[a] | 0.12 |
| 16:0 | 6.60 | 5,11,14,17-20:4 | 0.05 |
| 16:1n-7 | 3.33 | 20:4n-3 | 0.07 |
| 16:1n-5 | 0.09 | 20:5n-3 | 4.48 |
| iso-17:0[a] | 0.97 | 22:0 | 0.08 |
| anteiso-17:0 | 0.18 | 22:1n-11 + 22:1n-13 | 0.60 |
| Phytanic[a] | 1.32 | 22:1n-9 | 0.59 |
| 17:0 | 0.57 | 22:1n-7 | 0.15 |
| 17:1 | 0.04 | 7,13-22:2 | 0.72 |
| 17:1 | 0.50 | 7,15-22:2 | 0.47 |
| iso-18:0 | 0.29 | 22:2n-6 | 0.36 |
| 18:0 | 3.10 | 21:5n-3 | 0.03 |
| 18:1n-13 | 1.31 | 22:3n-6 | 0.26 |
| 18:1n-9 | 13.13 | 23:0 | 0.07 |
| 18:1n-7 | 2.48 | 9*E*,15*E*,19*Z*-22:3 (C)[b] | 5.61 |
| 18:1n-5 | 0.23 | 22:4n-6 + 23:1n-9 | 0.48 |
| 18:2n-6 + anteiso-19:0[a] | 0.50 | 4*Z*,9*E*,15*E*,19*Z*-22:4 (D)[b] | 3.01 |
| 19:0 | 0.13 | 22:5n-6 | 0.35 |
| 19:1[a] | 0.65 | 22:4n-3 | 0.70 |
| 19:1 | 0.14 | 22:5n-3 | 0.23 |
| 19:1 | 0.09 | 24:0 | 0.05 |
| 18:3n-3 | 0.17 | 22:6n-3 | 1.50 |
| 18:4n-3 | 0.12 | 24:1n-9 | 1.06 |
| 18:4n-1 | 0.01 | 24:6n-3 | 5.88 |
| 20:0 | 0.12 | Unidentified[c] | 6.29 |

[a]Minor component of the peak.
[b]Fatty acid identified in the present study.
[c]Each of unidentified components was less than 0.5% of total fatty acids.

Fatty acid composition of the Ophiuroidea sample is shown in Table 3. Lipids of Ophiuroidea contain high levels of 24:6n-3. About 3–15% of 24:6n-3 was found in total fatty acids from all of the samples previously reported (1–3). The Ophiuroidea sample analyzed in the present study also contained 5.9% of 24:6n-3 in total fatty acids. The other sample caught at a depth of 258–642 m in Toyama Bay contained 11.2% of this acid. Takagi *et al.* (1) suggested that 24:6n-3 in Ophiuroidea does not originate from diets. In contrast, 7*E*,13*E*-20:2, 7*E*,13*E*,17*Z*-20:3, 9*E*,15*E*,19*Z*-22:3, and 4*Z*,9*E*, 15*E*,19*Z*-22:4 have not been observed in Ophiuroidea at a significant level except for the sample analyzed in this study. Unidentified minor components amounting to 6.3% of total fatty acids were also found in the present sample (Table 3). NMI fatty acids identified in this study or their precursors seem to be related to diets characteristic of each sample.

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# Regioisomers of Octanoic Acid–Containing Structured Triacylglycerols Analyzed by Tandem Mass Spectrometry Using Ammonia Negative Ion Chemical Ionization

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**ABSTRACT:** Tandem mass spectrometry based on ammonia negative ion chemical ionization and sample introduction *via* direct exposure probe was applied to analysis of regioisomeric structures of octanoic acid containing structured triacylglycerols (TAG) of type MML, MLM, MLL, and LML (M, medium-chain fatty acid; L, long-chain fatty acid). Collision-induced dissociation of deprotonated parent TAG with argon was used to produce daughter ion spectra with appropriate fragmentation patterns for structure determination. Fatty acids constituting the TAG molecule were identified according to $[RCO_2]^-$ ions in the daughter ion spectra. With the standard curve for ratios of $[M - H - RCO_2H - 100]^-$ ions corresponding to each $[RCO_2]^-$ ion, determined with known mixtures of *sn*-1/3 and *sn*-2 regioisomers of structured TAG, it was possible to determine the proportions of different regioisomers in unknown samples. The method enabled quantification of MML- and MLM-type structured TAG. In the case of MLL- and LML-type TAG, it was possible to determine the most abundant regioisomer in the unknown mixture and estimate the proportions of regioisomers when there were more than 50% MLL-type isomers in the mixture.

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There is a great interest in research and production of structured triacylglycerols (TAG) such as cocoa butter equivalents, human milk fat substitutes, or lipids with enhanced nutritional properties (1,2). Methods for analysis of structured lipids are important in production process development and quality control (1–4) and may also be of importance when detecting adulterations of commercially valuable fats and oils such as cocoa butter (5,6). Structured TAG containing short- or medium-chain fatty acids have special properties that are often desired in food, nutritional, or pharmacological applications. TAG possessing short- or medium-chain fatty acids in *sn*-1/3 positions and a long-chain fatty acid in the *sn*-2 position are rapidly hydrolyzed by pancreatic lipase. The resulting short- or medium-chain fatty acids are effectively absorbed into the portal blood as free fatty acids, and the long-chain fatty acid containing monoacylglycerol is efficiently absorbed into mucosal cells. Such TAG have been reported to be useful in the treatment of lipid malabsorption and also to have beneficial effects on immune function, nitrogen balance, and lipid clearance from the bloodstream (1). On the other hand, these short- or medium-chain fatty acid–containing structured TAG have special mass spectromeric properties, which have to be considered when analyzing such samples. Mass spectrometry with ammonia negative ion chemical ionization has been used for analysis of TAG molecular weight distribution and regioisomeric structure in various natural fats and oils (7–15). High-temperature gas chromatography (GC) with electron impact and chemical ionization mass spectrometry (16–18), high-performance liquid chromatography (HPLC) with chemical ionization mass spectrometry (19), and capillary supercritical fluid chromatography with atmospheric pressure chemical ionization mass spectrometry (20) have been applied to analysis of milk fat TAG that naturally contain short-chain fatty acids. These methods produce sufficient fragmentation for identifying the fatty acid constituents of TAG molecular weight species but no information concerning regiospecific structure of TAG. By fast atom bombardment tandem mass spectrometry, it has been possible to determine also the regiospecific structure of bovine udder TAG containing two short-chain fatty acids (21). However, there are no reports describing the tandem mass spectrometric characterizaton of mixed-acid structured TAG containing medium-chain fatty acids. In the present study, negative ion chemical ionization tandem mass spectrometry utilizing a direct exposure probe in sample introduction was applied to analysis of regioisomeric structures of octanoic acid containing structured TAG.

## MATERIALS AND METHODS

*Preparation of TAG standards.* The MLM- and MLL-type (M, medium-chain fatty acid; L, long-chain fatty acid) (8:0-18:2-8:0 and 8:0-18:2-18:2) TAG standards were prepared by purification of the interesterified products of safflower oil (Róco, Copenhagen, Denmark) and octanoic acid (Sigma Chemical Co., St. Louis, MO). The LML- and MML-type (18:2-8:0-18:2 and 8:0-8:0-18:2) TAG standards were prepared by purification of the interesterified products of trioctanoylglycerol and linoleic acid (both were from Sigma Chemical). In this study 8:0-18:2-18:2 (MLL) is used to denote the mixture of stereoisomers *sn*-8:0-18:2-18:2 + *sn*-18:2-18:2-8:0, and 8:0-8:0-18:2 (MML) denotes *sn*-8:0-8:0-18:2 + *sn*-18:2-8:0-8:0. Both products were synthesized in a small-scale packed-bed reactor using reaction parameters that were

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Abbreviations: ACN, acyl carbon number; ESI, electrospray ionization; FAB, fast atom bombardment; GC, gas chromatography; HPLC, high-performance liquid chromatography; M, medium-chain fatty acid; L, long-chain fatty acid; TAG, triacylglycerol.

reported previously (3). The required TAG standards were isolated from the interesterified products with preparative HPLC. A Waters Delta Prep 3000 HPLC (Millipore Corporation, Milford, MA) was equipped with a Delta-Pak C18 column (47 × 300 mm, particle size 15 μm and pore size 300 Å, Waters Corporation, Milford, MA). Lambda-Max model 481 LC spectrophotometer (Waters) was used as the detector at 210 nm. The column was maintained at ambient temperature with a flow rate of 60 mL/min (22). A binary solvent system was applied: Solvent A was acetonitrile and solvent B was isopropanol/hexane (2:1, vol/vol). All solvents were of HPLC grade (BDH Laboratory Supplies, Poole, England). The gradient of solvent was changed according to the composition of interesterified products. Two milliliters of the product solution with a concentration of 0.5 g/mL was injected, and the required structured TAG were collected.

To determine the purity of standard TAG, they were methylated to fatty acid methyl esters with 2 M methanolic KOH solution, and the fatty acid methyl esters were analyzed with a gas chromatograph (HP 6890; Hewlett-Packard, Waldbronn, Germany) equipped with a fused-silica capillary column (SP-2380, 60 m × 0.25 mm i.d., $d_f$ 0.2 μm; Supelco Inc., Bellefonte, PA). Oven temperature was programmed from 70 to 160°C at a rate of 15°C/min, then to 180°C at a rate of 1°C/min, further to 185°C at a rate of 0.5°C/min, and finally to 200°C at a rate of 20°C/min and held for 10 min (22). A flame-ionization detector was used at 280°C, and the injector temperature was 250°C. The injector was used in split mode with a ratio 1:20. Carrier gas was helium with a column flow of 2 mL/min. The fatty acid methyl esters were identified by comparing their retention times with authentic standards (Sigma), and the resulting compositions were calculated using the actual response factors for each fatty acid.

*Grignard degradation.* About 10 mg of the purified TAG standards were dissolved in 10 mL diethyl ether. The reaction started after adding 0.3 mL allylmagnesium bromide (1 M in diethyl ether) and lasted for 1 min, and thereafter was stopped by adding acid buffer (0.27 M HCl in 0.4 M boric acid). The organic phase was washed twice with 0.4 M boric acid, dried with anhydrous sodium sulfate, and evaporated under nitrogen. The lipid residues were separated on a thin-layer chromatography plate that was precoated with boric acid, and the 2-monoacylglycerol fraction was scraped off and extracted with diethylether. After methylation with 2 M KOH in methanol, the fatty acid methyl esters were analyzed by GC.

*Mass spectrometry.* Negative ion chemical ionization with ammonia (≥99.998%; Prax Air, Oevel, Belgium) was used to analyze the structured TAG. An aliquot of 0.5 μL of hexane solution (0.5 mg/mL) of TAG was applied on the rhenium wire of a direct exposure probe. After evaporation of the solvent, the probe was introduced into the ion source and the rhenium wire was heated at a rate of 40 mA/s to vaporize the sample. All the mass spectrometric analyses were performed using Finnigan MAT TSQ-700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a combined EI/CI ion source. The pressure of ammonia (8500 mtorr), the ion source temperature (200°C), the electron energy (70 eV), and the filament current (400 μA) were selected based on method optimization that was done earlier in our laboratory (11). Quadrupole 1 of the instrument was used to select the parent ion that was fragmented by colliding it with argon in quadrupole 2, while the fragments formed were monitored with quadrupole 3 by scanning *m/z* values from 100 to 800. To produce appropriate fragmentation, the pressure of argon (≥99.998%; AGA, Lidingö, Sweden) was set to 1.4 mtorr and the collision energy was 15 eV. Scans containing the TAG fragment ions were averaged and displayed. Each sample was analyzed four times, and averaged results with standard deviations are presented.

## RESULTS AND DISCUSSION

Table 1 lists the fatty acid composition of structured TAG standards used to characterize the fragmentation of different regioisomers in the tandem mass spectrometric analysis. The results were obtained by GC and Grignard degradation as described in the Materials and Methods section.

Figure 1 shows the fragmentation pattern of TAG standards (A, 8:0-18:2-8:0; B, 8:0-8:0-18:2; C, 18:2-8:0-18:2; D, 8:0-18:2-18:2) in tandem mass spectrometric analysis. The unfragmented parent TAG $[M - H]^-$ ion (A,B, *m/z* 605; C,D, *m/z* 741) was detected in all cases, as well as $[RCO_2]^-$ ions corresponding to fatty acids 8:0 (*m/z* 143) and 18:2 (*m/z* 279). Diacylglycerol fragment ions $[M - H - RCO_2H]^-$ formed by loss

**TABLE 1**
**The Main Fatty Acid Composition of Structured Triacylglycerol (TAG) Standards[a]**

| | 8:0-18:2-8:0 | | 8:0-18:2-18:2 | | 18:2-8:0-18:2 | | 8:0-8:0-18:2 | |
|---|---|---|---|---|---|---|---|---|
| Fatty acid | TAG | *sn*-2 | TAG | *sn*-2 | TAG | *sn*-2 | TAG | *sn*-2 |
| 8:0 | 67.2 | — | 35.7 | — | 30.8 | 93.7 | 62.7 | 95.0 |
| 14:0 | — | — | 0.1 | — | 0.1 | — | — | — |
| 16:0 | 0.1 | | 0.2 | — | 0.2 | — | — | — |
| 18:0 | — | — | 0.3 | — | 0.2 | — | — | — |
| 18:1n-9 | 0.1 | 0.1 | 0.3 | 0.8 | 4.3 | 0.6 | 0.3 | 0.2 |
| 18:2 | 32.4 | 99.3 | 63.3 | 99.2 | 58.0 | 4.6 | 36.2 | 3.5 |
| 18:3 | — | -— | — | — | 3.3 | — | 0.2 | — |
| Others | 0.2 | 0.6 | 0.1 | — | 3.1 | 1.1 | 0.6 | 1.3 |
| Total | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

[a]Results expressed as molar percentages of total fatty acids.

of either of the fatty acids were also detected in all cases. The loss of 8:0 produced $[M - H - 8:0]^-$ ions (Figs. 1A,B, *m/z* 461; Figs. 1C,D, *m/z* 597) and correspondingly the loss of 18:2 produced $[M - H - 18:2]^-$ ions (Figs. 1A,B, *m/z* 325; Figs. 1C,D, *m/z* 461). In addition, $[M - H - RCO_2H - 56]^-$ and $[M - H - RCO_2H - 100]^-$ ions, the structure of which remained unidentified, were detected in spectra of all standard TAG analyzed. In Figures 1A and 1B $[M - H - 8:0 - 56]^-$ and $[M - H - 8:0 - 100]^-$ fragment ions occur at *m/z* 405 and 361 and $[M - H - 18:2 - 56]^-$ and $[M - H - 18:2 - 100]^-$ ions at *m/z* 269 and 225, respectively. In Figures 1C and 1D, the corresponding fragment ions were detected at *m/z* 541 and 497 as well as at *m/z* 405 and 361.

The fragment ions $[RCO_2]^-$ and $[M - H - RCO_2H - 100]^-$

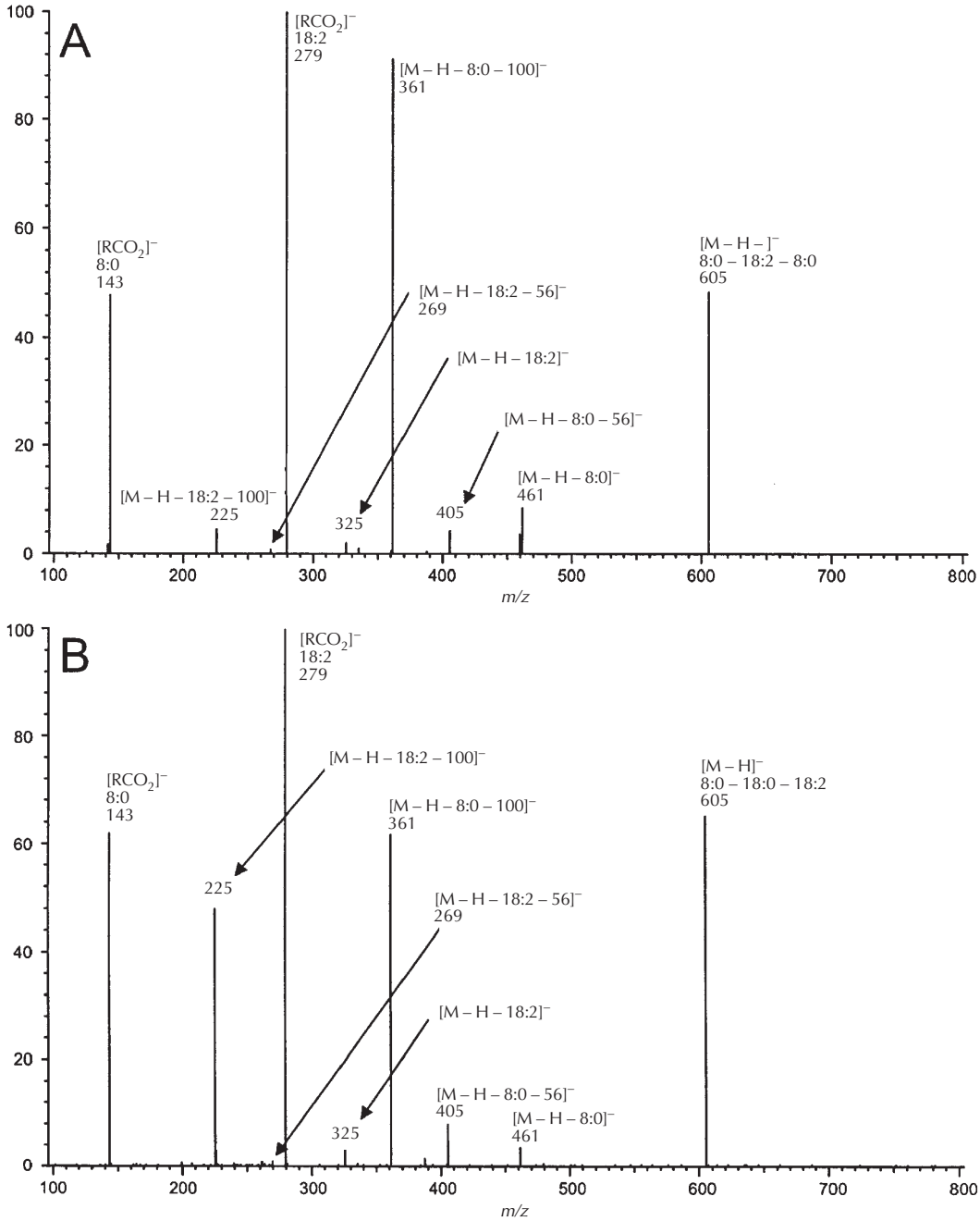


**FIG. 1.** Collision-induced dissociation spectra of MLM/MML (M, medium-chain fatty acid; L, long-chain fatty acid) type triacylglycerol (TAG) isomers 8:0-18:2-8:0 (A) and 8:0-8:0-18:2 (B) and the corresponding LML/MLL type isomers 18:2-8:0-18:2 (C) and 8:0-18:2-18:2 (D). The fragment ions are identified in the figure. In the MLM/MML type TAG $[M - H - 18:2 - 100]^-$ and $[M - H - 8:0 - 100]^-$ ions (*m/z* 225 and 361) are present in both spectra (A,B), but the ratio of the intensities of these ions increases by a factor of 18 when A is compared to B. This increase indicates the easier formation of $[M - H - 8:0 - 100]^-$ fragment ions when 8:0 is located in the primary positions in TAG molecule (A). In the LML/MLL type TAG $[M - H - 8:0 - 100]^-$ and $[M - H - 18:2 - 100]^-$ ions (*m/z* 497 and 361, respectively) are also present in both spectra (C,D). The ratio of intensities of these ions increases by a factor of 5 when C is compared to D. The result indicates that the fragmentation is less regiospecific in the case of LML/MLL type isomers than in the case of MLM/MML type TAG. See the Materials and Methods section for details of mass spectrometric analyses. (Continued)

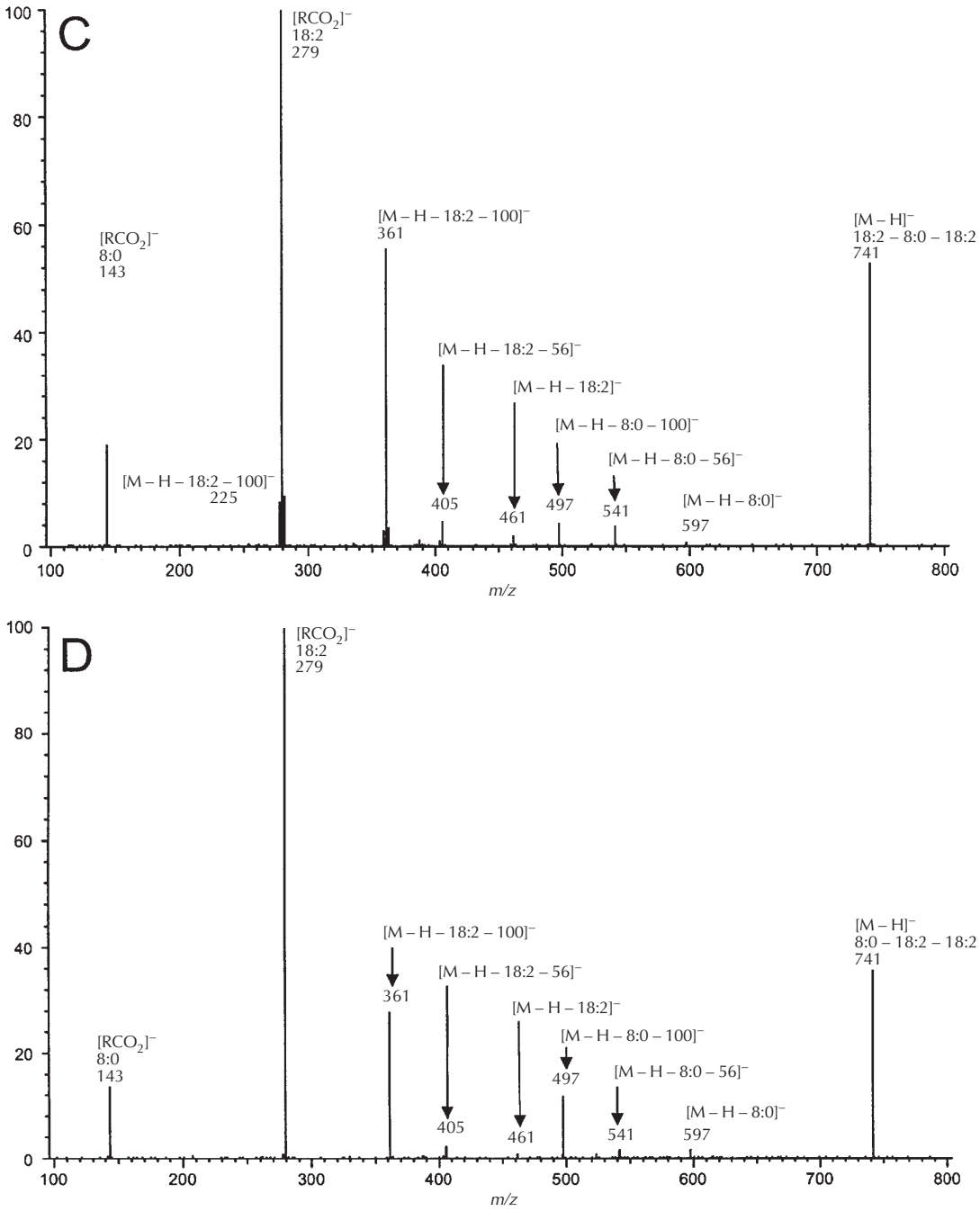


**FIG. 1.** (Continued)

were the most abundant and also gave the most information about the structure of the parent TAG as reported earlier in the case of long-chain fatty acid TAG (8,10). Theoretically, the intensities of $[RCO_2]^-$ ions would be expected to be proportional to the number of corresponding fatty acids in the parent TAG. As an example, in Figure 1A the ratio of $[RCO_2]$ ions of 8:0 (*m/z* 143) and 18:2 (*m/z* 279) would be expected to be 2:1, because there are two octanoic acid molecules and one linoleic acid molecule esterified to parent TAG. In the fragment ion spectrum of 8:0-18:2-8:0, the intensity of $[RCO_2]$ ions of 8:0 is lower than that of 18:2 (Fig. 1A). By analyzing a series of known structured TAG standards, it was calculated that the intensity of $[RCO_2]^-$ ions of 8:0 must be corrected by a factor of 3.0 to obtain the ratio of $[RCO_2]^-$ ions of 8:0 and 18:2 that corresponds to the number of fatty acids in parent TAG. By using this information, together with earlier determined correction factors for different fatty acids (9), it is possible to determine the fatty acid composition of an unknown 8:0-containing TAG molecule.

In the case of MLM- and MML-type structured TAG, the ratio of $[M - H - 18:2 - 100]^-$ and $[M - H - 8:0 - 100]^-$ ions was increased by a factor of 18 when 8:0-18:2-8:0 was compared with 18:2-8:0-8:0 (Figs. 1A,B). The ratio was further investigated by analyzing mixtures containing different molar

proportions of 8:0-18:2-8:0 and 18:2-8:0-8:0 (Fig. 2). The examination of the results showed a linear relationship ($y = 0.11x$, $R^2 = 0.93$) between the ratio of ion intensities and the molar proportion of 18:2-8:0-8:0 in the mixture (Fig. 2). This information enables the quantification of this type of regioisomers in an unknown mixture. When the same investigation of the ratio of $[M - H - 8:0 - 100]^-$ and $[M - H - 18:2 - 100]^-$ ions was performed with LML- and MLL-type structured TAG 18:2-8:0-18:2 and 8:0-18:2-18:2, a different result was obtained. Unexpectedly, the ratio of intensities of these ions increased only by a factor of five when 18:2-8:0-18:2 and 8:0-18:2-18:2 were compared (Figs. 1C,D). This result indicates that the fragmentation is less regiospecific in the case of LML-/MLL-type isomers than in the case of MLM-/MML-type triacylglycerols. The result of investigation of the ratios of $[M - H - 8:0 - 100]^-$ and $[M - H - 18:2 - 100]^-$ ions in mixtures containing different proportions of 18:2-8:0-18:2 and 8:0-18:2-18:2 is presented in Figure 3. The ratio is constant until the proportion of 8:0-18:2-18:2 in the mixture is about 50%, after which the ratio is increased with an increasing proportion of MLL-type isomer. This phenomenon makes the exact quantification of this type of isomers in an unknown mixture impossible. Nevertheless, the predominant isomer is possible to determine, and the proportion of isomers can be estimated providing that the proportion of 8:0-18:2-18:2 exceeds 50%.

The correction factors for the intensities of $[RCO_2]^-$ ions tend to increase with the increasing number of double bonds in the fatty acid and with the decreasing chain length of the fatty acid (9). Relative to 18:2, the correction factor for 8:0 was 3.0 in the present study, which is in accordance with the trend obtained for correction factors for different $[RCO_2]^-$ fragments in earlier studies (8,9). The lower abundance of short chain $[RCO_2]^-$ fragments may be postulated to be caused by the shielding effect of long-chain fatty acids in TAG or the lower probability of collision where the short-chain fatty acid fragment is formed. Instead of the fragmentation process, the lower abundance of unsaturated fatty acid fragments is more likely to be related to the different molecular response in mass spectrometric analysis.

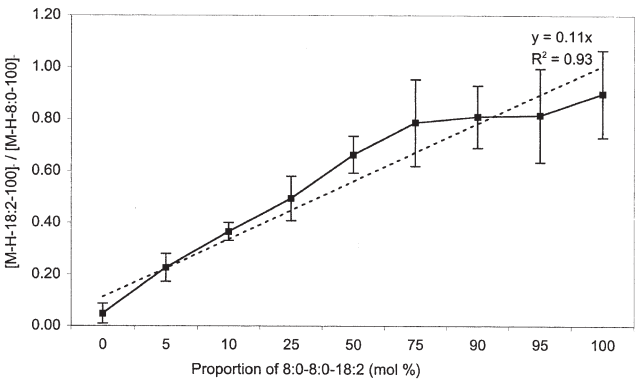


**FIG. 2.** The ratio of $[M - H - 18:2\text{-}100]^-/[M - H - 8:0 - 100]^-$ ions as a function of the molar proportion of 8:0-8:0-18:2 in the mixture of standard triacylglycerol isomers 8:0-8:0-18:2 (MML), and 8:0-18:2-8:0 (MLM) as determined on the basis of collision-induced dissociation spectra. An average and standard deviation of four replicate analyses are presented. The figure shows a linear relationship ($y = 0.11x$, $R^2 = 0.93$) between the ratio of ion intensities and the molar proportions of MLM-/MML-type TAG isomers in the mixture, which enables the quantification of isomers in an unknown mixture. For abbreviations see Figure 1.

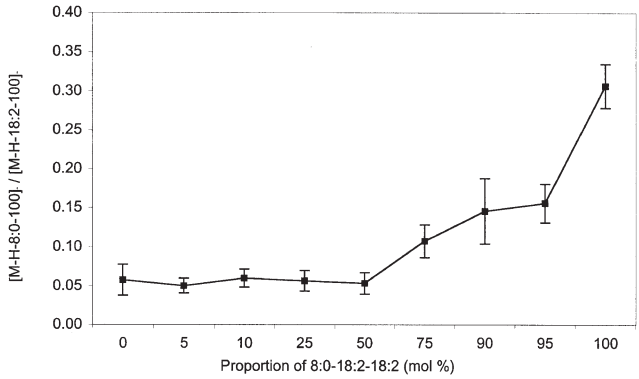


**FIG. 3.** The ratio of $[M - H - 8:0 - 100]^-/[M - H - 18:2 - 100]^-$ ions as a function of the molar proportion of 8:0-18:2-18:2 in the mixture of standard TAG isomers 8:0-18:2-18:2 (MLL) and 18:2-8:0-18:2 (LML) as determined on the basis of collision-induced dissociation spectra. An average and standard deviation of four replicate analyses are presented. The defined relationship between the ratio of ion intensities and proportions of LML-/MLL-type TAG in the mixture could not be found as in Figure 2, making the exact quantification of regioisomers impossible. Nevertheless, the predominant isomer can be determined and the proportions of isomers approximated if the proportion of MLL-type isomer exceeds 50%.

If the fragmentation obtained for medium-chain fatty acid TAG is compared with the fragmentation reported earlier for long-chain fatty acid TAG (7–10,12,13,15), some differences are found. As presented in Figures 2 and 3, the formation of $[M - H - RCO_2H - 100]^-$ ions is different to an extent that the calculation system for interpreting the results (10) cannot be applied if medium-chain fatty acids are present. Formation of $[M - H - RCO_2H - 74]^-$ ions was reported earlier parallel with the formation of $[M - H - RCO_2H - 100]^-$ ions in the case of long-chain fatty acid TAG (8,9). In the fragmentation of medium-chain fatty-acid–containing TAG, the $[M - H - RCO_2H - 56]^-$ ions instead of $[M - H - RCO_2H - 74]^-$ ions were observed as indicated in Figure 1. The formation of diacylglycerol fragment $[M - H - RCO_2H]^-$ ions was observed in both cases.

Previously, electrospray ionization (ESI)-produced $[M + NH_4]^+$ ions have been reported to produce $[M + NH_4 - R_nCOONH_4]^+$, $[R_nCO + 128]^+$, $[R_nCO + 74]^+$, and $R_nCO +$ fragment ions in high-energy collisional activation tandem mass spectrometry, but these fragments did not provide information about the position of acyl groups on the glycerol backbone (24). In the same study ESI- and fast atom bombardment (FAB)–produced $[M + Na]^+$ ions fragmented to eight types of ions, according to which the *sn*-1/3 vs. *sn*-2 position of acyl groups could also be determined. The fragmentation was concluded to proceed through charge-remote and charge-driven pathways, and proposed structures of product ions from $[M + Na]^+$ have been presented (24). A similar type of fragmentation pattern was obtained also when bovine udder TAG containing two short-chain fatty acids were analyzed (21). High-energy collision-induced dissociation of FAB-produced $[M + Na]^+$ ions resulted in

formation of fragment ions that allowed the determination of regiospecific positions of acyl groups according to known reference compounds (21). Negative ionization mass spectrometry has not been applied to analysis of TAG as widely as positive ionization mass spectrometry (25). In-source formation of $[RCO_2]^-$, $[RCO_2 - 18]^-$, and $[RCO_2 - 19]^-$ fragment ions has been reported when high-temperature GC was coupled to negative ion chemical ionization mass spectrometry (26). In the presence of 1% methylene choride in the mobile phase, $[M + Cl]^-$ ions were exclusively formed in HPLC negative ion chemical ionization mass spectrometry (19). The current method produces abundant $[M - H]^-$ ions of TAG under optimized conditions (11), and collision-induced dissociation of these ions produces simple product ion spectra containing informative fragments regarding the regiospecific structure of the parent TAG. Since the structure of the fragment ions $[M - H - RCO_2H - 100]^-$ and $[M - H - RCO_2H - 56]^-$ was not established in the present study, the fragmentation mechanism of $[M - H]^-$ ions produced by negative ion chemical ionization in low-energy collision-induced dissociation needs further investigation.

The method is applicable on regioisomeric structure analysis of simple mixtures of medium-chain fatty acids containing structured TAG provided that appropriate standard compounds are available. In all cases the exact quantification of all fatty acid combinations may not be possible as indicated by the data presented in this paper. Nevertheless, the method offers a considerable choice if a large number of samples need to be characterized because it requires very little sample preparation and is fast to perform, making it possible to analyze 50–100 samples per day.

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COMMUNICATION

# Dehydroepiandrosterone Alters Phospholipid Profiles in Zucker Rat Muscle Tissue

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**ABSTRACT:** Insulin-resistant muscle tissue contains low proportions of arachidonic acid (AA), and increased proportions of muscle AA correlate with improved insulin sensitivity. Dehydroepiandrosterone (DHEA) and AA, like the thiazolidinedione drugs that decrease insulin resistance (IR), are peroxisome proliferators. Long-chain fatty acids (FA) have been named the "one true" endogenous ligand for activating the peroxisome proliferator-activator receptor (PPAR), and DHEA has been named a "good candidate" as a naturally occurring indirect activator of PPAR. This study was conducted to determine DHEA's effects on lipid profiles of skeletal and cardiac muscle in lean and obese Zucker rats (ZR), a model of IR, type 2 diabetes mellitus, and obesity. We hypothesize that DHEA may alter long-chain FA profiles in muscle tissue of obese rats such that they more closely resemble that of the lean. In our experiments, we employed a DHEA and a pair-fed (PF) group ($n$ = 6) for 12 lean and 12 obese ZR. For 30 d, the diet of the two DHEA groups was supplemented with 0.6% DHEA; PF groups were given the average daily calories consumed by their corresponding treatment group. Hearts and gastrocnemius muscles were assayed for phospholipid (PL), free FA, and triglyceride (TG) FA profiles. The proportion of PL AA was significantly greater in both muscle types of lean compared to obese rats. Hearts from both DHEA groups had greater PL proportions of AA and less oleic (18:1) acid than their PF controls. Likewise, 18:1 proportions were significantly lower in the gastrocnemius; however, AA proportions were not significantly different. Similar phenotypic profile differences were observed in the TG fraction of both muscle types. There were no DHEA-related TG FA profile alterations.

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In Zucker rats (ZR), an animal model of youth-onset obesity and type 2 diabetes, dehydroepiandrosterone (DHEA) decreases dietary fat intake, body fat (1–4), adipocyte hyperplasia, and adipocyte hypertrophy (2,5). Recently, we demonstrated that DHEA treatment decreases serum free fatty acid (FFA) levels in lean and obese ZR (6).

DHEA treatment in rodents stimulates a dramatic increase in both the size and number of peroxisomes, inducing those catalyzing fatty acid (FA) β-oxidation (7,8). This may be initiated by activation of the peroxisome proliferator-activated receptors (PPAR-γ) (9–11) within nuclei of myocytes in both skeletal (red and white fibers) and cardiac muscle (12). Synthetic drugs that improve insulin sensitivity, "insulin sensitizers," act as PPAR-γ agonists in muscle and improve glucose utilization without stimulating insulin release.

Studies using ZR demonstrate that PPAR-γ agonists lower circulating triglyceride (TG) and FFA levels (13,14). In humans, these compounds enhance insulin sensitivity and improve glucose homeostasis in insulin-resistant individuals (15). Unfortunately, mechanisms by which PPAR-γ agonists regulate insulin sensitivity remain poorly defined.

DHEA has been termed a "good candidate" as a naturally occurring indirect activator of PPAR-γ (7). Polyunsaturated FA (PUFA) such as arachidonic acid (AA) have been termed "the one true endogenous ligand" for activating several PPAR classes (11,16–18). We have demonstrated that DHEA treatment in the ZR significantly increases hepatic tissue proportions of AA and other PUFA (6). Perhaps a mechanism by which DHEA functions is related to its effects on FA profiles. Because increased PUFA in skeletal muscle phospholipid (PL) have been correlated with an improved insulin sensitivity (19), perhaps FA flux in muscle represents crucial factors that govern the development of insulin resistance (IR).

Our goal in this study is to determine DHEA-related fractional lipid changes in PL, FFA, and TG FA profiles of skeletal and cardiac muscle of lean and obese ZR. Although this study does not measure IR, the identification of DHEA-induced changes in lipid pools may point to a mechanism for a DHEA role in decreasing IR.

## MATERIALS AND METHODS

The IACUS and IRB of the Louisiana State University Health Center approved the methodology of this study. There was a DHEA and a pair-fed (PF) group ($n$ = 6) for 12 lean and 12 obese ZR. For 30 d, the two DHEA groups' diet was supplemented with 0.6% DHEA. PF groups were given the average daily calories consumed by their corresponding treatment group. A specialized macronutrient diet, as described earlier (3), was modified such that the proportions of energy as carbohydrate, protein, and fat were 30, 20, and 50%, respectively. Complete descriptions of the diet, feeding, and ZR are published elsewhere (6).

After 30 d of DHEA treatment or pair-feeding, animals were fasted for 14 h and sacrificed by rapid decapitation.

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Abbreviations: AA, arachidonic acid; DHEA, dehydroepiandrosterone; FA, fatty acid; FFA, free fatty acid; IR, insulin resistance; LD, lean DHEA; LPF, lean pair-fed; MUFA, monounsaturated fatty acid; OD, obese DHEA; OPF, obese pair-fed; PF, pair-fed; PL, phospholipid; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated FA; TG, triglyceride; ZR, Zucker rat.

Their left gastrocnemius and hearts were excised, weighed, frozen in liquid nitrogen, and stored at −80°C until assayed for PL, free FA, and TG FA profiles *via* gas–liquid chromatography as previously described (6,20). Insulin levels were determined in duplicate using a rat insulin radioimmunoassay kit (Linco Research, Inc., St. Louis, MO).

*Statistical analysis.* Experimental measurements were examined by one-way analysis of variance on a Power Macintosh 7600/120 Superanova program. Significance for $P \leq 0.05$ was measured using Fisher's Protected Least Squares Difference, giving a *P*-value for each comparison.

**TABLE 1**
**Gastrocnemius and Heart Weights and Serum Insulin Levels at the Time of Sacrifice[a]**

| | OPF | ODHEA | LPF | LDHEA |
|---|---|---|---|---|
| Gastrocnemius (g) | 0.94 ± 0.1 a | 0.93 ± 0.1 a | 1.28 ± 0.2 b | 1.26 ± 0.2 b |
| Heart (g) | 0.89 ± 0.2 a | 0.87 ± 0.2 a | 0.83 ± 0.2 b | 0.84 ± 0.2 a |
| Insulin (ng/mL) | **4.2 ± 0.4 a** | **3.1 ± 0.2 b** | 1.4 ± 0.2 c | 1.2 ± 0.3 c |

[a]Mean ± standard error of the mean (SEM) for each group: obese pair-fed (OPF), obese dehydroepiandrosterone (ODHEA), lean pair-fed (LPF), and lean DHEA (LDHEA). Values in rows that do not share the same letter are significantly different ($P \leq 0.05$). DHEA effects are typed in bold.

## RESULTS

For all tables, values are presented as means ± standard error of the mean. Rows not sharing the same letter are significantly different ($P \leq 0.05$). Values in columns are not statistically compared. Group abbreviations are as follows: obese pair-fed (OPF), obese DHEA (OD), lean pair-fed (LPF), and lean DHEA (LD).

Table 1 presents left gastrocnemius and heart weights at the time of sacrifice and serum insulin levels. Body weights are recorded elsewhere (6) and were not different within phenotype groups; however, by day 14, both lean and obese DHEA groups weighed significantly less than their PF controls. While the lean gastrocnemius weighed significantly more than the obese, there were no DHEA-treatment differences in the weights of either muscle. Obese control insulin levels were significantly greater than lean. Insulin levels were significantly lower in obese but not lean DHEA-treated rats.

Tables 2 and 3 contain FA profiles for the left gastrocnemius and hearts of each group. For each profile, sums are given for saturated FA, monounsaturated fatty acids (MUFA), and PUFA. The sum of all identified FA (values not shown) was never less than 95% of total.

PL FA profiles of the hearts are recorded in Table 2. The proportion of AA was significantly greater in lean compared to obese controls, and the proportion of oleic acid (18:1) was significantly lower in lean than in obese controls. Hearts of both DHEA groups had greater proportions of AA and less oleic acid than their PF controls. These changes resulted in significantly lower proportions of MUFA and higher proportions of PUFA in OD vs. OPF groups.

PL FA profiles for gastrocnemius muscles are recorded in

**TABLE 2**
**Phospholipid Fatty Acid Profiles of Heart and Gastrocnemius Muscle[a]**

| | OPF | | ODHEA | | LPF | | LDHEA | |
|---|---|---|---|---|---|---|---|---|
| Fatty acid | Heart | Gastrocnemius | Heart | Gastrocnemius | Heart | Gastrocnemius | Heart | Gastrocnemius |
| 12:0 | 0.0 | 0.1 a | 0.0 | 0.1a | 0.0 | 0.1 a | 0.0 | 0.1 a |
| 14:0 | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a |
| 14:1 | 1.5 ± 0.2 a | 0.0 | 1.6 ± 0.2 a | 0.0 | 1.4 ± 0.1 a | 0.0 | 1.5 ± 0.2 a | 0.0 |
| 16:0 | 11.2 ± 0.8 a | 17.0 ± 1.2 a | 11.0 ± 1.2 a | 17.0 ± 0.8 a | 10.0 ± 1.0 a | 16.0 ± 0.9 a | 9.3 ± 0.7 a | 16.0 ± 1.0 a |
| 16:1 | 0.2 a | 0.6 ± 0.1 a | 0.2 a | 0.7 ± 0.2 a | 0.2 a | 0.5 ± 0.2 a | 0.2 a | 0.6 ± 0.2 a |
| 18:0 | 21.0 ± 0.7 a | 13.4 ± 0.7 a | 20.1 ± 0.9 a | 14.0 ± 0.7 a | 19.6 ± 1.2 a | 13.0 ± 0.8 a | 18.9 ± 1.1 a | 13.1 ± 0.8 a |
| 18:1 | **13.0 ± 0.8 a** | **10.0 ± 0.8 a** | **9.0 ± 9.5 b** | **8.6 ± 0.8 b** | **8.1 ± 0.7 b,c** | **9.0 ± 0.9 b** | **6.9 ± 0.5 d** | **11.1 ± 0.7 a** |
| 18:2 | 10.1 ± 0.8 a | 16.4 ± 0.7 a | 10.2 ± 0.7 a | 17.0 ± 0.7 a | 11.3 ± 0.9 a | 14.0 ± 1.0 b | 10.8 ± 1.1 a | 15.0 ± 1.2 b |
| 20:0 | 0.3 ± 0.1 a | 0.2 a | 0.3 ± 0.1 a | 0.2 a | 0.2 ± 0.1 a | 0.2 a | 0.3 ± 0.1 a | 0.2 a |
| 18:3 | 0.0 | 0.2 a | 0.0 | 0.2 a | 0.0 | 0.2 a | 0.0 | 0.2 a |
| 20:1 | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a |
| 20:2 | 0.3 ± 0.1 a | 0.4 ± 0.1 a | 0.3 ± 0.1 a | 0.4 ± 0.1 a | 0.3 ± 0.1 a | 0.6 ± 0.2 a | 0.4 ± 0.1 a | 0.5 ± 0.1 a |
| 20:3 | 0.7 ± 0.1 a | 0.9 ± 0.1 a | 0.8 ± 0.1 a | 1.0 ± 0.1 a | 0.7 ± 0.1 a | 1.0 ± 0.2 a | 0.8 ± 0.1 a | 0.9 ± 0.1 a |
| 22:0 | 0.5 ± 0.1 a | 0.4 ± 0.1 a | 0.7 ± 0.1 a | 0.3 ± 0.1 a | 0.6 ± 0.2 a | 0.4 ± 0.1 a | 0.4 ± 0.2 a | 0.3 ± 0.1 a |
| 20:4 | **18.0 ± 0.8 a** | 11.4 ± 0.5 a | **23.0 ± 1.1 b** | 12.9 ± 1.0 a | **23.1 ± 1.1 b** | 14.3 ± 1.0 b | **27.0 ± 0.8 c** | 15.3 ± 0.9 b |
| 22:1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 20:5 | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a |
| 22:4 | 0.5 ± 0.1 a | 0.6 ± 0.1 a | 0.4 ± 0.1 a | 0.7 ± 0.1 a | 0.4 ± 0.1 a | 0.7 ± 0.2 a | 0.5 ± 0.1 a | 0.7 ± 0.1 a |
| 24:1 | 0.4 ± 0.1 a | 0.4 ± 0.1 a | 0.4 ± 0.1 a | 0.4 ± 0.1 a | 0.6 ± 0.2 a | 0.6 ± 0.2 a | 0.5 ± 0.1 a | 0.5 ± 0.1 a |
| 22:5 | 2.3 ± 0.3 a | 2.9 ± 0.5 a | 2.2 ± 0.2 a | 3.6 ± 0.5 a | 2.0 ± 0.2 a | 2.4 ± 0.3 a | 2.0 ± 0.2 a | 3.2 ± 0.4 a |
| 22:6 | 17.3 ± 1.0 a | 19.9 ± 1.6 a | 16.4 ± 0.8 a | 18.6 ± 1.4 a | 17.8 ± 1.2 a | 19.1 ± 1.6 a | 16.3 ± 1.1 a | 21.1 ± 1.2 a |
| SFA | 33.0 ± 1.2 a | 31.9 ± 1.3 a | 32.2 ± 1.4 a | 32.4 ± 0.9 a | 31.0 ± 2.1 a | 30.6 ± 0.7 a | 30.0 ± 1.6 a | 29.6 ± 0.7 a |
| MUFA | **15.2 ± 0.8 a** | 11.2 ± 0.9 a | **11.3 ± 0.7 b** | 1.0 ± 0.8 a | 10.2 ± 0.8 b,c | 12.2 ± 0.7 a | 9.0 ± 1.0 c | 9.5 ± 1.1 a |
| PUFA | **48.9 ± 1.1 a** | 52.1 ± 1.5 a | **53.4 ± 0.9 b** | 52.8 ± 1.9 a | 55.3 ± 2.9 b,c | 53.0 ± 1.2 a | 56.7 ± 1.0 c | 55.7 ± 1.5 a |

[a]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; for other abbreviations and other explanations, see Table 1.

**TABLE 3**
**Triglyceride Fatty Acid Profiles for Heart and Gastrocnemius Muscle[a]**

| | OPF | | ODHEA | | LPF | | LDHEA | |
|---|---|---|---|---|---|---|---|---|
| Fatty acid | Heart | Gastrocnemius | Heart | Gastrocnemius | Heart | Gastrocnemius | Heart | Gastrocnemius |
| 12:0 | 0.1 a | 0.1 a | 0.1 a | 0.1 a | 0.1 a | 0.1 a | 0.1 a | 0.1 a |
| 14:0 | 1.2 ± 0.7 a | 1.2 ± 0.1 a | 1.2 ± 0.7 a | 1.1 ± 0.1 a | 0.8 ± 0.2 a | 1.2 ± 0.7 a | 0.8 ± 0.2 a | 1.5 ± 0.4 a |
| 14:1 | 0.4 a | 0.3 a | 0.4 a | 0.3 a | 0.4 a | 0.3 a | 0.4 a | 0.3 a |
| 16:0 | 24.7 ± 0.9 a | 22.2 ± 1.1 a | 22.9 ± 1.1 a | 22.5 ± 1.1 a | 15.1 ± 0.9 b | 17.1 ± 0.9 b | 14.5 ± 1.5 b | 16.1 ± 1.1 b |
| 16:1 | 4.1 ± 0.8 a | 6.1 ± 0.4 a | 3.2 ± 0.6 a | 5.9 ± 0.3 a | 0.6 ± 0.1 a | 2.1 ± 0.5 b | 0.8 ± 0.2 a | 2.0 ± 0.4 b |
| 18:0 | 6.6 ± 0.4 a | 5.3 ± 0.5 a | 6.3 ± 0.4 a | 5.2 ± 0.3 a | 7.1 ± 0.8 a | 5.7 ± 0.7 a | 7.2 ± 0.5 a | 6.2 ± 0.8 a |
| 18:1 | 34.4 ± 1.4 a | 37.8 ± 1.0 a | 37.3 ± 1.2 a | 37.8 ± 1.2 a | 32.4 ± 1.5 a | 36.3 ± 2.2 a | 35.9 ± 3.0 a | 35.1 ± 2.4 a |
| 18:2 | 14.3 ± 0.3 a | 14.8 ± 0.3 a | 14.4 ± 0.4 a | 14.9 ± 0.4 a | 27.9 ± 1.2 b | 23.3 ± 2.3 b | 25.5 ± 1.2 b | 21.6 ± 1.8 b |
| 20:0 | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a |
| 18:3 | 0.6 ± 0.1 a | 0.8 ± 0.1 a | 0.6 ± 0.1 a | 0.8 ± 0.1 a | 0.7 ± 0.2 a | 0.8 ± 0.1 a | 0.8 ± 0.2 a | 1.0 ± 0.2 a |
| 20:1 | 0.4 a | 0.3 a | 0.4 a | 0.3 a | 0.4 a | 0.3 a | 0.4 a | 0.3 a |
| 20:2 | 1.5 ± 0.5 a | 1.2 ± 0.5 a | 1.7 ± 0.2 a | 1.2 ± 0.2 a | 1.8 ± 0.6 a | 1.3 ± 0.2 a | 1.7 ± 0.3 a | 1.4 ± 0.3 a |
| 20:3 | 1.6 ± 0.3 a | 0.6 ± 0.1 a | 1.5 ± 0.2 a | 0.6 ± 0.1 a | 1.2 ± 0.7 a | 0.7 ± 0.2 a | 1.1 ± 0.2 a | 0.9 ± 0.2 a |
| 22:0 | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a | 0.2 a | 0.1 a |
| 20:4 | 2.7 ± 0.4 a | 0.9 ± 0.1 a | 2.7 ± 0.9 a | 0.8 ± 0.1 a | 2.6 ± 0.3 a | 2.1 ± 0.4 b | 2.8 ± 0.8 a | 2.5 ± 0.6 b |
| 22:1 | 0.1 a | 0.0 | 0.1 a | 0.0 | 0.1 a | 0.0 | 0.1 a | 0.0 |
| 20:5 | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a | 0.2 a |
| 22:4 | 0.3 ± 0.1 a | 0.6 ± 0.1 a | 0.3 ± 0.1 a | 0.4 ± 0.1 a | 0.1 ± 0.1 a | 0.5 ± 0.2 a | 0.1 ± 0.7 a | 0.7 ± 0.3 a |
| 24:1 | 0.4 ± 0.1 a | 0.3 a | 0.5 ± 0.1 a | 0.3 a | 0.5 ± 0.2 a | 0.3 a | 0.5 ± 0.2 a | 0.3 a |
| 22:5 | 0.9 ± 0.1 a | 1.3 ± 0.3 a | 1.1 ± 0.2 a | 1.4 ± 0.2 a | 0.6 ± 0.1 b | 1.1 ± 0.3 a | 0.6 ± 0.1 b | 1.1 ± 0.3 a |
| 22:6 | 1.4 ± 0.3 a | 2.0 ± 0.4 a | 1.6 ± 0.7 a | 2.1 ± 0.3 a | 1.6 ± 0.2 a | 1.4 ± 0.4 a | 1.7 ± 0.3 a | 1.5 ± 0.4 a |
| SFA | 33.3 ± 0.8 a | 29.0 ± 1.4 a | 31.3 ± 1.2 a | 28.4 ± 0.8 a | 23.8 ± 1.0 b | 24.9 ± 0.8 b | 23.3 ± 1.2 b | 24.9 ± 0.7 b |
| MUFA | 39.8 ± 1.2 a | 44.7 ± 1.8 a | 41.8 ± 2.0 a | 44.7 ± 1.0 a | 36.3 ± 2.1 b | 39.3 ± 2.09 a,b | 38.0 ± 2.4 a,b | 38.3 ± 1.9 b |
| PUFA | 23.3 ± 0.9 a | 23.2 ± 1.2 a | 23.8 ± 1.5 a | 23.2 ± 1.1 a | 36.7 ± 0.7 b | 31.9 ± 2.5 b | 34.4 ± 2.3 b | 32.5 ± 2.2 b |

[a]For abbreviations and other explanations see Tables 1 and 2.

Table 2. AA was proportionally greater in lean compared to obese controls. Proportions of oleic acid were significantly decreased in OD and LD groups when compared to their controls; values for OD and LD controls were not different.

Table 3 presents the TG FA profiles for the hearts. The proportions of palmitic acid (16:0), palmitoleic acid (16:1), saturated FA, and MUFA were significantly lower in lean compared to obese controls. Proportions of linoleic acid (18:2) and PUFA were greater in lean compared to obese controls. DHEA treatment did not alter TG FA profiles in either muscle type.

Table 3 also presents the TG FA profiles for gastrocnemius muscles. The same phenotypic profile differences that are observed in hearts are also seen in the gastrocnemius with an additional significantly greater proportion of AA in the LPF compared to the OPF group. DHEA treatment did not alter TG FA profiles in either muscle type.

## DISCUSSION

In agreement with our results, other investigators have shown that hearts of obese ZR contain significantly lower proportions of AA than lean ZR (21).

Increased proportions of PL FA in skeletal muscle PL has been correlated with improved insulin sensitivity, whereas lower proportions, specifically AA, correlate with IR development (22). Perhaps beneficial effects of DHEA in obese ZR are related to DHEA-specific PL FA profile alterations in skeletal muscle that result in lower serum insulin levels.

Insulin sensitizers, such as the thiazolidinediones, improve skeletal muscle IR *via* a mechanism that involves activation of myocyte PPAR-γ (12). Skeletal muscle PPAR-γ expression is higher in IR patients (23). PPAR-γ levels are initially low in IR tissue but become significantly elevated in muscle during treatment with a peroxisome proliferating agent (24). Because DHEA is also a peroxisome proliferator, it may alter tissue FA *via* a similar mechanism.

This study demonstrates that DHEA treatment alters PL FA profile in ZR muscle tissue. The proportions of muscle PL FA, such as AA, "the one true endogenous ligand" for activating several PPAR classes (11,16–18), may be increased by DHEA, a "good candidate" for a naturally occurring indirect activator of PPAR-γ (7). We therefore hypothesized that DHEA may indirectly decrease IR by altering FA profiles of muscle tissue *via* PPAR activation.

This study demonstrates that the proportion of heart PL AA increases significantly with a corresponding decrease in oleic acid in both lean and obese ZR after 30 d of DHEA treatment. Similar oleic acid changes are seen in the gastrocnemius. AA is proportionally increased in the gastrocnemius; however, these increases did not reach statistical significance as they did in the hearts.

This and other studies show that obese ZR have lower proportions of muscle PUFA than lean ZR. Furthermore, our study implicates the PL fraction as the location of this difference. Increased proportions of muscle PL AA correlates with improved insulin sensitivity, and ZR treated with DHEA for 30 d had increased muscle proportions of PL PUFA. Because both DHEA and PUFA are known PPAR-γ activators, this

study may prove to be an important step to understanding the development of IR in muscle tissue.

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LETTER TO THE EDITOR

# Chromatographic Methods for Ceramide Identification

Sir:

In regard to a recent review in *Lipids* about methods for identification and quantification of ceramides (1), we would like to present some additional comments on methods of molecular species analysis of ceramides. The study of chromatographic systems in order to develop tools for ceramide identification is one of the themes of our laboratory. The ceramide class of lipids is highly heterogeneous in structure, i.e., the polar head varies with the nature of the sphingoid base, and two alkyl chains can have a variable number of carbon atoms and varying degrees of unsaturation.

The main idea of our work was to study the retention behavior of these lipids in various chromatographic systems and then model the relationship between their retention and their structure in order to both identify peaks and/or to predict the retention of an unknown structure. This approach was developed because the large number of ceramide structures and the lack of commercially available standards frequently prevent identification by comparison of retention times to those of known standards. Toward this goal, three systems were studied and are briefly discussed here. The development of chromatographic methods was a high priority since these techniques could be applicable in routine analysis for biological samples, and we attempted to construct a simple model of the retention-structure relationship as a linear retention chart diagram (RCD).

First, reversed-phase (RP) liquid chromatography was studied with an octadecyl grafted silica column. The optimized conditions were found in gradient elution from acetonitrile/tetrahydrofuran 95:5 to acetonitrile/tetrahydrofuran/propanol-1 65:35:5 in 30 min (2), where the criteria were the best separation with a high correlation between retention and increased numbers of methylene units. Thereby, a linear model was derived between the retention and the structure, which constitutes our tool for ceramide identification. The basis of this model was knowledge of the retention behavior. In order to accomplish this, structurally well-defined samples were needed. Gas chromatography coupled with mass spectrometric analysis was thus performed in parallel with RP–high-performance liquid chromatography, which allowed direct structural identification (3). Next, a more hydrophobic stationary phase was investigated: Hypercarb® columns (100 × 2.1 mm i.d.; Hypersil, Runcorn, United Kingdom) (4) led to a significantly improved separation with a gradient elution of chloroform/methanol 45:55 to 85:15 in 15 min. This method was also modeled in a linear RCD. Finally, the third linear RCD came from a system with an octadecyl grafted silica stationary phase and a supercritical mobile phase. The optimal condition was 6% of methanol in $CO_2$ at 130 MPa and 31°C (5). Although this chromatographic apparatus is less commonly used, it provided powerful ceramide separation in isocratic conditions with hazardless solvent.

Optimizing the detection was an essential point in our approach, to detect and therefore identify trace amounts of sample. A detection technique suitable for lipid analysis is evaporative light-scattering detection (ELSD), but one of its drawbacks is its low detection limit. We have shown that adding 0.1% vol/vol of triethylamine along with a chemically equivalent amount of formic acid to the mobile phase greatly increased the response of the detector to ceramides (6). Therefore, a limit of detection of 5 ng injected was reached. The response of this detector is, unfortunately, not 1inear, which complicates quantitative analysis. We then studied fluorescence detection without derivatization by adding a molecule in the postcolumn that fluoresces in a hydrophobic environment. The same limit of detection was achieved as with ELSD, and a linear response was obtained between 0 to 1000 ng vs. only two orders of magnitude with ELSD (7).

In conclusion, our different RCD were finally compared and assessed on the basis of their precision and exactitude to provide an identification result. We have concluded that a single model was not sufficient to provide an unambiguous result. The ambiguities, however, can be overcome by combining results from two RCD.

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Abbreviations: ELSD, evaporative light-scattering detection; RCD, retention chart diagram; RP, reversed phase.

# INTRODUCTION

# Vascular Disease and Nutrition

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Women who eat fish five or more times per week have a markedly reduced probability (relative risk 72% compared with a control cohort) of suffering a cerebral stroke. This is the salient finding in the latest analysis of the Nurses' Health Study, which followed 79,839 nurses in the United States in the years 1980 through 1994 (1). The observation adds another piece to the evidence that nutritional intervention can greatly influence the incidence and natural history of disease.

The influence of nutritional factors on disease, in particular, vascular disease, is the thread that connects a series of five scientific workshops organized by the editors of this supplement during the past decade. The first of these workshops, Fish Oil and Vascular Disease, was held in Pisa, Italy, in 1991 within the framework of the Annual Meeting of the European Society of Clinical Investigation (as were all subsequent workshops); follow-up meetings took place in Heidelberg, Germany, in 1993; in Cambridge, England, in 1995; and in Crakow, Poland, in 1998. The most recent workshop, Prevention and Treatment of Vascular Disease, was held in Aarhus, Denmark, in May 2000.

For three of the previous workshops, the proceedings have been published as books by Springer Academic Publishing, New York: De Caterina *et al.; Fish Oil and Vascular Disease,* 1992; De Caterina *et al., n-3 Fatty Acids and Vascular Disease*, 1993; and Kristensen *et al., n-3 Fatty Acids: Prevention and Treatment in Vascular Disease*, 1995.

Following the tradition of these publications, and in line with the rapid growth and influence of this field of therapy and prevention, the editors are very pleased to present this supplement: *Prevention and Treatment of Vascular Disease—A Nutrition-Based Approach*. The publication brings together leading experts on three aspects of the theme: (i) homocysteinemia, now acknowledged as an independent risk factor for vascular disease; (ii) the large and heterogeneous area of antioxidant therapy; and (iii) the area of n-3 polyunsaturated fatty acids.

In Part I, Falk *et al.* give an overview of the pathophysiology of homocysteine and atherothrombosis; Cattaneo then summarizes the clinical studies that have established hyperhomocysteinemia as an independent risk factor for cardiovascular disease. Malinow *et al.* examine the effect of folic acid supplementation on plasma homocysteine concentrations; and Ueland *et al.* present findings of the Hordaland Homocysteine study, a large (18,043 men and women) epidemiologic study confirming the association of homocysteine with cardiovascular risk factors and identifying a new association with definite complications of pregnancy.

Part II of the supplement, devoted to antioxidants and the Mediterranean diet, is introduced by Iuliano, with a presentation of the oxidant stress hypothesis of atherogenesis. Praticò then describes methods for the *in vivo* measurement of the redox state. Visioli and Galli examine the role of antioxidants in the Mediterranean diet. A negative conclusion, the absence of a relevant clinical effect of vitamin E on the risk of cardiovascular events, was drawn from the results from the antioxidant treatment groups of the GISSI-Prevenzione and the HOPE trials, presented by Marchioli.

The third and largest part of the supplement addresses n-3 polyunsaturated fatty acids, starting with an overview of this lipid class by Schmidt *et al.* De Caterina and Zampolli summarize the evidence for antiatherosclerotic effects. Kristensen *et al.* then review the effects of n-3 fatty acids on coronary thrombosis. Strategies for public health policy based on n-3 fatty acid studies are defined by Simopoulos. Griffin then summarizes findings on the effect of n-3 fatty acids on LDL subfractions. von Schacky *et al.* and Arnesen present intervention studies in patients with coronary artery disease. The topic of n-3 fatty acids and cardiac arrythmias, which has drawn particular attention over the past few years, is addressed by the papers of Leaf, McLennan, and Hagstrup. Finally, the findings of the GISSI-Prevenzione trial, which, in 1999, proved a reduction in coronary events by the long-term intake of 1 g of n-3 fatty acids, are presented by Marchioli, the study's principal investigator. In a concluding roundtable session, summarized by Nordøy, the indication, doses, and preparations of n-3 fatty acids are discussed.

The editors are proud to have brought together clinical and scientific experts on these three pillars of nutrition-based therapy and prevention of cardiovascular disease. The editors are grateful to the individual authors for the diligent and up-to-date papers they provided. Particular thanks are extended to the sponsors who made the workshop and the publication of this supplement possible: Pronova Healthcare, Denmark; Pronova Biocare, Norway; Pharmacia-Upjohn and SPA-Società Prodotti Antibiotici, Italy. Additional support came from Roche Vitamins, Switzerland, and BASF, Germany.

Overall, the field of nutritional intervention for vascular disease has grown over the past two decades from prudent, epidemiology-based hypotheses to *in vitro* mechanistic and animal (*in vivo*) pathophysiologic studies. These were

followed by a number of small, phase II clinical studies that have strengthened some hypotheses and rejected others. Finally, during the past seven years, large phase III intervention studies have identified patient groups that definitely profit from nutritional intervention, in particular that based on n-3 polyunsaturated fatty acids. A recent *in vivo* animal study (2) strikingly illustrates how long in life a transient perinatal n-3 fatty acid deficiency can affect the vascular system.

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# Homocysteine and Atherothrombosis

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**ABSTRACT:** Atherosclerosis with or without thrombosis superimposed is the most frequent cause of ischemic heart disease (IHD), peripheral arterial disease, and a main cause of stroke. Conflicting results have been reported in genetic, observational, and experimental studies on the relationship between homocysteine and these atherothrombotic diseases. Although cardiovascular complications are common in homocystinuric patients (severe hyperhomocysteinemia), IHD, the most frequent manifestation of atherothrombosis in the general population, appears to be rare. On the basis of findings in individuals with hyperhomocysteinemia of genetic origin, there is in fact no clear evidence for a causal role of homocysteine in the pathogenesis of atherothrombotic disease, and the positive association between plasma homocysteine and IHD observed in many, but not all epidemiologic studies does not prove causality. To infer causality from observational studies, there should be a temporal, consistent, strong, independent, graded (dose-response effect), and duration-dependent relationship between exposure and outcomes, and a biologically plausible mechanism should exist. The relationship between plasma homocysteine levels and IHD does not fulfill these criteria beyond reasonable doubt. In the general population, plasma homocysteine levels are to a great extent determined by dietary habits, and plasma homocysteine could be a marker, or a consequence, of atherothrombosis and/or risk-associated behavior (e.g., a diet low in fruits and vegetables) rather than a cause of atherothrombosis. Experimentally, hyperhomocysteinemia is not in itself atherogenic in normal animals with relatively low plasma cholesterol levels. The homocysteine theory of atherosclerosis should be tested more thoroughly in hypercholesterolemic animals that develop atherosclerosis spontaneously to determine whether elevated plasma homocysteine levels are harmful under atherogenic conditions. A causal role of homocysteine in atherothrombotic disease remains to be established.

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Hyperhomocysteinemia has been proposed as an independent risk factor for ischemic heart disease (IHD), stroke, and peripheral artery disease (1–3). The underlying vascular pathology is usually atherosclerosis, often with thrombosis superimposed. Therefore, if homocysteine plays a causal role in IHD, it probably promotes the development of atherosclerosis or arterial thrombosis, or both. The evidence for a causal role of homocysteine in atherothrombosis will be reviewed in a historical perspective. But first, what is atherothrombosis?

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Abbreviations: CBS, cystathionine β-synthase; IHD, ischemic heart disease; MGH, Massachusetts General Hospital; smc, smooth muscle cell.

### *IHD vs. Stroke and Cardiovascular Disease*

Atherothrombosis is an occlusive arterial disease that develops over decades and ultimately may lead to luminal narrowing due to a variable mix of atherosclerosis and thrombosis (4). Thrombosis plays no role in the initiation of atherosclerosis and its early development, but thrombosis, superimposed on a ruptured or eroded atherosclerotic plaque, is by far the most frequent precipitating cause of acute coronary syndromes (unstable angina, myocardial infarction, sudden coronary death) and carotid artery–related stroke (5,6). In adults, atherothrombosis is responsible for nearly all cases of IHD but only ~20% of strokes (6). That is, in contrast to IHD, stroke is not a homogenous entity but has many different etiologies besides atherothrombosis; these include cardiogenic embolism (e.g., atrial fibrillation, valve disease, or ventricular thrombi), venous thrombosis (e.g., intracranial embolism through a patent foramen ovale), hypertensive small vessel disease (lacunar stroke), dysplasia and dissection of the arteries of the neck, and cerebral hemorrhage (6,7). Therefore, in the study of potential causal factors in the pathogenesis of atherothrombosis, IHD is a reliable clinical end point; this holds true also for intermittent claudication (peripheral artery disease), but not for stroke. Stable angina pectoris is assumed to reflect the underlying coronary atherosclerosis (chronic luminal narrowing), whereas acute heart attacks, particularly myocardial infarction, reflect superimposed thrombosis (4,5).

From a pathogenetic point of view, even more ambiguous than "stroke" is the term "cardiovascular disease." Cardiovascular disease includes an extremely heterogenous group of diseases of quite different etiologies; for this discussion, it is of particular importance to realize that well-established causal factors in atherosclerosis, arterial thrombosis, and venous thromboembolism differ substantially. Particularly, no single factor has convincingly been shown to promote both arterial and venous thrombosis by a common mechanism.

### *Homocystinuria in Mental Retardation*

In 1962, a new and rare inborn error of methionine-homocysteine metabolism associated with increased urinary excretion of homocystine (homocysteine-homocysteine disulfide) in mentally retarded children was described simultaneously by investigators in Europe and North America (8). Two years later, Mudd and colleagues (9) identified the underlying cause as a defect in the homocysteine-degrading enzyme cystathionine β-synthase (CBS) (Fig. 1); the first description of widespread vascular changes and thrombosis in this syndrome appeared the same year (11).

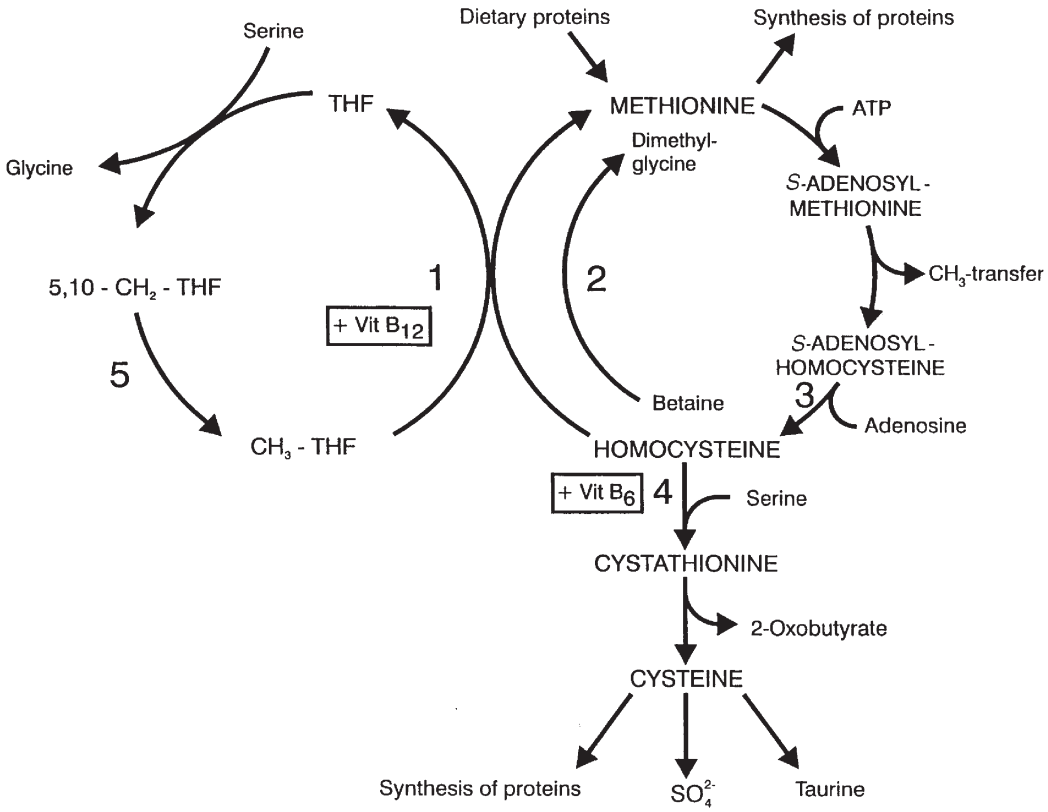


**FIG. 1.** The methionine-homocysteine metabolism. *S*-Adenosylmethionine is the methyl donor in a wide range of transmethylation reactions. The loss of the methyl group results in the formation of *S*-adenosylhomocysteine, which is subsequently converted to homocysteine by *S*-adenosylhomocysteine hydrolase (**3**). 5-Methyltetrahydrofolate: homocysteine methyltransferase (**1**), which uses cobalamin (vitamin $B_{12}$) as a coenzyme, transfers a methyl group from 5-methyltetrahydrofolate ($CH_3$-THF) to homocysteine to form methionine (***remethylation***). 5-Methyltetrahydrofolate is made by reduction of 5,10-methylenetetrahydrofolate (5,10-$CH_2$-THF), the compound of central importance in folate metabolism, by 5,10-methylenetetrahydrofolate reductase (**5**). An alternative pathway for the methylation of homocysteine to methionine is mediated by betaine:homocysteine methyltransferase (**2**) using betaine as methyl donor. In the ***transsulfuration*** pathway, homocysteine is condensed with serine to form cystathionine by the pyridoxal phosphate (vitamin $B_6$)-dependent enzyme cystathionine β-synthase (**4**). (Adapted from Ref. 10 with permission.)

### *McCully's Theory: A Shaky Foundation*

In 1969, Harvard pathologist Kilmer McCully at Massachusetts General Hospital (MGH) in Boston put forward the homocysteine theory of arteriosclerosis, initiated by the findings of widespread arterial changes in a newborn child with hyperhomocysteinemia caused by a vitamin $B_{12}$–dependent remethylation defect (12). The child died at the age of 7.5 wk. At autopsy, no gross lesions or occlusions were found in the cardiovascular system, but microscopic examination of large, medium-sized, and small arteries and arterioles in many organs of the body and the pulmonary artery revealed focal nonspecific changes, including swelling and hyperplasia of endothelial cells, fibrous intimal thickenings, disruption of the internal elastic membrane, and perivascular fibrosis (12). Lipid accumulation (atherosclerosis) was not present, nor was thrombosis or venous disease.

In the files of MGH, McCully found another child who, retrospectively, was assumed to have had hyperhomocysteinemia caused by the genetic CBS deficiency identified by Mudd and colleagues in 1964 (9), i.e., a vitamin $B_6$-dependent transsulfuration defect (12). The child, previously reported as a case record in the *New England Journal of Medicine* already in 1933 (13), was retarded and died of stroke at the age of 8 yr. Autopsy revealed fibrous thickening of the carotid arteries, and the right carotid was almost occluded by an organized thrombus. The designation arteriosclerosis (arterial hardening) was used to characterize the thickened vessel wall; lipid accumulation, atherosclerosis, was not described (12). In addition, widespread focal nonspecific arterial changes as described above were seen by microscopic examination. Venous disease was not found.

These two cases, a neonate who survived <2 mon and an 8-yr-old child, formed the basis for McCully's homocysteine theory of arteriosclerosis. Both had homocystinuria but due to different metabolic defects in methionine-homocysteine metabolism. They shared markedly elevated homocysteine, whereas the concentrations of other metabolites differed (e.g., methionine). This led McCully to conclude that homocysteine (or one of its derivatives) was the common factor responsible

for the arterial damage (12). It should be stressed, however, that McCully used the term arteriosclerosis, not atherosclerosis, in his early landmark article published in 1969 as well as in a recent review on the vascular pathology of hyperhomocysteinemia, in which he summarized that "the vascular pathology of hyperhomocysteinemia caused by deficiency of cystathionine synthase consists of fibrous arteriosclerotic plaques with little lipid deposition, associated with severe narrowing and thrombotic occlusions of aorta, major muscular arteries and arterioles in organs throughout the body" (14).

Recently, Rubba *et al.* (15) updated the existing knowledge on vascular complications of homocystinuria (severe hyperhomocysteinemia), including autopsy findings in 12 cases of premature cardiovascular death. Both arterial and venous thrombosis are frequent and occur at an early age but apparently often without concomitant atherosclerosis, and "heart disease does not represent a prominent feature of this condition. . . . It is unlikely that typical atherosclerotic lesions precede thrombus formation in homocystinuric patients of young age. Pathology studies rarely show typical atherosclerotic lesions in young homocystinuric patients who succumbed to thrombosis" (15). And Wilcken (16), who has been in the forefront of this area for decades, recapitulated in 2000 that ". . . the underlying process as seen in children before the advent of effective therapy was in the nature of a 'non-lipid' model for arteriosclerosis. . . ." Thus, it may indeed be questioned whether these arteriosclerotic and lipid-poor vascular changes seen in homocystinuric patients with severe hyperhomocysteinemia, apparently involving both pulmonary and systemic arteries of all sizes, have anything to do with atherosclerosis or represent a distinct and unique thrombosis-prone disease.

### *Homocysteine and Atherosclerosis: A Misconception*

At the time McCully formulated his homocysteine theory of arteriosclerosis, it fit very well into the contemporary understanding of the response-to-injury hypothesis of atherosclerosis (17–19). Atherosclerosis was believed to evolve through the following sequence of events: focal loss of endothelium (due to "toxic" injury), exposure of subendothelial tissue, and adherence of platelets followed by release of factors (e.g., platelet-derived growth factor) that stimulate intimal smooth muscle cell (smc) proliferation—a fibrous healing response initiated by a denuding endothelial injury and mediated by platelets and smc (17–19). As clearly stated in 1973 in the title of a famous article in the journal *Science* (17), proliferation of smc was believed to be a key event in atherogenesis. Therefore, when it was shown that homocystine infusion in primates caused patchy endothelial desquamation and smc-mediated intimal fibrosis (no or sparse lipid), it was considered strong supportive evidence for McCully's theory (20,21). The response-to-injury hypothesis of atherosclerosis typical of the period was, however, based on a misconception, i.e., de-endothelialization and platelets play no role in atherogenesis until the more advanced stages; early lesion formation occurs under an intact but activated endothelium (4). Today we know that atherosclerosis is a lipid-driven inflammatory disease characterized by endothelial activation, expression of adhesion molecules leading to macrophage recruitment, and lipid accumulation, followed by healing and repair mediated by the vascular smc (22), in which the last-mentioned stabilizes the lesion and is thus beneficial, not detrimental (23,24). Of note, recent observations indicate that plasma homocysteine is unrelated to restenosis after coronary angioplasty, which is an smc-mediated phenomenon (25).

### *Homocysteine and Coronary Thrombosis: Another Misconception*

In 1985, Mudd and co-workers (26) published their classical article on the natural history of homocystinuria due to CBS deficiency, based on a worldwide questionnaire survey of 629 cases. The study confirmed that homocystinuria, i.e., severe hyperhomocysteinemia, is indeed associated with premature vascular diseases but, despite the widely held view, it did not provide evidence for a causal role of hyperhomocysteinemia in the pathogenesis of IHD. Of the 253 thromboembolic events recorded in 158 homocystinuric patients, 51% affected peripheral veins, 32% gave rise to cerebrovascular accidents [probably often of venous origin (27)], 11% involved peripheral arteries, and only 4% ($n$ = 10) led to myocardial infarctions (26). No data on atherosclerosis were provided. Thus, venous thrombosis prevailed, and myocardial infarction was strikingly rare, which is in sharp contrast to atherosclerosis-mediated coronary thrombosis in the general population.

### *Hyperhomocysteinemia and IHD in the General Population*

In 1976, Wilcken and Wilcken (28) studied patients with angiographically proven coronary artery disease without any known metabolic defects such as CBS deficiency and found evidence of a reduced ability to metabolize homocysteine in some of these patients. It was the first study to suggest an association between high plasma homocysteine levels near the average range (after oral methionine) of a population and premature IHD. Subsequently, numerous epidemiologic studies have identified raised plasma homocysteine as an independent risk factor for atherothrombotic disease, including IHD, stroke, and peripheral vascular disease (1–3). A frequently quoted meta-analysis suggests that plasma homocysteine elevation is a graded risk factor; an increment of only 5 μmol/L in plasma homocysteine level is associated with a 60 and 80% increased risk of IHD for men and women, respectively, and homocysteine may account for 10% of the population's IHD risk (1). A strong association between plasma homocysteine and atherothrombotic disease has, however, been observed predominantly in cross-sectional and retrospective case-control studies, in contrast to a weak or absent association in many nested case-control and prospective studies (3). These conflicting results may suggest that plasma homocysteine is a marker or a consequence of atherothrombotic disease rather

than one of its causes (3,27,29). In the case of causality, the epidemiologic data suggest that homocysteine plays a more important role in patients with established disease than in persons without symptoms of vascular disease. That is, mildly elevated homocysteine could promote complications to atherosclerosis (e.g., thrombosis) rather than atherosclerosis itself (30–34), and hyperhomocysteinemia is, in fact, linked to thrombosis of venous origin (31,35). Plasma homocysteine and stable angina pectoris (atherosclerosis-related) did not correlate in the single prospective study that examined the question (36).

### *Homocysteine and IHD: Cause, Consequence, or Marker?*

The mere presence of a positive relation between elevated plasma homocysteine levels and IHD, as seen in many observational studies, does not indicate whether the former is a cause of, related to, or a consequence of the latter. Noticeably, elevated plasma homocysteine levels are associated with many of the well-known conventional risk factors, including old age, male sex, smoking, high blood pressure, elevated cholesterol level, lack of exercise, and a diet low in fruits and vegetables (37). To answer the question about causality and, if present, its strength, studying IHD risk in persons with genetically elevated plasma homocysteine levels (present from birth, precedes the disease, long exposure, and not a consequence of the disease or a marker of something else) provides important information. As mentioned, severe elevation of plasma homocysteine levels caused by homozygous CBS deficiency (>100 μmol/L) is clearly associated with premature venous thromboembolism and possibly with arterial thrombosis with a character of its own, but not with premature atherosclerosis and IHD (15,16,26). Coronary thrombosis appears to be extraordinarily rare (26). Treatment with pharmacologic doses of vitamin $B_6$ appears to reduce the cardiovascular risk dramatically but, strangely enough, not only in biochemically well-controlled patients but also in $B_6$ nonresponders and in responders with persistently high plasma homocysteine levels (~50 μmol/L and higher), i.e., levels that are associated with a markedly increased risk of cardiovascular events in the normal population (38). In a survey among heterozygotes (394 parents of homocystinuric children), no significantly increased risk of heart attack or stroke was identified, although many heterozygotes are known to have abnormally high plasma homocysteine levels after methionine loading (27); it was concluded that the result of this study failed to support McCully's theory (39,40). Heterozygosity for CBS deficiency appears, in fact, to be rare in premature IHD (41), although contrasting results have been reported (42). Similarly, no increased risk of either arterial or venous disease has been documented convincingly with modestly elevated plasma homocysteine levels (e.g., from 15 to 20 μmol/L) caused by milder genetic "defects" such as the TT genotype for the common C677T methylenetetrahydrofolate reductase polymorphism (27). Hyperhomocysteinemia associated with the TT genotype, even in the presence of low folate status, seems to be benign and not related to the risk or severity of cardiovascular disease (27). This mutation did not correlate with IHD in a meta-analysis of >6,000 persons (43). These observations in hyperhomocysteinemia of genetic origin argue against a causal role of homocysteine itself in the pathogenesis of atherothrombotic disease.

It should be remembered that normal (but not necessarily optimal) levels of fasting plasma homocysteine are considered to be <15 μmol/L (37,44), and that epidemiologic observations indicate that a 5-μmol/L increase in plasma homocysteine concentrations is associated with a significant increase in IHD risk (1). Therefore, the question arises concerning why a relatively small life style–mediated increase in plasma homocysteine (~5 μmol/L) appears to be associated with enhanced risk of atherothrombosis, whereas a much larger increase of genetic origin is not. How can these conflicting observations be explained?

It has been suggested that much, if not all of the epidemiologic association between mild hyperhomocysteinemia and atherothrombotic disease could be explained by homocysteine being a consequence of atherosclerosis/IHD or a marker of risk-associated behavior (27). For example, impaired renal function due to hypertension and atherosclerosis (nephrosclerosis) could be an important cause of the elevated plasma homocysteine found in patients with vascular disease, called the reverse causality hypothesis (Fig. 2) (27). Furthermore, several lifestyle factors that lower homocysteine levels are also of proven cardiovascular benefit through other mechanisms (37).

There is, however, a reasonably strong argument for a proatherogenic effect of hyperhomocysteinemia, i.e., it causes endothelial dysfunction (45,46), probably mediated by increased oxidant stress (47), and is seen even with the small physiologic increment in plasma homocysteine (~2 μmol/L) induced by a meal rich in animal protein (46). However, the long-term implications of homocysteine-induced endothelial dysfunction in atherogenesis are not known.

### *Diet, Homocysteine, Vitamins, and IHD*

Foods contain only trace amounts of homocysteine. The primary source of homocysteine in the body is methionine (essential amino acid) in dietary proteins. Although dietary intake of total protein and methionine does not correlate significantly with blood homocysteine (44), meals may transiently affect plasma homocysteine levels (48). Vitamins do not influence the conversion of methionine to homocysteine, but they do determine its fate (Fig. 1) (49). In high-methionine states, homocysteine is degraded predominantly through the transsulfuration pathway, which requires vitamin $B_6$ to function optimally. In low-methionine states, methionine is recirculated *via* homocysteine and the remethylation pathway, which requires folate and vitamin $B_{12}$ to function optimally. Therefore, $B_6$, folate, and $B_{12}$ deficiencies may lead to hyperhomocysteinemia but *via* different mechanisms; the plasma homocysteine level is in fact a very sensitive marker of folate, $B_{12}$, and $B_6$ status (50). Homocysteine levels start to rise as plasma concentrations of

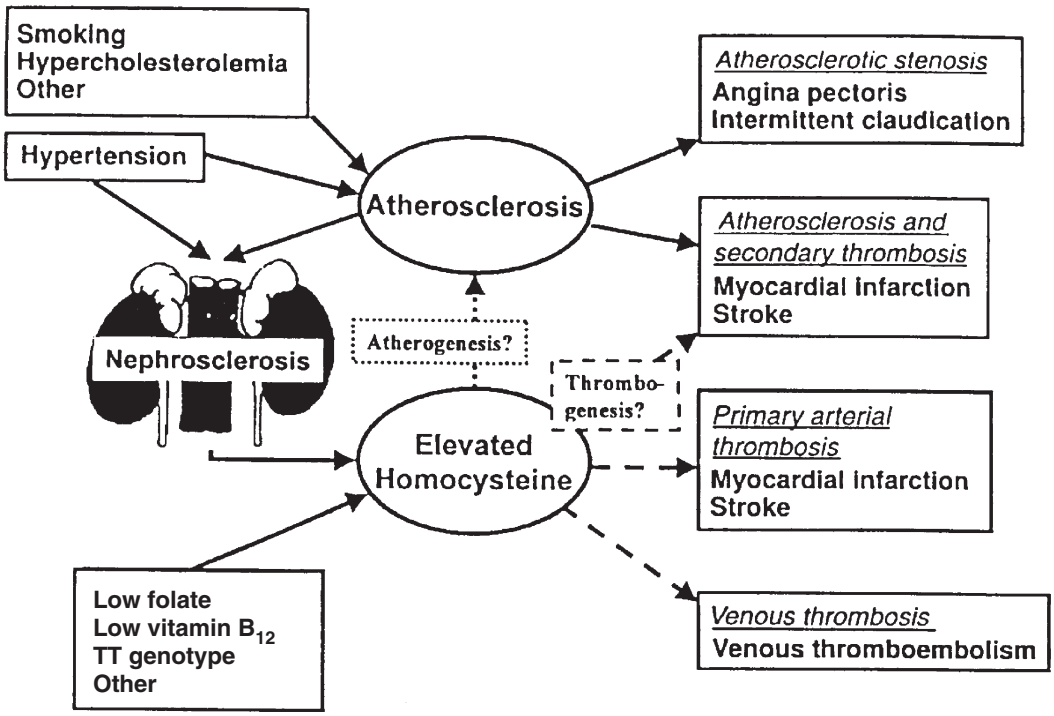


**FIG. 2.** Impaired renal function, poor folate or vitamin $B_{12}$ status, and the TT genotype of the C677T methylenetetrahydrofolate reductase polymorphism are common causes of elevated plasma homocysteine. Hypertension (and age and atherosclerosis?) leads to nephrosclerosis and decline in renal function, which in turn leads to elevated plasma homocysteine. This mechanism may explain much of the association between plasma homocysteine and atherothrombotic disease. Because there is no consistent evidence that elevated homocysteine due to low folate, low vitamin $B_{12}$, or the TT genotype is associated with increased risk of atherosclerotic or thromboembolic disease, the role of a modest elevation in plasma homocysteine in cardiovascular disease remains unclear. (Adapted from Ref. 27 with permission.)

folate or $B_{12}$ fall below the mean value and long before overt vitamin deficiency is present (51). Supplementation with these factors, particularly folate, can reduce homocysteine levels by perhaps 30% in most subjects (51).

One of the most consistent findings in dietary research is that those who consume higher amounts of fruits and vegetables have lower rates of heart disease and stroke as well as cancer (52,53). The precise mechanisms for these apparent protective effects are not clear. A typical cardioprotective diet is low in saturated fat and animal proteins, relatively high in plant proteins (methionine-poor), n-3 fatty acids, and wine, and high in fresh fruits and vegetables, which are major sources of natural antioxidants (vitamins C and E, carotenoids, and polyphenols), minerals (e.g., selenium), dietary fiber, and homocysteine-lowering vitamins (folic acid and $B_6$). One of many consequences of such a cardioprotective diet is a low plasma homocysteine level, and the latter may simply be a marker of the former.

Some observational studies suggest that, to a large extent, the association between hyperhomocysteinemia and vascular disease is explained by a vitamin deficit, especially with regard to vitamin $B_6$ and folate (54–56). Other studies, however, are less consistent, and it has even been suggested that elevation of plasma homocysteine concentrations due to poor folate status is benign with regard to risk or severity of cardiovascular disease (27). Thus, low plasma homocysteine could be a marker of the consumption of a low-risk diet (57), and cardiovascular protection could be mediated by mechanisms independent of the homocysteine-lowering effect. If a reduction in cardiovascular events is seen in ongoing clinical trials of vitamin supplementation, a possible homocysteine-independent vasculoprotective effect of folate will certainly cloud the issue of causality (58–60). For example, randomized trials have shown that periconceptional folic acid supplementation not only reduces the plasma homocysteine levels but also prevents neural tube defects; nevertheless, the mechanism of action remains unclear. Thus, it is still being debated whether this beneficial effect is the result of homocysteine lowering itself or caused by other folate-dependent mechanisms (61).

### *Homocysteine and Atherothrombosis: Experimental Observations*

Experimental models of hyperhomocysteinemia have produced conflicting results regarding the contribution of homocysteine to the development of atherosclerosis. Generally, however, hyperhomocysteinemia is not in itself atherogenic in experimental animals fed a normal low-fat diet, including nonhuman primates (20,21,62,63), rabbits (64), rats (65), pigs

(66), and mice (67). However, all of these animals have relatively low plasma cholesterol levels and none develop atherosclerosis spontaneously to any significant degree. Therefore, the homocysteine theory of atherosclerosis has to be tested more thoroughly in hypercholesterolemic animals to determine whether elevated plasma homocysteine levels are harmful under atherogenic conditions. This possibility is currently under intense investigation.

As long as a causal role of homocysteine in atherothrombotic disease remains to be established, it may be premature to discuss potential pathogenetic mechanisms; however, various mechanisms have been proposed (68). *In vivo*, hyperhomocysteinemia is associated with endothelial dysfunction in animals (69–71) [as in humans (45–47)], and it appears to promote endothelial-dependent neointima formation in rat carotid arteries after balloon injury (72); however, as mentioned, homocysteine itself has not proved to be atherogenic in animal studies (29,73,74). *In vitro*, many potentially proatherothrombotic effects of homocysteine have been reported, including endothelial toxicity (75,76) and stress (77), promotion of smc growth and collagen production (78,79), enhanced low density lipoprotein peroxidation (80), platelet activation (81), downregulation of thrombomodulin on endothelial cells (82), and upregulation of tissue factor activity on both endothelial cells (83) and macrophages (82,84). The culprit, if one exists, has been suggested to be the prooxidant activity of homocysteine (85,86).

Many of these homocysteine-mediated effects, however, have been demonstrated only at very high supraphysiologic concentrations (>1000 μmol/L), and a biological plausible mechanism of action at normal and mildly elevated homocysteine levels (<30 μmol/L) has been more difficult to identify (69,84). It is, in fact, just the opposite of the real world experience in which a small increase (1–2 μmol/L) in plasma homocysteine appears to enhance the risk of atherothrombosis in the general population, whereas no clear-cut effect is seen with the much higher levels in CBS deficiency.

In conclusion, conflicting results have been reported in genetic, observational, and experimental studies on the relation between homocysteine and atherothrombotic disease. McCully's homocysteine theory of arteriosclerosis, evoked by autopsy findings in two homocystinuric children (severe hyperhomocysteinemia), was based on the misconception that arteriosclerosis is equated with atherosclerosis, the disease responsible for IHD in the general population. Another misconception derived from studies of homocystinuric children that continues to prevail is that their high risk of cardiovascular complications includes atherothrombotic disease. Coronary thrombosis, the most frequent manifestation of atherothrombosis in the general population, appears to be rare in homocystinuric patients. On the basis of findings in individuals with hyperhomocysteinemia of genetic origin, there is, in fact, no clear evidence for a causal role of homocysteine in the pathogenesis of atherothrombotic disease, and the positive association between plasma homocysteine and IHD observed in many, but not all epidemiologic studies does not prove causality. For example, considerable epidemiologic evidence exists for a protective role of β-carotene, vitamin E, and hormone replacement therapy in IHD; nevertheless, recent randomized trials failed to prove protection (87–89), and some of these "promising" interventions may, in some cases, even be harmful.

To infer causality from observational studies, there should be a temporal, consistent, strong, independent, graded (dose-response effect), and duration-dependent relation between exposure and outcomes, and a biologically plausible mechanism should exist. The relation between plasma homocysteine levels and IHD does not fulfill these criteria beyond reasonable doubt (90), and particularly troublesome is the lack of consistency among studies. In the general population, plasma homocysteine levels are to a great extent determined by dietary habits, and plasma homocysteine could be a marker, or a consequence of atherothrombosis and/or risk-associated behavior (e.g., a diet low in fruits and vegetables) rather than a cause of atherothrombosis. It is indeed difficult to reconcile the low risk of IHD in severe hyperhomocysteinemia of genetic origin (plasma homocysteine >100 μmol/L) with a strong and graded causal effect of plasma homocysteine in mild hyperhomocysteinemia of dietary origin (<30 μmol/L) in the general population.

Atherosclerosis is a lipid-driven disease and homocysteine cannot in itself initiate the process. It may, however, accelerate the process although we do not know if this is the case. What we do know is that a cardioprotective diet rich in vitamins lowers the plasma homocysteine level and the risk of atherothrombosis, but it remains to be shown whether the beneficial effect is homocysteine mediated. The ongoing clinical trials testing the effect of homocysteine-lowering vitamins on cardiovascular outcomes will probably not answer this question. It has, for example, been documented that folic acid can prevent neural tube defects; nevertheless, the mechanism of action remains unclear. Thus, causality of homocysteine in atherothrombotic disease may be disproved, but never proved, by vitamin supplementation.

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# Hyperhomocysteinemia and Thrombosis

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**ABSTRACT:** Homocysteine (Hcy) is a sulfhydryl amino acid derived from the metabolic conversion of methionine, which is dependent on vitamins (folic acid, $B_{12}$, and $B_6$) as cofactors or cosubstrates. In 1969, McCully first reported the presence of severe atherosclerotic lesions in patients with severe hyperhomocysteinemia and hypothesized the existence of a pathogenic link between hyperhomocysteinemia and atherogenesis. Several case-control and cross-sectional studies were consistent with the initial hypothesis of McCully, showing that moderate hyperhomocysteinemia is also associated with heightened risk of occlusive arterial disease. Less consistent results have been reported by prospective cohort studies of subjects who were healthy at the time of their enrollment, whereas prospective cohort studies of patients with overt coronary artery disease or other conditions at risk consistently confirmed the association between moderate hyperhomocysteinemia and cardiovascular morbidity and mortality. More recently, an association between moderate hyperhomocysteinemia and heightened risk of venous thromboembolism has been documented, suggesting that hyperhomocysteinemia might be involved not only in atherogenesis, but also in thrombogenesis. The mechanisms by which hyperhomocysteinemia might contribute to atherogenesis and thrombogenesis are incompletely understood. The mainstay of treatment of hyperhomocysteinemia is folic acid, alone or in combination with vitamin $B_{12}$ and vitamin $B_6$. Although it is quite clear that vitamins effectively reduce the plasma levels of total homocysteine (tHcy), we do not yet know whether they will decrease the risk of vascular disease. The results of ongoing randomized, placebo-controlled, double-blind trials of the effects of vitamins on the thrombotic risk will help in defining whether the relationship between hyperhomocysteinemia and thrombosis is causal, and will potentially have a dramatic effect in the prevention of thromboembolic events.

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Homocysteine (Hcy) is a nonprotein sulfhydryl amino acid derived from the metabolic conversion of methionine. It exists in both free and protein-bound forms and is oxidized in plasma to the disulfides homocysteine-homocysteine (homocystine) and homocysteine-cysteine (mixed disulfide). Free and protein-bound Hcy and its disulfides are globally referred to as total homocysteine (tHcy) or homocyst(e)ine. The intracellular metabolism of Hcy occurs through two pathways of remethylation to methionine and one pathway of trans-sulfuration to cysteine. In the remethylation pathway, which is catalyzed by methionine synthase, cobalamin acts as a cofactor and the methyl group is donated by 5-methyltetrahydrofolate, the major form of folate in plasma, which derives from the reduction of 5,10-methylenetetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR). In the other remethylation pathway, which is relatively less important, betaine is the methyl donor and the reaction is catalyzed by betaine-homocysteine methyltransferase. In the trans-sulfuration pathway, homocysteine is transformed by cystathionine-β-synthase (CBS) to cystathionine, with pyridoxal-5′-phosphate, a vitamin $B_6$ derivative, acting as a cofactor. Vitamin $B_6$ is also necessary for transformation of cystathionine to cysteine and α-ketobutyric acid. Under conditions of methionine excess, homocysteine is largely metabolized through the trans-sulfuration pathway; in contrast, under conditions of negative methionine balance, homocysteine is transformed primarily to methionine (1,2).

## CAUSES OF HYPERHOMOCYSTEINEMIA

### *Determinants of Total Homocysteine Levels in Plasma*

The plasma levels of tHcy increase with age, are lower in fertile women than in men, and increase after menopause (3–7). Major determinants of plasma tHcy levels include genetic abnormalities (see below), diet (vitamin $B_{12}$, $B_6$, and folate intake), renal function, cigarette smoking, and coffee consumption (5–11). In a large population study, a lifestyle that included low folate intake, smoking, and heavy coffee consumption was characterized by high median tHcy levels and pronounced skewness toward high tHcy, whereas the contrasting lifestyle profile (high folate intake, no smoking, and low coffee consumption) was associated with low tHcy levels and normal distribution of the tHcy values (11). Two recent reports showed that strict vegetarians tend to have high tHcy levels, probably due to vitamin $B_{12}$ depletion (12,13). Other, less well-established determinants of tHcy are race (14,15), arterial hypertension, hypercholesterolemia, and physical exercise (16).

### *Severe Hyperhomocysteinemia (Homocystinuria)*

The most frequent cause of severe hyperhomocysteinemia (characterized by fasting levels of tHcy in plasma higher than 100 μmol/L) is the homozygous deficiency of CBS, which

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Abbreviations: CBS, cystathionine-β-synthase; CI, confidence interval; Hcy, homocysteine; HUVEC, human umbilical vein endothelial cells; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; PML, postmethionine loading; RR, relative risk; SLE, systemic lupus erythematosus; tHcy, total homocysteine.

has a prevalence in the general population of ~1 in 335,000, varying between 1:65,000 (Ireland) and 1:900,000 (Japan) (17). Affected individuals develop the classical syndrome of homocystinuria, characterized by ectopic lens, skeletal abnormalities, premature vascular disease, thromboembolism, and mental retardation (18). Approximately 5–10% of cases of severe hyperhomocysteinemia are caused by inherited defects of remethylation (18,19). Homozygous deficiency of MTHFR, which catalyzes the reduction of methylenetetrahydrofolate to methyltetrahydrofolate, is the most common inherited defect of the remethylation pathway and is characterized by neurological dysfunction, psychomotor retardation, seizures, peripheral neuropathy, premature vascular disease, and thromboembolism. Rare cases of homocystinuria have been described in subjects with errors of cobalamin metabolism, resulting in decreased activity of methionine synthase (20).

### *Mild-to-Moderate Forms of Hyperhomocysteinemia*

Mild-to-moderate forms of hyperhomocysteinemia (fasting levels of tHcy between 15 and 100 μM) are encountered in phenotypically normal subjects with genetic defects, acquired conditions, or, more frequently, a combination of both. Genetic defects associated with moderate hyperhomocysteinemia cause approximately a 50% reduction in activities of the corresponding enzymes, such as heterozygosity for CBS or MTHFR deficiency, whose cumulative prevalence in the general population is between 0.4 and 1.5% (21). Another genetic defect that is associated with a 50% reduction of the enzymatic activity is characterized by the presence of a thermolabile mutant of MTHFR (22), which is due to the homozygous C to T substitution at nucleotide 677 of the encoding gene, converting the codon for alanine to that for valine (23). The prevalence of homozygosity for the C677T mutation is between 5 and 20% in subjects of Caucasian descent. Moderate elevations of plasma tHcy levels do not cause a 50% reduction of the corresponding enzyme activities in all subjects with genetic defects, indicating that their phenotypic expression can be influenced by other factors. For instance, homozygotes for the thermolabile form of MTHFR and heterozygotes for CBS deficiency have high homocysteine levels mainly in the presence of low serum concentrations of folic acid (24–26). Another common mutation in the MTHFR gene, A1298C, is not associated with hyperhomocysteinemia, but might interact with the C677T mutation in raising tHcy levels in plasma (27).

Causes of acquired hyperhomocysteinemia include deficiencies of folate, cobalamin, and pyridoxine, which are essential cosubstrates or cofactors for homocysteine metabolism, and chronic renal insufficiency. Vitamin deficiencies are the most frequent cause of hyperhomocysteinemia (7), especially in elderly people (8,28,29). Drugs interfering with the metabolism of folate, such as methotrexate, trimethoprim, and anticonvulsants, of cobalamin, such as nitrous oxide, and of vitamin $B_6$, such as theophylline, can cause moderate hyperhomocysteinemia (21,30–32). The increase in plasma tHcy after therapy with fibrates (33–35) may be related in part to an induced functional reduction in renal function (34,35). Estrogens (36,37), tamoxifen (38,39), penicillamine (40), and acetylcysteine (41) reduce the plasma tHcy levels.

## DIAGNOSIS OF HYPERHOMOCYSTEINEMIA

Of plasma tHcy, ~70% is bound to albumin, 30% is oxidized to disulfides, and only ~1% is present as free homocysteine. For measurement of total homocysteine in plasma, immediate deproteinization of the sample is necessary to avoid its gradual binding to plasma proteins, and very sensitive methods are required. Hence, before the discovery of protein-bound homocysteine, the diagnosis of moderate elevations of plasma homocysteine levels was very cumbersome. For this reason, early epidemiologic studies of the prevalence of moderate hyperhomocysteinemia in health and disease were based on the measurement of plasma homocysteine levels after an oral methionine loading, which temporarily increases the plasma homocysteine levels (see below). Since the introduction of methods that measure tHcy in blood, which imply the treatment of samples with reducing agents, fasting blood levels of homocysteine can be reliably measured to discriminate between normal subjects and patients with mild impairments of its metabolism. Current methods to assay plasma tHcy include gas chromatography–mass spectroscopy and high-performance liquid chromatography with fluorometric or electrochemical detection (reviewed in Ref. 42). Recently, manual or fully automated enzyme immunoassays have become commercially available, which should allow tHcy measurements in nonspecialized clinical laboratories (43,44). Two recent multicenter studies, which compared plasma tHcy measurements in different laboratories, pointed to the need to improve analytical imprecision and to establish an international plasma standard to harmonize tHcy measurement across laboratories (45,46).

### *The Postmethionine Loading (PML) Test*

Measurement of plasma homocysteine 4–8 h after a standardized oral methionine loading (3.8 g/m$^2$ body surface area or 0.1 g/kg body), which was initially developed to detect heterozygosity for CBS deficiency (47,48), improves the ability of distinguishing between normal individuals and subjects with mild abnormalities of homocysteine metabolism (18, 48–52). A shortened 2-h protocol has been validated, and may offer advantages of patient acceptability over the 4- or 8-h protocols (53). However, the equivalence of the 2- and 4-h protocol has been questioned recently (54). It appears that the PML test is most sensitive to the trans-sulfuration pathway of homocysteine (55,56). However, it is also abnormal in individuals who are homozygous for the C677T mutation of the MTHFR gene, who have a selective defect of the remethylation pathway of homocysteine (57).

### *Normal Ranges*

The cutoff point for hyperhomocysteinemia is usually, but arbitrarily, set at the 95th percentile of the homocysteine

distribution in healthy subjects, which, for fasting tHcy levels, corresponds to ~15 μmol/L. Normal ranges vary widely in different populations because they are affected by several lifestyle determinants (see above). When determined in a population with adequate vitamin intake (58,59), the upper limit of the normal range may be as low as 12 μmol/L. Because fasting tHcy levels are affected by gender, different cutoff points should be used for men and women. Reference ranges for the PML increase of tHcy above fasting levels (the PML absolute levels of tHcy should not be considered because they are affected in part by the fasting levels) have been determined less extensively. The effect of gender on PML tHcy increase is controversial (52,60).

## HYPERHOMOCYSTEINEMIA IN ATHEROTHROMBOTIC DISEASE

There are three autosomal recessive inborn errors of homocysteine metabolism associated with very high tHcy levels in plasma and homocystinuria, i.e., deficiency of CBS, deficiency of MTHFR, and errors of cobalamin metabolism resulting in impaired methionine synthase activity (18). All three genetic disorders are characterized by very high risk for early-onset cardiovascular complications of either the arterial or the venous circulation (18). Because high tHcy levels are the only known biochemical abnormality that is common to these conditions, it is likely that Hcy is in some way involved in the pathogenesis of thromboembolic complications in these disorders. In 1969, McCully (61) first reported the presence of severe arteriosclerotic lesions in patients with homocystinuria and hypothesized the existence of a pathogenic link between hyperhomocysteinemia and atherogenesis. Several epidemiologic studies have yielded results consistent with the initial hypothesis of McCully, showing that moderate hyperhomocysteinemia may also be associated with heightened risk of arterial disease (16,62).

### *Case-Control Studies*

In 1976, Wilcken and Wilcken (63) first showed that patients with coronary artery disease have elevated concentrations of plasma cysteine-homocysteine disulfide after an oral methionine load. Increased plasma levels of homocysteine species before and/or after an oral methionine load were subsequently reported by several other groups concerning patients with atherothrombosis (coronary artery disease, cerebrovascular disease, and peripheral arterial occlusive disease) (16,62).

A meta-analysis of 27 studies, most having a case-control design, published before 1994, revealed that the summary odds ratio (OR) as an estimate of the relative risk (RR) in subjects with hyperhomocysteinemia was 1.7 [95% confidence interval (CI) 1.5–1.9] for CAD, 2.5 (2.0–3.0) for cerebrovascular disease, and 6.8 (2.9–15.8) for peripheral arterial disease (64). The results of this meta-analysis were heavily influenced by a high prevalence of case-control studies. For each increase in tHcy concentration of 5 μmol/L, there was an increase of ~40% in the relative risk for CAD. The association of hyperhomocysteinemia with arterial occlusive diseases remained significant after adjustment for known risk factors such as smoking, cholesterol, hypertension, and diabetes. Many other case-control studies have been published since then, most of which confirmed the association of hyperhomocysteinemia with cardiovascular diseases. Among them, a multicenter study of 750 patients with vascular disease and 800 controls confirmed that hyperhomocysteinemia conferred a graded risk of vascular disease, which was similar to and independent of that of other risk factors, such as smoking and hypercholesterolemia (52). In addition, the study demonstrated that, for both sexes combined, high fasting homocysteine levels showed a more than multiplicative effect on risk in smokers and in hypertensive subjects (52). A more recent analysis of the same study revealed that, in addition to plasma homocysteine levels, concentrations of red cell folate below the 10th centile and of vitamin $B_6$ below the 20th centile for control subjects were also associated with increased risk (65). This risk was independent of conventional risk factors and, for folate, it was explained in part by high levels of tHcy. In contrast, the association between vitamin $B_6$ and the risk of vascular disease was independent of tHcy levels both before and after methionine loading (65). These results agree with those of those of other studies, which showed that low pyridoxal-5′-phosphate confers an independent risk for coronary artery disease (66,67).

### *Cross-Sectional Studies*

Two studies reported the correlation between tHcy and carotid arterial wall thickness measured by B-mode ultrasound in subjects without clinically apparent atherosclerosis (29,68). In most of the cases, hyperhomocysteinemia was associated with low concentrations of pyridoxine and folic acid. Low plasma levels of folic acid and pyridoxine were associated with increased risk for carotid artery stenosis (29) also independently of tHcy plasma levels. The association of hyperhomocysteinemia and the risk of atherosclerosis of the carotid and peripheral arteries was later shown in subjects aged 55–74 y, but not in older subjects (69). Other cross-sectional studies demonstrated the existence of a relationship between homocysteine level and the extent of atherosclerosis in the aorta (70), coronary (71,72), and peripheral arteries (73,74).

### *Prospective Studies*

*Prospective studies of healthy subjects.* The results of 13 prospective studies, all with a nested case-control design, of the relationship between tHcy and risk of cardiovascular disease in subjects who were healthy at the time of their enrollment are controversial. Six studies demonstrated that tHcy levels at baseline could predict the risk of future cardiovascular and/or cerebrovascular events, whereas the other seven failed to demonstrate such an association (67–86).

More recently, the results of six prospective studies of elderly healthy subjects, one including postmenopausal women, were published (87–92). All of them showed that high tHcy levels are associated with an increased risk not only for future cardiovascular and cerebrovascular events (87–92) but also for all-cause and cardiovascular mortality (91,92).

*Prospective studies of patients with overt coronary artery disease or other conditions at risk.* The only prospective study of patients with overt coronary artery disease showed a strong, graded, and significant relationship between tHcy levels and overall mortality that was independent of other risk factors (93). When death due to cardiovascular disease was considered as the end point, the relation between tHcy levels and mortality was even stronger. Subgroup analysis revealed that tHcy predicted the risk of death independently of age, gender, serum cholesterol, smoking, blood pressure, and serum creatinine. In a study of 337 patients with systemic lupus erythematosus (SLE), Petri *et al.* (94) found that high tHcy concentrations were significantly associated with stroke (OR, 2.24; 95% CI, 1.22–4.13) and arterial thrombotic events (3.71; 95% CI, 1.96–7.13). The association remained significant after adjustment for established risk factors.

Patients with chronic renal failure have markedly increased plasma levels of tHcy. In addition to case-control and cross-sectional studies (95,96), two prospective studies of maintenance peritoneal dialysis or hemodialysis patients revealed that the adjusted hazard ratios for nonfatal and fatal cardiovascular events were 3.0–4.4 in patients with tHcy levels in the upper quartile, compared with those of patients with tHcy levels in the three lowest quartiles (97) and that the relative risk for cardiovascular events, including death, increased 1% per μM increase in tHcy concentration (RR, 1.01; 95% CI, 1.00–1.01) (98). More recently, hyperhomocysteinemia was shown to be a predictor of mortality in type-2 diabetes (99,100).

In conclusion, in contrast with case-control studies, prospective studies of the association of hyperhomocysteinemia and the risk of arterial disease in otherwise healthy subjects at the time of randomization have given contrasting results. Although the negative results of some prospective studies might weaken causal inference and support the hypothesis of Hcy being a risk marker rather than a causal risk factor, other interpretations could be considered.

The within-person consistency in tHcy levels over time might attenuate the association of a one-time measure with the future vascular events. As a matter of fact, both the Physicians' Health Study (79) and the prospective study by Kark *et al* (92) showed that the association of tHcy levels and incidence of MI or death became weaker after the first 5 yr of follow-up.

In addition, the effects of genetic and/or nutritional differences (101) and of different cardiovascular risk profiles among the populations studied could be considered. In fact, Alfthan *et al.* (102), in an elegant ecological study, showed that mean basal levels of plasma tHcy differ among 11 different countries and are positively correlated with cardiovascular mortality.

The findings that hyperhomocysteinemia consistently proved to be predictive of cardiovascular events in patients affected by pathologies associated with high cardiovascular risk (93–100) suggest that the basal cardiovascular risk profile of the population studied may be relevant. In line with this hypothesis, all of the most recent prospective studies of healthy elderly subjects, who are at higher cardiovascular risk than young individuals, showed consistently that hyperhomocysteinemia is predictive of future cardiovascular and cerebrovascular morbidity and mortality (87–92).

### *Case-Control and Cross-Sectional Studies of Genetic Abnormalities of Hcy Metabolism*

Two studies showed that subjects with heterozygous mutations of the CBS gene are not at increased risk for cardiovascular disease (67,103). The negative data on the association of CBS mutations with cardiovascular disease agree with the findings of an early questionnaire survey on obligate heterozygotes for CBS deficiency, which failed to demonstrate an increased risk for vascular disease (104) and with those of a more recent study of obligate heterozygotes for CBS deficiency (105). However, they are in contrast with the results of other studies, that showed a high prevalence of abnormal methionine loading and decreased CBS activity in cultured fibroblasts of patients with early-onset vascular disease (106,107). It must be noted, however, that the results of decreased CBS activity in thrombotic patients, which were generated by one and the same laboratory for both studies, could not be reproduced when retested in a different laboratory (103).

In contrast to heterozygous CBS deficiency, the homozygous C677T mutation of MTHFR was associated with a threefold increase in cardiovascular risk in the initial study by Kluijtmans *et al.* (103). After this initial report, several additional studies of the frequency of the C677T MTHFR mutation in cardiovascular patients were published. A meta-analysis of eight studies revealed that the homozygous genotype for the C677T mutation was present in 299 of 2476 patients (12.1%) and in 257 of 2481 controls (10.4%), resulting in a significant OR of 1.22 (95% CI, 1.01–1.47) relative to the normal genotype (108). In contrast, a subsequent meta-analysis of 13 studies including a total of 3281 patients with cardiovascular disease and 3218 healthy controls revealed no difference between patients and control subjects in either the allele frequency (33.7 vs. 35.6, respectively) or the frequency of mutant homozygotes (12.2 vs. 13.2, respectively) (109). More recent studies suggest that the C677T mutation of MTHFR may be associated with increased risk for early-onset coronary artery disease (110) or childhood stroke (111).

A common 844ins68 insertion variant in the CBS gene, which was independently described by two groups (112,113), is not associated with hyperhomocysteinemia or with heightened risk of vascular disease (114). In line with similar studies (see above), two recent cross-sectional studies showed that carotid atherosclerosis was associated with high Hcy

levels; however, no such association was found with the C677T mutation (115,116). The lack of associations between genetic determinants of hyperhomocysteinemia and cardiovascular risk or carotid atherosclerosis is in marked contrast with the results of case-control studies of hyperhomocysteinemia in cardiovascular diseases. Although some explanations have been attempted (62), this paradox questions the hypothesis of the existence of a causal relationship between hyperhomocysteinemia and cardiovascular diseases (see below).

## HYPERHOMOCYSTEINEMIA IN VENOUS THROMBOSIS

### *Case-Control Studies*

After the publication of two negative studies of small series of patients (117,118), the association of moderate hyperhomocysteinemia with venous thrombosis was demonstrated by Falcon *et al.* in 1994 (49). They showed a high prevalence of moderate hyperhomocysteinemia in patients with early-onset venous thrombosis, in whom other congenital or acquired causes of thrombophilia had been ruled out (49). In all but one patient, who had low serum levels of folates and vitamin $B_{12}$, acquired causes of hyperhomocysteinemia were excluded and family studies showed that most of the studied probands had at least one first-degree relative with the same abnormality. In this, as well as in subsequent confirmatory studies (50,51), the measurement of tHcy after an oral methionine loading allowed the detection of a greater number of patients with abnormal Hcy metabolism than did measurement of fasting levels alone. In 1995, the association between hyperhomocysteinemia and venous thrombosis was reported in a study of patients with a history of recurrent venous thrombosis (119). This study showed that the prevalence of hyperhomocysteinemia among cases was similar whether it was diagnosed by measuring fasting or PML levels of tHcy. It must be noted, however, that the two measurements were not always concordant, i.e., several patients with abnormal PML tHcy levels had normal fasting levels, and *vice versa*. It is evident, therefore, that in this study the combination of the two tests would also have identified a larger number of patients with impaired homocysteine metabolism than either test alone. Therefore, in laboratory screening of patients at increased risk of thrombosis, plasma tHcy should be measured both before and after an oral methionine loading (51). A high prevalence of hyperhomocysteinemia was later found in patients presenting with first episodes of deep-vein thrombosis of the lower extremities (51,120–122), although results were significant in only two studies (120,121). No association was found between hyperhomocysteinemia and the risk of deep-vein thrombosis of the upper extremities (123). In contrast to arterial thrombosis, the frequency distribution histograms found in a study relating tHcy to the risk for deep-vein thrombosis of the lower extremities indicated the existence of a threshold effect rather than a continuous dose-response relationship (120). Two meta-analyses of published case-control studies of hyperhomocysteinemia in venous thrombosis have been published. Both showed that both fasting and PML hyperhomocysteinemia are associated with a two- to threefold relative risk of venous thrombosis (124,125).

Whether hyperhomocysteinemia is associated with increased risk for venous thrombosis by itself or only when combined with other congenital risk factors has been a matter of debate, although the bulk of evidence now suggests that the thrombotic risk associated with hyperhomocysteinemia is independent of the coexistence of abnormalities of the natural anticoagulant system. Two studies of patients with homocystinuria gave conflicting results, i.e., in one, factor V Leiden did not prove to be a major determinant of thrombosis in patients with homocystinuria due to CBS deficiency (126), whereas the other study observed thrombotic complications only in those homocystinuria patients with concomitant heterozygous or homozygous factor V Leiden (127). Three reports showed that the association of mild-to-moderate hyperhomocysteinemia and venous thrombosis persisted after exclusion of patients with known congenital risk factors, such as deficiencies of natural inhibitors of coagulation and/or resistance to activated protein C due to factor V Leiden (49,120,121).

### *Prospective Studies*

*First episodes of venous thromboembolism.* Three prospective studies of tHcy as predictor of the risk for first episodes of venous thromboembolism gave essentially negative results (94,128,129). There was no association of hyperhomocysteinemia and the risk of venous thromboembolism in SLE patients (94), or in patients who underwent elective hip replacement surgery and were screened for postoperative deep-vein thrombosis with bilateral phlebography (129). Ridker *et al.* (128), in their nested case-control study of a subset of 22,071 male physicians participating in the Physicians' Health Study, did find an association between hyperhomocysteinemia and an increased risk of developing future episodes of idiopathic venous thrombosis, but only when hyperhomocysteinemia was associated with factor V Leiden, an established risk factor for venous thromboembolism. The association of hyperhomocysteinemia with idiopathic venous thrombosis in the absence of factor V Leiden tended to be significant ($P = 0.06$). The inclusion of men only and of patients with cancer in this prospective study are among the possible reasons accounting for the differences with case-control studies, which demonstrated an association between hyperhomocysteinemia and venous thrombosis that was independent of the coexistence of resistance to activated protein C or factor V Leiden (Table 3 in Ref. 130).

*Recurrent episodes of thromboembolism.* In a prospective, multicenter study of 264 patients with an objectively documented single episode of idiopathic venous thromboembolism, Eichinger *et al.* (131) recently showed that the risk of recurrent venous thromboemblism is higher (RR, 2.7; 95% CI, 1.3–5.8) in patients with hyperhomocysteinemia than in patients with normal tHcy levels.

### *Case-Control Studies of Genetic Abnormalities of Hcy Metabolism*

Studies of the prevalence of mutant C677T MTHFR in patients with venous thrombosis gave conflicting results (57,132–138). In three studies, C677T was shown to be a risk factor for venous thrombosis (132,136,137). The other studies failed to demonstrate an increased prevalence of C677T MTHFR among patients compared with controls. The coexistence of factor V Leiden or the G20210A mutation of the gene encoding for factor II and mutant C677T MTHFR conferred a particularly high risk for venous thrombosis in some studies (57,136,139) but not in others (133,134,140).

## IS HYPERHOMOCYSTEINEMIA A CAUSAL RISK FACTOR FOR ARTERIAL AND VENOUS THROMBOSIS?

The demonstration of an association between hyperhomocysteinemia and thrombosis has relied mainly on case-control and cross-sectional studies, which consistently showed that patients with previous episodes of arterial or venous occlusive diseases have higher tHcy plasma levels than healthy controls. However, prospective studies of individuals who were healthy at the time of their enrollment gave conflicting results. In addition, the lack of association of genetic abnormalities responsible for moderate hyperhomocysteinemia with an increased risk for thrombosis does not support the concept that hyperhomocysteinemia is causally related to the development of thrombotic events. Intervention studies with agents that lower the tHcy plasma levels and further prospective cohort studies on healthy individuals are necessary to shed more light on the problem of hyperhomocysteinemia as an independent and causal risk factor for arterial and venous thrombotic diseases. Screening for plasma tHcy levels is probably not yet justified in healthy individuals at low cardiovascular risk but may be advisable in individuals at high risk (141,142).

## HYPERHOMOCYSTEINEMIA IN OTHER PATHOLOGIC CONDITIONS

In addition to renal failure and cobalamin and folate deficiencies, hyperhomocysteinemia can be found in other pathologic conditions, such as hypothyroidism (143), inflammatory bowel disease (144), and rheumatoid arthritis (145), suggesting a potential mechanism for the high incidence of thrombotic complications in these patients. Hyperhomocysteinemia was also found in patients with renal or heart transplantation (reviewed in 146) with lymphoproliferative disorders (147) but not in patients with polycythemia (148). It is common in insulin-dependent diabetes mellitus if it is complicated by nephropathy and may contribute to increased mortality from cardiovascular disease in these patients (149); in addition, it is a very strong risk factor for cardiovascular complications in patients with noninsulin-dependent diabetes mellitus (150).

## MECHANISMS BY WHICH HYPERHOMOCYSTEINEMIA MIGHT PREDISPOSE TO ATHEROSCLEROSIS AND THROMBOSIS

The mechanism(s) by which hyperhomocysteinemia might contribute to atherogenesis and thrombogenesis are incompletely understood. *In vivo* studies in baboons showed that homocysteine causes endothelial cell desquamation, smooth muscle cell proliferation, and intimal thickening (151). *In vitro* studies showed that homocysteine-induced endothelial injury requires copper and oxygen and is prevented by catalase but not superoxide dismutase, suggesting that production of hydrogen peroxide is responsible for the toxic effect on endothelial cells (152). Other *in vitro* effects of homocysteine include the following: activation of factor V (153) and interference with protein C activation and thrombomodulin expression (154–156); inhibition of tissue plasminogen activator binding (157) and modulation of tissue plasminogen activator binding to annexin II tail domain (158); impaired generation and decreased bioavalability of endothelium-derived relaxing factor/nitric oxide (157–160) and prostacyclin (162), which are potent antiaggregating agents and vasodilators; induction of tissue factor activity (163); suppression of the expression on the vessel wall of the anticoagulant substance heparan sulfate (164); inhibition of ecto-ADPase (165); increase of DNA synthesis in aortic smooth muscle cells and inhibition of DNA synthesis in human umbilical vein endothelial cells (HUVEC) (166); enhanced collagen production and accumulation by smooth muscle cells (167); endoplasmic reticulum stress and growth arrest in HUVEC (168); and acceleration of endothelial cell senescence (169). It must be noted, however, that most *in vitro* effects of homocysteine on endothelial cells have been demonstrated for very high homocysteine concentrations, usually at least one order of magnitude higher than the plasma concentrations of homocysteine that can be found in patients with homozygous homocystinuria. The lack of control samples in which the effects of other thiols, such as cysteine, were studied casts doubt on the specificity of the observed effects. Therefore, the pathophysiologic relevance of most *in vitro* studies awaits confirmation from *in vivo* and *ex vivo* experiments.

The data of an *in vivo* study of the effects of diet-induced hyperhomocysteinemia on vascular functions in monkeys showed that Hcy decreases vascular relaxation in response to various stimuli and inhibits thrombomodulin-dependent protein C activation in aortic endothelial cells (170). These findings are consistent with the observation that *in vitro* exposure of endothelial cells to Hcy decreases the activity of nitric oxide and the activation of protein C. However, the finding that circulating plasma levels of activated protein C are not decreased in subjects with hyperhomocysteinemia and are increased in hyperhomocysteinemic patients with previous deep-vein thrombosis, questions the pathogenic role of the thrombomodulin-protein C pathway in Hcy-induced thrombosis (171). In agreement with these data, another *ex vivo* study showed that patients with homocystinuria due to CBS deficiency have high plasma levels of activated protein C

(172). These patients also had high plasma levels of markers of thrombin generation, and abnormally high *in vivo* biosynthesis of thromboxane $A_2$, as reflected by increased urinary excretion of its metabolite 11-dehydro-thromboxane $B_2$ (172,173).

Compelling evidence indicates that both chronic (174,175) and acute (induced by an oral methionine loading) (176–181) hyperhomocysteinemia impair the endothelium-dependent flow-mediated dilation of the brachial artery. The inhibitory effects of homocysteine were inhibited by antioxidant vitamins (178–180), suggesting that the adverse effects of homocysteine on vascular endothelial cells are mediated through oxidative stress mechanisms. However, this hypothesis has recently been questioned (181).

## TREATMENT OF HYPERHOMOCYSTEINEMIA

Estrogens reduce the mean plasma tHcy levels in postmenopausal women (36). The estrogen agonist/antagonist tamoxifen, which is the standard endocrine treatment for breast cancer, reduces tHcy levels not only in patients with advanced breast cancer (38), but also in healthy women (39), indicating that its lowering effect is due not only to its antitumoral activity, but also to its direct effect on estrogen-regulated targets. This finding may explain the observed reduction in coronary artery disease associated with tamoxifen treatment (182) and bears potentially important implications for the outcome of the ongoing trials of breast cancer prevention.

L-Thyroxine administration normalizes the high tHcy levels in patients with hypothyroidism (183).

The mainstay of treatment of hyperhomocysteinemia is folic acid, alone or in combination with cobalamin and vitamin $B_6$ (184). Although the three vitamins are often administered in combination, it appears that folic acid is the most effective agent because it also dramatically reduces the plasma tHcy fasting levels when given alone (185,186). A meta-analysis of 12 trials of reduction of tHcy by dietary supplementation with folic acid alone or in combination with vitamin $B_6$, vitamin $B_{12}$, or both, including 1114 individuals, was recently published (187). It showed that the proportional and absolute reductions in blood fasting tHcy produced by folic acid supplements were greater at higher pretreatment blood tHcy concentrations and lower pretreatment serum folic acid levels. After standardization to pretreatment tHcy levels of 12 μM and of folate of 12 nM, folic acid, at doses ranging between 0.5 and 5 mg/d, significantly reduced plasma tHcy levels by 25% (95% CI, 23–28%) (187). A lower dose of folic acid (0.5 mg every second day) decreased the tHcy levels by only 11% in 50 healthy women (188). Higher doses of folic acid (2.4 mg) may be necessary to treat hyperhomocysteinemia in renal transplant recipients (189,190). Vitamin $B_{12}$ (mean 0.5 mg/d) caused an additional reduction of 7% (3–10%), whereas vitamin $B_6$ (mean 16.5 mg/d) did not have a significant additional effect (187). However, vitamin $B_6$ should be added to folic acid and vitamin $B_{12}$ because it effectively reduces the PML tHcy levels (189). This finding corroborates the hypothesis that the PML test is most sensitive to the trans-sulfuration pathway of Hcy metabolism, in which vitamin $B_6$ acts as a cofactor (32,55,56).

Although it is quite clear that vitamins effectively reduce the plasma levels of tHcy, we do not yet know whether they will decrease the risk of vascular disease. On the basis of the graded effect of Hcy on coronary risk, a meta-analysis of 11 studies of the effects of increasing folic acid intake calculated that 13,500–50,000 deaths due to coronary artery disease could be avoided annually in the United States by fortification of food with folic acid (64). Cereal-grain products in the U.S. food supply are being fortified with folic acid to prevent neural tube defects. Cereals providing 127 μg of folic acid daily, which approximates the levels of folic acid fortification recommended by the Food and Drug Administration (140 μg/100 g cereal grain products), decreased plasma tHcy levels by only 3.7% in a recent controlled trial, which is probably insufficient to prevent cardiovascular disease (191). A subsequent trial showed that a meal providing mean folate intakes of 601 ± 143 μg/d decreased the serum tHcy concentration from 10.8 ± 5.8 to 9.3 ± 4.9 μM, whereas the normal diet, providing mean folate intakes of 270 ± 107 μg/d had no effects on tHcy levels (192). The first published study of the effects of folic acid fortification in the United States was done within the frame of the Framingham Offspring Study (193). It showed that folic acid fortification decreased the mean tHcy levels from 10.1 to 9.4 μM and the prevalence of high tHcy concentrations (>13 μM) from 18.7 to 9.8%. The most marked effect of folic acid fortification on tHcy levels was seen in subjects with hyperhomocysteinemia at baseline, whose mean levels decreased from 18.7 to 9.8 μM (193).

Recently, some interesting data from the Nurses' Health Study, including 80,082 women followed up for 14 yr, were published (194). All participants completed a detailed food-frequency questionnaire when they were enrolled in the study in 1980. From these data the usual intake of folate and vitamin $B_6$ was derived. After controlling for other cardiovascular risk factors, the RR of coronary heart disease between the extreme quintiles of total energy-adjusted daily intakes were 0.69 (95% CI, 0.53–0.87) for folate (median intake 696 vs. 158 μg/d), and 0.67 (0.53–0.85) for vitamin $B_6$ (median intake 4.6 vs. 1.1 mg/d). The risk of coronary heart disease was reduced among women who regularly used multiple vitamins (RR = 0.76, 0.65–0.9). These data strongly suggest that intake of folate and vitamin $B_6$ may be important in the primary prevention of cardiovascular disease.

In an uncontrolled study, the supplementation of vitamins (folic acid 2.5 mg/d, pyridoxine 25 mg/d, and cyanocobalamin 250 μg/d) to 50 patients with vascular disease and hyperhomocysteinemia for ~2 yr decreased the rate of progression of carotid plaque (measured by two-dimensional B-mode ultrasound) from 0.21 ± 0.41 $cm^2$/y to –0.049 ± 0.24 $cm^2$/yr (193). A lesser effect of the same vitamin regimen was also seen in 51 vascular disease patients with normal tHcy concentrations (195).

The recommended treatment of hyperhomocysteinemia today should include folic acid (at least 0.5 mg/d) and vitamin

$B_6$, with the addition of vitamin $B_{12}$ to secure full folic acid responsiveness and to avoid the risk of deteriorating cobalamin neuropathy in deficient patients, due to masking of hematologic changes by folic acid (196). The doses of vitamin $B_6$ and $B_{12}$ to be added in addition to folic acid have not yet been clearly defined (62). Ongoing prospective, placebo-controlled clinical trials will tell us whether the hypothetical preventive effect of vitamin supplementation on thrombotic diseases is real.

In conclusion, case-control and cross-sectional studies clearly indicated that mild-to-moderate hyperhomocysteinemia is associated with heightened risk of both arterial and venous thrombosis. On the other hand, additional studies are required to define unequivocally whether hyperhomocysteinemia is a causal risk factor for thrombosis, especially of the venous circulation. Among these, prospective cohort studies will clarify better the temporal relationship between high homocysteine levels and the thrombotic event. Most important, however, randomized, placebo-controlled, double-blind trials of the effects of vitamins on the thrombotic risk are urgently needed. They will help to define whether the relationship between hyperhomocysteinemia and thrombosis is causal (197).

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# Short-Term Folic Acid Supplementation Induces Variable and Paradoxical Changes in Plasma Homocyst(e)ine Concentrations

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**ABSTRACT:** Folic acid is presently the mainstay of treatment for most subjects with elevated plasma homocyst(e)ine concentrations [Plasma or serum homocyst(e)ine, or total homocysteine, refers to the sum of the sulfhydryl amino acid homocysteine and the homocysteinyl moieties of the disulfides homocystine and homocystein-cysteine, whether free or bound to plasma proteins.] Changes in homocyst(e)ine in response to folic acid supplementation are characterized by considerable interindividual variation. The purpose of this study was to identify factors that contribute to heterogeneity in short-term responses to folic acid supplementation. The effects of folic acid supplementation (1 or 2 mg per day) for 3 wk on plasma homocyst(e)ine concentrations were assessed in 304 men and women. Overall, folic acid supplementation increased mean plasma folate 31.5 ± 98.0 nmol/L and decreased mean plasma homocyst(e)ine concentrations 1.2 ± 2.4 μmol/L. There was evidence of substantial interindividual variation in the homocyst(e)ine response from –18.5 to +7.1 μmol/L, including an increase in homocyst(e)ine in 20% of subjects (mean increase 1.5 ± 1.4 μmol/L). Basal homocyst(e)ine, age, male gender, cigarette smoking, use of multivitamins, methylene tetrahydrofolate reductase, and cystathionine β-synthase polymorphisms accounted for 47.6% of the interindividual variability in the change in homocyst(e)ine after folic acid supplementation, but about 50% of variability in response to folic acid was not explained by the variables we studied.

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Biochemical and pathological studies in homocystinuric children led McCully to propose that moderately elevated blood levels of homocyst(e)ine may cause arteriosclerosis in adults who lack other characteristics of homocystinuria (1). The association of moderately high levels of homocyst(e)ine [plasma or serum homocyst(e)ine, or total homocysteine, refers to the sum of the sulfhydryl amino acid homocysteine and the homocysteinyl moieties of the disulfides homocystine and homocysteine-cysteine, whether free or bound to plasma proteins] and vascular disease has been confirmed in numerous studies (2). A recent review of about 80 clinical and epidemiological studies suggested that a moderately elevated level of homocyst(e)ine is a common risk factor for atherosclerosis and venous thromboembolism (3).

It could be questioned whether it is necessary or appropriate to treat moderately elevated levels of homocyst(e)ine before the results of ongoing clinical trials are available (4). However, the overwhelming positive association of high homocyst(e)ine levels and cardiovascular diseases (3), together with the accepted clinical practice of treating modifiable, established risk factors, makes us believe it is indeed justifiable to attempt to lower homocyst(e)ine levels with an inexpensive and generally innocuous vitamin therapy prior to the availability of supporting data from clinical trials.

Since the pioneering observation of Kang and colleagues (5) of the association between high blood levels of homocyst(e)ine and low blood folate, supplemental folic acid has been considered to be the mainstay of the treatment for an elevated level of homocyst(e)ine, with the addition of vitamins B-6 and B-12 as needed (6–8).

A metaanalysis of 12 studies showed that group averages of plasma homocyst(e)ine concentrations could be comparably reduced by folic acid supplementation at mean doses ranging from 0.5 to 5.7 mg per day (8). Although group mean plasma homocyst(e)ine levels are consistently decreased by folic acid supplementation (6–8), individual responses vary substantially. For example, Santhosh-Kumar *et al.* (9) reported "unpredictable inter-individual variation" in the response of homocyst(e)ine to folic acid supplementation. In one "unusual case," homocyst(e)ine increased when folate intake was raised from 286 to 516 μg/day (10), and a limited number of patients showed a small increase in the homocyst(e)ine concentration, following a 5-wk period ingesting folic acid-fortified breakfast cereal (11). Furthermore, Worley and Turi (12) reported that 30 to 50% of patients with homocyst(e)ine levels >10.5 μmol/L or post-methionine load levels >28 μmol/L failed to respond to pharmacological doses of folic acid, pyridoxine, and vitamin

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Abbreviations: BMI, body mass index; CBS, cystathionine β-synthase; CI, confidence intervals; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; ORPRC, Oregon Regional Primate Research Center.

B-12. In view of these observations, the present study was conducted to identify factors that contribute to interindividual heterogeneity in the effects of folic acid supplementation on levels of plasma homocyst(e)ine.

## SUBJECTS AND METHODS

The study population of 304 men and women included 242 subjects previously treated with folic acid (13) and a subset of 22 subjects who were retested 3 yr later, when all subjects in the upper and lower quintiles of plasma homocyst(e)ine changes following the folic acid supplementation were invited for a continuation of the study. Twenty-two subjects agreed to participate and received identical folic acid supplements as in the initial test. The present study population included new data on methylene tetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS) gene polymorphisms and excluded subjects receiving nicotinic acid. Subjects were recruited from internists or family physicians associated with Providence St. Vincent Hospital (Portland, OR), from a cohort of patients discharged with the diagnosis of ischemic heart disease (ICD 9 code 410-414), or were self-referred in response to advertisements. The study subjects were men and women 45–85 yr of age at the time of the initial interview. Subjects were excluded if they received medication(s) that might affect homocyst(e)ine levels (i.e., methotrexate, tamoxifen, anticonvulsants, niacin, bile acid sequestrants, or recent exposure to nitrous oxide anesthesia). Other exclusion criteria were missed lab appointments, ingesting ≥0.8 mg folic acid daily, or basal plasma creatinine levels ≥1.7 mg/dL. All subjects were advised to continue with their usual medications, including multivitamins, throughout the intervention. The study was approved by the Institutional Review Boards of Providence St. Vincent Hospital and Oregon Regional Primate Research Center (ORPRC).

Case subjects were diagnosed more than 3 mon previously with a history of acute myocardial infarction, angina pectoris documented by a cardiologist, percutaneous transarterial coronary angioplasty, or coronary bypass graft surgery. Control subjects had no self-reported or documented history of coronary heart disease. Case and control subjects reported having no history of stroke, intermittent claudication, or peripheral arterial revascularization. All subjects completed a medical history form, signed an informed consent form, and were then randomized to receive either 1 or 2 mg folic acid orally per day for 3 wk. The amount of folic acid selected accords with the data in the metaanalysis of the Trialists' Collaboration (8). The length of folic acid supplementation was sufficient to observe an effect on plasma homocyst(e)ine. Lowering in plasma homocyst(e)ine concentrations was reported after only 2 wk of supplementation with smaller doses than 1 or 2 mg per day by Seltzer *et al.* (14) and Brouwer *et al.* (15) as well as with larger doses by Brattstrom *et al.* (6). The need for a placebo group may be obviated by previous studies that demonstrated stability of homocyst(e)ine plasma levels during a 4-wk interval by Garg *et al.* (16) and Brouwer *et al.* (15). Subjects were instructed to arrive for laboratory appointments from 7:00 to 10:30 A.M., Monday through Friday, in the fasting state (i.e., no food after midnight) and were instructed not to take any vitamins on the morning of phlebotomy. During the first appointment, 1-mg folic acid tablets were given to the subjects, with appropriate instructions (1 or 2 tablets daily). During the second visit, subjects returned their remaining folic acid tablets for assessment of compliance.

Within 30 min of venous blood drawing, plasma was separated in a refrigerated centrifuge at 4°C for clinical chemistry; then plasma was frozen and stored at –20°C for duplicate analyses of homocyst(e)ine by high-pressure liquid chromatography and electrochemical detection as described (17,18) (performed at ORPRC by M.R. Malinow). Plasma aliquots were protected from light, frozen, and stored at –20°C for single radioassays of folic acid and vitamin B-12 (Bio-Rad Quantaphase II; Bio-Rad Diagnostics, Hercules, CA) (performed at ORPRC by D.L. Hess). The blood buffy-coat layer was separated, mixed with three drops of dimethylsulfoxide, and the blood cell pellets were frozen and stored at –20°C for genotype analyses (performed at Fox Chase Cancer Institute, Philadelphia, PA, by W.D. Kruger). The inter-run coefficients of variation for plasma samples were 6% for homocyst(e)ine, 13.5% for plasma folate, and 14.9% for plasma vitamin B-12.

*Statistical analysis.* The frequency distributions of participants' characteristics were examined. Comparisons of categorical variables were made using chi-square or Fisher's exact tests. Unadjusted mean differences in continuous variables such as body mass index (BMI) and levels of homocyst(e)ine, folate and vitamin B-12 were assessed using the Student's *t*-test statistic. The absolute change in homocyst(e)ine was calculated as the difference between the homocyst(e)ine level of post- minus pre-folic acid supplementation [Δ homocyst(e)ine in μmol/L]. Because we had previously demonstrated that 1 or 2 mg of folic acid supplementation has similar effects on plasma homocyst(e)ine (13), in agreement with the Trialist metaanalysis mentioned above (8), results from subjects receiving 1 or 2 mg folic acid supplementation were combined in our statistical analyses.

A least-squares multiple linear regression analysis was used to evaluate the relationships among the dependent variable, Δ homocyst(e)ine, and independent variables (Table 1). A modified stepwise selection algorithm was used for development of the final model. The level of statistical significance was fixed at $\alpha = 0.05$. Variables of *a priori* interest, but which did not reach statistical significance (e.g., multivitamin use, current cigarette smoking status, and MTHFR genotype) were forced in the final model. The adjusted $R^2$ values from the final models are reported and used as a measure of the total variation in Δ homocyst(e)ine that is explained by the final multivariate model.

Logistic regression analyses were conducted to identify characteristics that predict Δ homocyst(e)ine after folic acid supplementation. The study population for this analysis included subjects in the top [increased homocyst(e)ine] and bottom [decreased homocyst(e)ine] quintiles of the distribution of Δ homocyst(e)ine, thus containing only the extreme

**TABLE 1**
**Association in a Multivariate Linear Regression Model Between Several Characteristics and the Change in Homocyst(e)ine After Folic Acid Supplementation in 247 Subjects[a]**

| Covariate | Parameter estimate ± SE | *P* value |
|---|---|---|
| Intercept | –0.551 ± 2.16 | 0.799 |
| Age (yr) | 0.035 ± 0.012 | 0.003 |
| Male gender | 0.721 ± 0.230 | 0.002 |
| Current smoker | 0.837 ± 0.472 | 0.078 |
| Basal homocyst(e)ine | –0.478 ± 0.036 | 0.0001 |
| CBS (699 T/T or C/T and 1080 C/C) | 0.681 ± 0.244 | 0.006 |
| Multivitamins | –0.043 ± 0.230 | 0.853 |
| MTHFR 677 C/T | –0.243 ± 0.229 | 0.288 |
| MTHFR 677 T/T | –0.602 ± 0.359 | 0.096 |

[a]Negative sign indicates variable is associated with lower postsupplementation homocyst(e)ine level. Adjusted *R*-squared = 47.6%. 47.6% of the total variation in homocyst(e)ine change after supplementation is explained by these variables. CBS, cystathionine β-synthase; MTHFR, methylene tetrahydrofolate reductase. T/T, C/T, and C/C refer to genetic polymorphism.

responses (data not shown in tables). The odds ratio (OR) was used as a measure of association between extreme responses to folic acid supplementation and various participants' characteristics. Logistic regression procedures were used to calculate maximum likelihood estimates for the coefficient; their standard errors were used to calculate OR and 95% confidence intervals (CI) adjusted for confounding factors. Confounding was assessed by comparing the adjusted and unadjusted OR, after entering variables into a logistic regression model one at a time (19). Other statistical analyses were performed with Microsoft Excel 7.0 (Redmond, WA), PRISM GraphPad 2.0 Software, Inc. (San Diego, CA), and SAS Statistical Software (Cary, NC).

**TABLE 2**
**Characteristics of 304 Subjects[a]**

| Characteristics | Mean ± SD or *n* | % |
|---|---|---|
| Age (yr)[b] | 63 ± 10 | — |
| CHD[b] | 165 | 54.3 |
| Male gender[b] | 184 | 60.5 |
| BMI (kg/m$^2$)[b] | 27.5 ± 5.0 | — |
| Hypertension[b] | 121 | 39.8 |
| Current smoker[b] | 24 | 7.9 |
| Folic acid tablets (2 mg/d) (*n*)[b] | 154 | 49.3 |
| No multivitamins[b] | 178 | 58.6 |
| MTHFR 677 T/T (%)[b] | 27 [248] | 10.9 |
| CBS 699 T/T (%)[b] | 25 [246] | 10.2 |
| CBS 1080 T/T (%)[b] | 31 [246] | 12.6 |
| Basal homocyst(e)ine (μmol/L)[b] | 10.0 ± 3.6 | — |
| Homocyst(e)ine post-folic acid (μmol/L) | 8.8 ± 2.6 | — |
| Δ Homocyst(e)ine (μmol/L)[b] | –1.24 ± 2.4 | — |
| Basal folate (nmol/L)[b] | 19.7 ± 12.2 | — |
| Folate post-folic acid (nmol/L) | 51.1 ± 97.17 | — |
| Δ Folate (nmol/L) | 31.52 ± 98.0 | — |
| Basal vitamin B-12 (pmol/L)[b] | 350 ± 142 | — |
| Vitamin B-12 post-folic acid (pmol/L) | 321 ± 137 | — |
| Δ Vitamin B-12 (pmol/L) | –28.8 ± 74 | — |

[a]Values are mean ± SD or percentage; *n* = 304 subjects unless otherwise noted, i.e., figures in brackets indicate number of subjects studied.
[b]Variables entered into the multiple linear regression analysis. CHD, coronary heart disease; BMI, body mass index; See Table 1 for other abbrevia-

## RESULTS

Table 2 shows the characteristics of the 304 subjects. Consumption of folic acid tablets administered during the intervention (estimated from returned pill counts) was 99.7% (13). The substantial heterogeneity in the change in homocyst(e)ine after folic acid supplementation in the 304 subjects is evident in Figure 1.

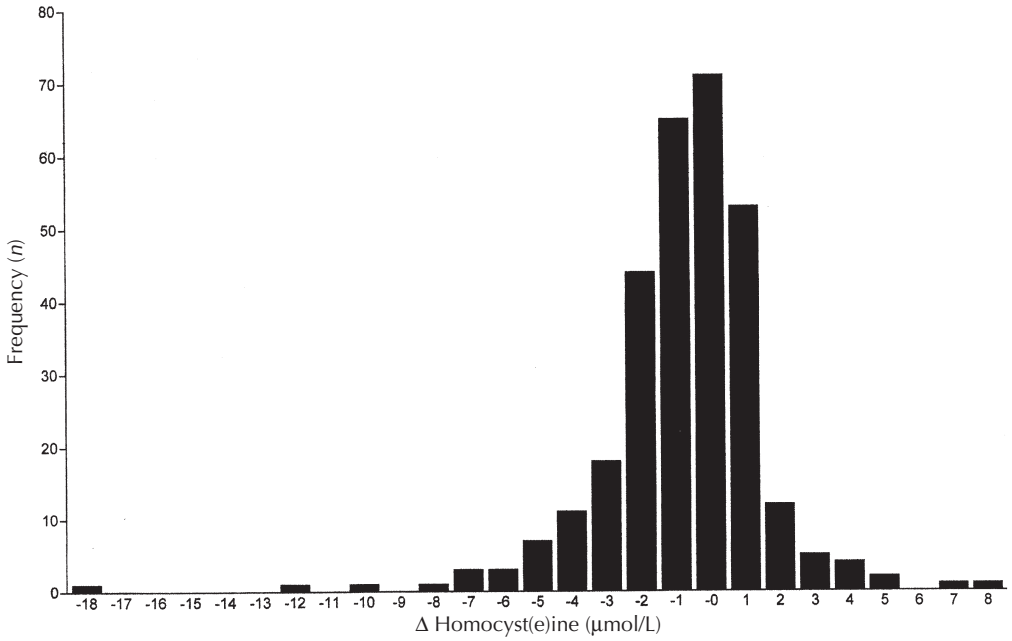


**FIG. 1.** Frequency distribution of changes in plasma homocyst(e)ine after folic acid supplementation for 3 wk in 304 subjects. Values vary between decreases of 19 μmol/L to increases of 8 μmol/L.

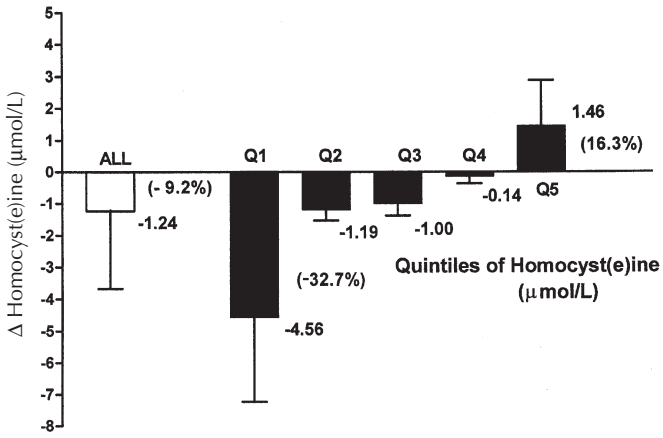


**FIG. 2.** The data of Figure 1 categorized by quintiles of change in plasma homocyst(e)ine in μmol/L. Although the overall effect was a decrease in homocyst(e)ine, an increase was observed in 20% of the subjects, whereas in the fourth quintile, only minor differences were observed that could be explained by the coefficient of variation of the method. Selected percentages indicate relative change in homocyst(e)ine. Quintiles were formed as follows: [Q1 < –2.5; Q2 = –2.49 to –1.38; Q3 = –1.37 to –0.48; Q4 = –0.47 to 0.29; Q5 > 0.30 to 7.3 (units are Δ homocyst(e)ine in μmol/L)].

There was a –1.24 ± 2.43 μmol/L mean decrease in homocyst(e)ine. The unadjusted data of Figure 1 were categorized by quintiles of changes in homocyst(e)ine to allow further analysis (Fig. 2). However, the change in homocyst(e)ine ranged from a mean of –4.56 μmol/L in the bottom quintile [decreased homocyst(e)ine] to a mean increase of 1.46 (μmol/L in the top quintile [increased homocyst(e)ine].

Males had a mean decrease of homocyst(e)ine after intervention of 0.98 μmol/L (± 1.74), whereas for women the decrease was 1.16 μmol/L (± 2.51). This difference was statistically insignificant in univariate analysis, but the result was confounded by the significantly different baseline homocyst(e)ine measurements in males and females (10.54 ± 3.14 vs. 8.53 ± 2.66, respectively; $P = 0.0001$). After adjustment for baseline homocyst(e)ine and the other factors in the model (Table 1), the difference in Δ homocyst(e)ine between males and females was 0.72 μmol/L ($P = 0.002$), with males having a smaller Δ homocyst(e)ine. As indicated in Table 1, the final regression model explained 47.6% of the total variation in homocyst(e)ine response.

Among users and nonusers of multivitamins, the mean decreases in homocyst(e)ine in the present study were –0.4 ± 1.67 μmol/L and –1.7 ± 2.74 μmol/L, respectively ($P < 0.0001$) (data not shown in tables). Because the univariate analyses suggested that the homocyst(e)ine response differs for users and nonusers of multivitamins (13), all least-square regression modeling was done for both groups separately. Results showed that although statistical significance was less frequently achieved, the β-coefficient for most of the independent variables was slightly greater for nonusers of multivitamins as compared with users of multivitamins (data not shown in tables).

Genetic polymorphisms in genes encoding two different enzymes involved in homocyst(e)ine metabolism were included in the model. These polymorphisms included the C 677T alteration in MTHFR, and the C699T and C1080T polymorphisms in CBS. Since the two polymorphisms in CBS are in strong linkage disequilibrium with each other, as shown by Kruger *et al.* (20), we decided to treat these two polymorphisms as a single variable. Therefore, individuals were grouped according to their combined genotype, i.e., individuals homozygous for C1080 and containing at least one 699T allele in one group, and all other genotypes in the other group. The MTHFR genotype was also divided into three groups, C/C vs. C/T and vs. T/T for MTHFR. When all other factors in the model were controlled for, subjects with the MTHFR genotype T/T experienced a mean 0.602 μmol/L (±0.359) greater reduction in homocyst(e)ine compared to those with the wild-type C/C genotype. Those with the MTHFR C/T genotype had a mean 0.243 μmol/L (±0.229) greater reduction.

Results from logistic regression analyses for Δ homocyst(e)ine, restricted to subjects in the top [increased homocyst(e)ine] and bottom quintiles [decreased homocyst(e)ine], were generally consistent with those from least-squares multiple linear regression analyses, although the analysis was hindered by the relatively small sample size. After adjusting for age, hypertension, gender, BMI, and cigarette smoking status, there was a very strong inverse relation between basal homocyst(e)ine and risk of increased homocyst(e)ine concentration after folic acid supplementation. For each unit increase in basal homocyst(e)ine, there was a 54% reduction in the likelihood of an increase in homocyst(e)ine after folic acid supplementation (OR = 0.46; 95% CI 0.35–0.62). Compared to females, males were 3.82 times more likely to experience an increase in homocyst(e)ine after folic acid supplementation (95% CI 1.14–12.85). Subjects above age 69 yr (OR = 1.92; 95% CI 0.60–6.15), with a history of chronic hypertension (OR = 1.57; 95% CI 0.50–4.88), or who reported cigarette smoking (OR = 4.41; 95% CI 0.82–23.51) also were more likely to experience an increase in homocyst(e)ine in response to folic acid supplementation (data not shown in tables).

A subgroup of 22 subjects was studied initially in 1995 and restudied in 1998. The basal homocyst(e)ine and folate levels in 1995 and 1998 were 9.6 ± 3.6 and 9.0 ± 2.7 μM ($P = 0.290$) and 18.1 ± 9.4 and 30.2 ± 11.8 μM ($P = 0.001$), respectively. The differences in basal folate may be related to the mandatory fortification of grain products with folic acid in the United States. since 1998 (26). Figure 3 shows the homocyst(e)ine responses in the 22 subjects studied in 1995 and 1998. Results document the marked intrasubject variability in responses.

## DISCUSSION

Following the demonstration of the inverse correlation between plasma homocyst(e)ine and folate levels (5), numerous authors have documented that folic acid supplementation in doses between 0.2 and 15 mg/d can lower plasma homocyst(e)ine concentration without apparent toxicity (4,21,22)

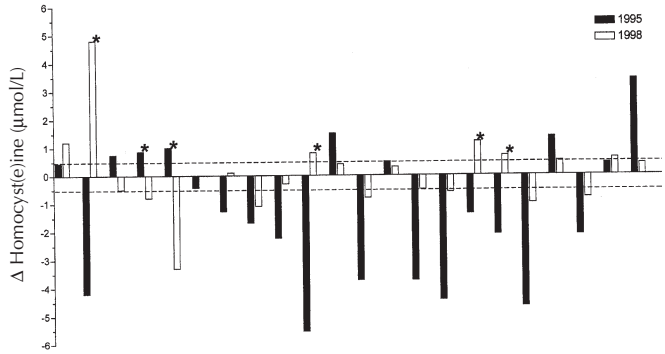


**FIG. 3.** Individual changes in homocyst(e)ine in 22 subjects studied in 1995 and 1998. Each pair of bars depicts data for a single subject in 1995 (■) and 1998 (□). Opposite responses of homocyst(e)ine in retesting, beyond the coefficient of variation of the method (indicated by dotted lines), were observed in six subjects (*).

and might beneficially modify a potentially important risk factor for vascular disease. Boushey *et al.* (23) estimated that an increased intake of folic acid ~400 μg/day would potentially prevent 50,000 deaths from vascular causes per year as a consequence of homocyst(e)ine lowering. A metaanalysis of 12 clinical studies, which included results from 1,114 patients, reported that supplementation with a mean dosage of 0.5 to 5.7 mg of folic acid per day reduced homocyst(e)ine levels by a mean of 25% (8). The smaller reduction of homocyst(e)ine (9.1%) following supplementation of 1 or 2 mg of folic acid/day in our study may be partly due to the use of multivitamins in 39.6% of the population (13). We previously have shown that the homocyst(e)ine-lowering effects of 1 or 2 mg of folic acid are similar, and that the effects of additional folic acid supplementation in users of multivitamins are minimal in most individuals (13).

In spite of the overwhelming evidence that folic acid supplementation lowers mean homocyst(e)ine levels in groups of subjects, there are occasional references of "unpredictable interindividual variation" in the response of homocyst(e)ine to folic acid supplementation (9,11). Moreover, Worley and Turi (12) demonstrated that a combination of pharmacological doses of folic acid and vitamins B-6 and B-12 failed to normalize homocyst(e)ine levels in 30 to 50% of patients with fasting homocyst(e)ine >10.5 (μmol/L, or post-methionine load homocyst(e)ine >28 μmol/L.

An apparent increase in homocyst(e)ine levels in response to folic acid fortification of grain products was also observed in recent reports of Framingham cohorts. Subjects studied before and after fortification of grain products experienced "a slight upward shift in homocyst(e)ine concentrations between examinations among those who used supplements" (24). Rader *et al.* (25) analyzed the folate content of folic acid–fortified foods and found that the measured amounts frequently exceeded the concentration recommended by the FDA (26). Their data suggest that grain products fortified with folic acid can easily provide ≥1 mg of folic acid per day in some individuals.

The results from our study demonstrated that basal homocyst(e)ine concentration, gender, CBS genotypes, and cigarette smoking influenced the response of homocyst(e)ine to folic acid supplementation. Male subjects with the lowest baseline homocyst(e)ine levels had the greatest risk of increasing their homocyst(e)ine level after treatment with folic acid. Previous univariate analyses of the response of the MTHFR genotype demonstrated a strong influence of the MTHFR T/T homozygote mutant, as well as the intake of multivitamin supplementation, on the responses of plasma homocyst(e)ine to folic acid supplementation (13). However, these effects did not attain statistical significance in the present multivariate model. Further research is needed to identify mechanisms responsible for the association between these factors and heterogeneity in responses of homocyst(e)ine to folic acid supplementation.

It is unknown whether pharmacological doses of B vitamins (including folic acid) will affect the course of atherosclerotic disease. Data from current clinical trials will probably provide an answer to this important question. Additional data are required to delineate the differential effects on atherosclerosis of changes in homocyst(e)ine levels after folic acid supplementation vs. those possibly independent, direct effects of B vitamins on atherothrombosis, or other aspects of atherosclerosis.

If the paradoxical finding of increased homocyst(e)ine in response to folic acid supplementation is confirmed, it may also have implications for the health and well-being of pregnant women and their fetuses. For example, pregnant women commonly have low homocyst(e)ine levels (i.e., <5.0 μmol/L) (27), but slightly elevated homocyst(e)ine levels (i.e., 8.7 μmol/L) may be associated with abnormal pregnancy outcomes (27). Increases in homocyst(e)ine after folic acid supplementation were observed in our study, especially when initial plasma homocyst(e)ine levels were low. If pregnant women respond similarly to routine administration of folic acid as nonpregnant women in our study, folic acid supplementation may cause mild increases in homocyst(e)ine in some individuals. If elevated levels of homocyst(e)ine are confirmed to contribute to the pathogenesis of abnormal pregnancy outcomes, an increase in homocyst(e)ine secondary to folic acid supplementation and/or fortification might have detrimental effects. Further studies are needed to evaluate this possibility.

Although folic acid supplementation lowered the mean homocyst(e)ine concentration in the present cohort, our data suggest there is considerable heterogeneity in responses to folic acid supplementation, as measured by absolute changes in homocyst(e)ine. In addition, 20% of the subjects responded to folic acid supplementation by increasing their mean homocyst(e)ine level 1.4 μmol/L. The homocyst(e)ine responses were not reproducible when subjects were retested 3 yr later. Thus, our observations suggest that repeat testing may be required to verify responsiveness of homocyst(e)ine to folic acid. The factors we studied accounted for 47.6% of the variation in the change in homocyst(e)ine after folic acid supplementation, yet about 50% of the response was unexplained by the factors studied here. Further follow-up studies are needed to (i) identify determinants of homocyst(e)ine responses to folic acid,

including the mechanism(s) responsible for a paradoxical increase in homocyst(e)ine concentration, and (ii) determine whether sustained increases in homocyst(e)ine after folic acid supplementation are associated with adverse effects on health.

## ACKNOWLEDGMENTS

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# The Hordaland Homocysteine Studies

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**ABSTRACT:** The Hordaland Homocysteine Study is a population-based screening of total plasma homocysteine (tHcy) in ~18,000 men and women aged 40–67 yr that took place in 1992–1993 in the county of Hordaland in Western Norway. In this cohort, tHcy was associated with several physiologic and life-style factors, including age and gender, blood pressure, serum cholesterol, smoking, alcohol and coffee consumption, physical activity, diet, and vitamin status. All associations with established cardiovascular risk factors were in the direction expected to confer increased risk. In a subset of 5,883 women aged 40–42 yr, tHcy was associated with previous pregnancy outcomes, including preeclampsia, placental abruption, and neural tube defects. This article reviews the published results from the Hordaland Homocysteine Study in the light of relevant literature. The Hordaland Homocysteine cohort will be used for future investigations of the stability of tHcy and vitamin status over time, and to investigate associations with mortality and morbidity including cancer incidence.

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The Hordaland Homocysteine Study is an investigation of 18,043 men and women, aged 40–67 y, living in Hordaland County in Western Norway (Fig. 1). The study was a collaboration between the University of Bergen and the National Health Screening Service. Eligible subjects were selected from the National Population Register, identified by site of residence and age on December 31, 1992, and plasma total homocysteine (tHcy) was determined in all subjects in 1992–1993. The aim of the study was to obtain cross-sectional data on the relationship between tHcy and life style and risk factors related to chronic diseases, in particular, cardiovascular disease (CVD). Furthermore, the long-term prospect of this study was to relate the tHcy concentration to future all-cause and cardiovascular mortality and morbidity. Finally, we used the collected data to assess relationships between tHcy level in 1992–1993 and previous pregnancy outcomes in the female participants of the Hordaland Study.

### *Recruitment and Data Collection*

A total of 24,815 subjects from three different age groups were invited to participate; the overall acceptance rate was 72.7%. The younger and the largest group ($n$ = 12,594) included all subjects in the county aged 40–42 yr. The older group ($n$ = 4,766) covered all subjects aged 65–67 yr in the city of Bergen. A third group ($n$ = 683), aged 43–64 yr, was a 2% random sample of residents in Bergen. Men and women in the younger and older age group represent the four main groups.

Data on type of work, physical activity, smoking habits, medical history of CVD, hypertension, diabetes mellitus, and food and vitamin intake were obtained by questionnaire. Blood samples from nonfasting subjects were collected into evacuated EDTA tubes and placed in a refrigerator (4–5°C) for 15–30 min; the plasma fraction was isolated within 1–3 h and stored at –20°C until analysis. tHcy was determined in plasma with high-performance liquid chromatography (HPLC) and fluorescence detection (1).

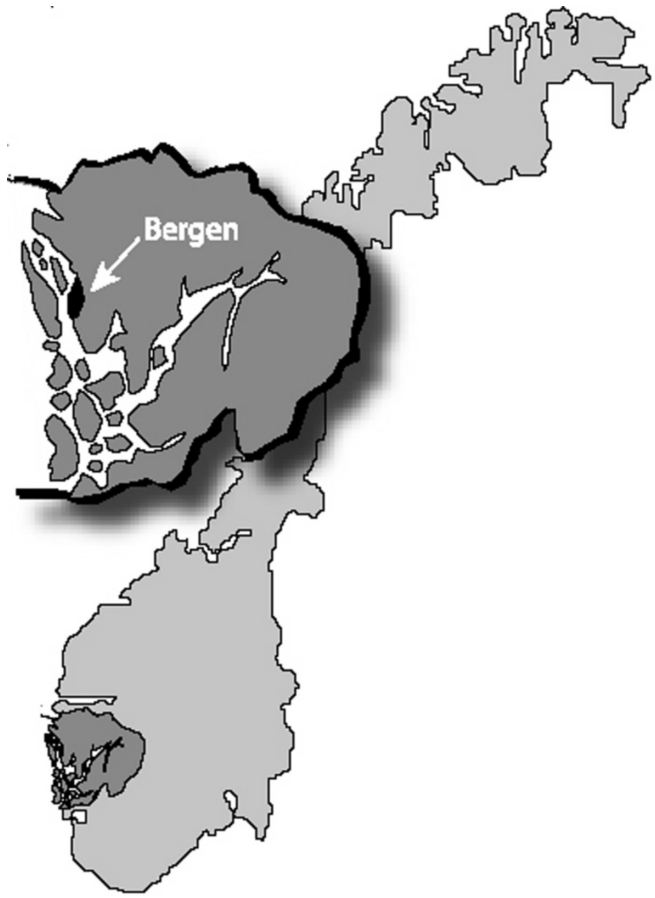


**FIG. 1.** The county of Hordaland is located on the western coast of Norway.

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Abbreviations: CVD, cardiovascular disease; HPLC, high-performance liquid chromatography; MTHFR, methylenetetrahydrofolate reductase; tCys, total cysteine; tHcy, total homocysteine.

### *Age, Gender, Blood Pressure and Cholesterol*

Plasma tHcy showed a skew distribution, with a tail toward higher levels in all four main age and sex groups investigated. The arithmetic means were 11.4 μM in 5,918 healthy men and 9.6 μM in 6,348 women aged 40–42 yr, demonstrating that tHcy is ~2 μM higher in men than in women. In the oldest age group (65–67 yr), the corresponding values were 13.0 and 11.6 μM. Thus, there was an increase in tHcy by age, which was more pronounced in women than in men (2).

There are consistent data based on large populations (3,4), demonstrating age- and sex-related differences in tHcy. Higher tHcy in men than in women has been explained by difference in muscle mass and hormonal effects, whereas the age-related increase is probably due to deterioration of renal function and impaired vitamin status with age (4,5).

The Hordaland study demonstrates a positive relation between tHcy and diastolic and systolic blood pressure. tHcy also showed a positive relation to serum cholesterol. These relations were essentially confined to the younger age group, and the difference in tHcy between the extreme groups was <1 μM after multivariate adjustments (2). A weak relation between tHcy and serum cholesterol has been demonstrated in some studies (6–8). Conceivably, thyroid status is a strong determinant of both tHcy and serum cholesterol (9), and may explain the correlation between these indices. The weak association between tHcy and blood pressure is not a consistent finding (10–12).

### *Life-Style Factors*

*Smoking and alcohol.* The first large study to address these issues was the Hordaland Homocysteine Study (13). There was a strong graded relationship between the number of cigarettes and tHcy levels, independent of age and sex (2), and folate intake (14). Notably, smoking was associated with an increased mean tHcy and a shift of the whole tHcy distribution curve to higher levels, similar to that observed in populations with low folate intake (14). The effect from smoking was more pronounced in women than in men. In women aged 40–42 yr, the estimated tHcy increase corresponded to 2% per cigarette/d. Comparing never smokers with heavy smokers (≥20/d), plasma tHcy levels were 8.7 and 10.7 μM in 40- to 42-yr-old women, and 10.5 and 11.7 μM in 40- to 42-yr-old men. There was no significant difference in tHcy between former smokers and subjects who had never smoked. Among smokers, the increase in tHcy was independent of the number of years of smoking (14).

A positive relation between smoking and tHcy has been demonstrated in most (12,15–17) but not all (18) smaller studies. Smokers in general consume a less healthy diet than nonsmokers, and elevated tHcy may be related to the effect of smoking on homeostasis of B-vitamins involved in homocysteine metabolism, including folate, vitamin $B_6$, and vitamin $B_{12}$ (5).

In the Hordaland cohort, alcohol consumption showed a weak U-shaped relation to tHcy with a negative association with consumption up to 14 units per week. This effect was markedly stronger in smokers (19).

The first studies on alcohol effect on tHcy were done in abusers and chronic alcoholics, and in these subjects, a marked increase in tHcy was observed (13). A positive relation between tHcy and blood alcohol concentration in alcohol abusers has recently been reported (20). The effect of moderate alcohol consumption was investigated in the large population-based Caerphilly cohort (21), and in this study, alcohol intake was negatively associated with tHcy and showed a positive relation to folate intake. The authors suggest that the tHcy reduction is mediated by folate (21). Smaller studies have demonstrated increased tHcy or no effect from moderate alcohol consumption (13). A recent intervention trial demonstrated that consumption of liquor and red wine, but not beer, increases the tHcy level (22). The beverage specificity may be related to the large amount of folate and vitamin $B_6$ in beer and negligible amounts in red wine and spirits (22).

*Physical activity.* There was a negative relation between physical activity in leisure time and plasma tHcy in the Hordaland cohort. After multivariate adjustment, individuals reporting heavy exercise had tHcy ~0.5 μM lower than individuals who characterized themselves as sedentary (2). Other observational studies noted no (11,23) or only a minor (12) effect from physical activity. One experimental study demonstrated a moderate increase in tHcy after acute exercise, which paralleled hemoconcentration (24), whereas another study demonstrated no tHcy response (25).

*Coffee.* Daily coffee consumption was registered for 89% of the Hordaland study participants, and among these, 95% used filtered coffee. There was an unexpected and strong positive association between plasma tHcy and consumption of various types of coffee, except decaffeinated coffee. This relation was almost as strong as that observed between tHcy and smoking, and was also found in nonsmokers and at both high and low folate intake. Heavy coffee consumption increases mean tHcy by decreasing the proportion with low and intermediate tHcy (<17 μM), and in this respect can be distinguished from folate deficiency and cigarette smoking (26).

The strong effect of heavy coffee consumption on plasma tHcy has been confirmed in several recent studies (27,28). Moderate coffee consumption in the Atherosclerosis Risk In Communities (ARIC) cohort was not associated with elevated tHcy (29), but recent intervention trials demonstrated that 1 L/d of unfiltered (30) as well as filtered coffee (31) increased tHcy by ~1.5 M. The coffee effect was observed within 2 wk and was reversible (31). Serum levels of folate, vitamin $B_6$, or vitamin $B_{12}$ did not change during the coffee consumption period (31). In the Framingham offspring cohort, the tHcy rising effect was observed from coffee and other caffeine-containing beverages (e.g., cola) but not from decaffeinated coffee (28). The elevation of tHcy by coffee is probably mediated by caffeine, which may influence the cardiovascular system or kidney function, or possibly interfere with vitamin $B_6$ function (5).

*Diet and vitamins.* In the Hordaland study, we constructed and verified folate and cobalamin intake scores, based on con-

sumption of various dietary items and vitamin supplements (14). Folate derived from food showed a weaker negative relation to plasma tHcy than folate taken as supplements. We could distinguish between the reduction of high tHcy to normal levels, which is usually conferred by folate derived from food, and the reduction from normal to subnormal levels, which is attributable to intake of folic acid supplements (14).

A large and consistent literature describes the effect of B-vitamins involved in homocysteine metabolism on plasma concentration of tHcy. In folate- or cobalamin-deficient subjects, tHcy is markedly reduced by treatment with the deficient vitamin (32). In subjects with no overt vitamin B deficiency, folate supplementation induces an average reduction of tHcy by ~25% (33), and there is essentially no additional effect from vitamin $B_6$ or cobalamin (33,34). Vitamin $B_6$ supplementation alone has no or only a modest effect on fasting tHcy (35). In several large population-based studies, tHcy shows a strong, inverse relation with serum folate concentration or folate intake, a weaker relation to serum cobalamin concentration, and often no relation to estimated intake of cobalamin. Both serum level and intake of vitamin $B_6$ show a significant relation to tHcy (13,28,36). Thus, folate status in particular but also cobalamin status are established determinants of fasting tHcy, whereas the observed vitamin $B_6$ effect may represent confounding from folate, and vitamin $B_6$ seems important to control postmethionine load tHcy.

*Combined effects and tail effects.* A life-style profile that reflects the combined effect of three major modifiable tHcy determinants, i.e., folate intake, smoking, and coffee consumption, is strongly correlated with tHcy (14). Subjects with a contrasting lifestyle with respect to these factors have a difference of 3–5 μM in tHcy, a difference that is larger than the effect attributable to each factor alone. This supports the notion of different mechanisms underlying the tHcy elevating effects of smoking, low folate intake, and heavy coffee consumption. Furthermore, tHcy is essentially normally distributed in a population characterized by a high folate intake, low coffee consumption, and nonsmoking (14).

The major tHcy determinants (age, gender, coffee consumption, smoking, and no intake of vitamin supplements) show different relationships with the extremes of the tHcy distribution curve (13). Male gender, old age, and heavy coffee consumption decreased the likelihood of having low tHcy (<7 μM), but had essentially no effect on the likelihood of having moderately (15–30 μM) or markedly elevated (30–100 μM) tHcy. Thus, these factors had their main effect on the lower part of the tHcy distribution curve. In contrast, smoking and no intake of vitamin supplements decreased the likelihood of low tHcy and increased the likelihood of moderately and markedly elevated tHcy, and thereby were associated with a displacement/shift of the whole tHcy distribution curve to a higher level (Fig. 2). These distribution effects suggest different mechanisms whereby age, gender, coffee consumption, smoking, and no intake of vitamin supplements affect the tHcy concentration, but also raise the possibility that the tHcy elevation related to coffee consumption and increasing age has less adverse effects than the high levels associated with smoking and impaired vitamin status (13).

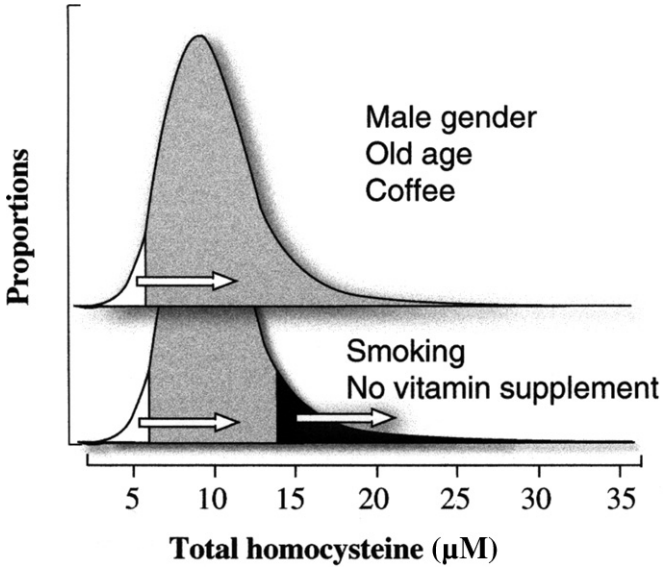


**FIG. 2.** Scheme of tail effects of total homocysteine (tHcy) determinants. The determinants gender, age, and coffee consumption, presented in the upper panel, have a selective effect on the lower part of the tHcy distribution curve. Smoking and no use of vitamin supplements (lower panel) cause a shift of the whole distribution curve to higher levels.

### *Intermediate Hyperhomocysteinemia and the 677C→T Methylenetetrahydrofolate Polymorphism*

Among the 18,043 subjects investigated in the Hordaland Homocysteine Study, 67 (0.4%) had tHcy ≥40 μM (37). Compared with controls, these subjects had lower plasma folate and cobalamin levels, lower intake of vitamin supplements, consumed more coffee, and were more frequently (60%) smokers. When seven subjects with cobalamin deficiency were excluded, 92% of these hyperhomocysteinemic subjects (compared with 10.4% of controls) were homozygous for the 677C→T methylenetetrahydrofolate reductase (MTHFR) polymorphism. These findings demonstrate a strong positive interaction between MTHFR genotype and life-style determinants of tHcy (37).

The 677C→T MTHFR polymorphism has been studied extensively during the last 5 yr because it is a genetic determinant of tHcy. MTHFR catalyzes the irreversible formation of 5-methyletrahydrofolate which serves as a methyl donor in the remethylation of Hcy to methionine (38). The T-allele codes for a thermolabile enzyme variant with low catalytic activity and reduced affinity for 5-methyltetrahydrofolate and the MTHFR co-factor, FAD. This explains the finding of elevated tHcy in subjects with the combination of the TT genotype and impaired folate status, and also the recent observation that low plasma riboflavin (vitamin $B_2$) increases tHcy in subjects with the T-allele (39). However, there is effect modification by the MTHFR polymorphism of several factors predisposing to hyperhomocysteinemia, which cannot readily be

explained by the enzymic properties of the thermolabile MTHFR variant. Thus, low serum cobalamin, renal failure, and drugs such as L-dopa and antifolate agents cause higher tHcy in subjects with the TT-genotype than those with the CC genotype (38). It therefore seems that the T-allele is associated with increased propensity toward hyperhomocysteinemia, which is in accordance with the high prevalence of the TT genotype and negative life-style factors in hyperhomocysteinemic subjects in the Hordaland cohort.

### *Total Cysteine vs. the Cardiovascular Risk Profile*

The relationship between the cardiovascular risk profile (which included life-style factors, age, gender, blood pressure, and cholesterol) and total concentration of another plasma aminothiol, cysteine, was investigated in the Hordaland cohort (40). Total cysteine (tCys) and tHcy showed a distinct and differential relation to components of the risk profile. Age, cholesterol, diastolic blood pressure, and coffee were positively related to both tCys and tHcy; body mass index showed a strong positive relation only to tCys, whereas smoking, folate and vitamin intake, heart rate, and physical activity (which were associated with tHcy) showed no relation to tCys (40).

There are a few studies (17,41–43) demonstrating significantly higher levels of plasma tCys in vascular patients than in healthy controls, suggesting that tCys is associated with cardiovascular risk. Furthermore, cysteine and homocysteine are interactive components of the plasma redox thiol status (44). Therefore, knowledge of tCys determinants is important to understand the possible pathogenic role of cysteine and to assess confounding of the observed disease-tCys relationships.

### *Pregnancy Outcomes*

Adverse pregnancy outcomes were investigated in 5,883 women from the age group 40–42 yr in the Hordaland cohort (45). Records of 14,492 pregnancies in the period from 1967 to 1996 were retrieved from the Medical Birth Registry of Norway, and outcomes were related to the tHcy level measured in 1992–1993.

The levels of tHcy in 1992–1993 showed a strong, concentration-dependent association with preeclampsia, prematurity, and low birth weight, whereas a moderate association was observed with stillbirth. An increased frequency of placental abruption was observed at tHcy > 15 μM. Plasma tHcy was also related to malformations such as neural tube defects and clubfoot, but not to orofacial cleft (45).

Of the pregnancies investigated, ~80% occurred more than 10 yr before the tHcy measurements. The associations with preeclampsia, prematurity, low birth weight, and stillbirth were strongest in the time interval closest to the tHcy determinations. Thus, the long time interval weakens the association between plasma tHcy and pregnancy outcomes.

The relationship among folate status, plasma tHcy, and pregnancy outcome has been investigated in several smaller studies (46–48). Most have a case-control design and demonstrate that hyperhomocysteinemia is associated with habitual miscarriage, preeclampsia, placenta abruption, thromboembolic events, neural tube defects, and perhaps with fetal death *in utero* and intrauterine growth retardation (46–48).

### *Prospective Analysis of Mortality*

The ability of tHcy to predict mortality was investigated in a population of 587 patients with angiographically verified coronary artery disease recruited from the Haukeland University hospital. The median follow-up time was 4.6 yr. There was a strong, graded relation between tHcy and both cardiovascular and overall mortality. After 4 yr, 3.8% of the patients with tHcy < 9 μM had died, compared with 24.7% of those with tHcy ≥ 15 μM (49). Notably, mortality rate was highest in patients with other established CVD risk factors, in particular diabetes mellitus, low ejection fraction, and elevated fibrinogen (Fig. 3). These data have inspired investigations of the association between tHcy and future CVD events and overall mortality and morbidity in the Hordaland cohort, and two Norwegian ongoing secondary intervention studies with tHcy-lowering B-vitamins in coronary patients

By the end of year 2000, there were ~35 prospective studies on the association between tHcy and CVD (50–61). About half (9 of 18) of the population-based studies and most (14 of 17) studies in clinical cohorts or in elderly demonstrated a significant positive relation. Thus, tHcy seems to be a particularly strong predictor of cardiovascular events or death in subjects with a preexisting illness, such as coronary heart disease, diabetes, or renal failure (62).

### *Conclusion and Perspectives*

The first part of the Hordaland Homocysteine Study provided cross-sectional data on the relation between plasma tHcy and several established cardiovascular risk factors and life style in a large general population ($n$ = 18,043) of men and women. The study demonstrated for the first time a positive relation between tHcy and coffee consumption, and affirmed the as-

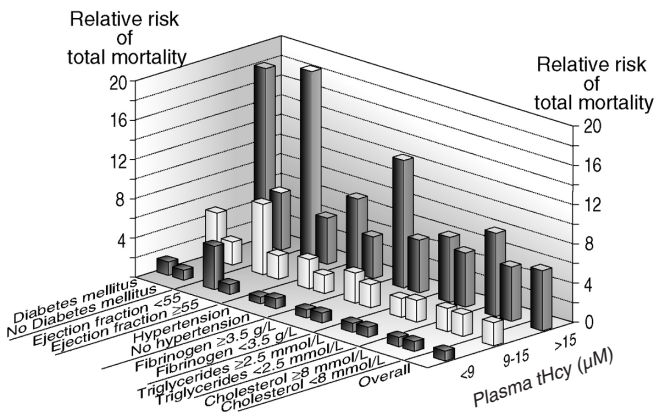


**FIG. 3.** Total mortality according to plasma total homocysteine (tHcy) and other established risk factors for cardiovascular disease. Data from Reference 49.

sociations between tHcy and age, sex, smoking, and blood pressure. The magnitude of this cohort allows the investigation of a diversity of clinical conditions related to tHcy in the same population, as demonstrated by the study on pregnancy outcomes. Ongoing and future investigations include studying the relationship between baseline tHcy and all-cause and cause-specific mortality, cardiovascular morbidity, and cancer incidence. Finally, on-going reinvestigation in 1998–1999 of ~6000 subjects from the age groups 40–42 and 65–67 yr in 1992–1993 allows assessment of the stability of tHcy and folate status over time, the relation to single nucleotide polymorphisms, and the association of two measurements with mortality and morbidity due to common diseases.

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# The Oxidant Stress Hypothesis of Atherogenesis

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**ABSTRACT:** Atherosclerosis is the commonest lesion of blood vessels and is responsible for life-threatening events such as myocardial infarction and stroke. In the last two decades a series of excellent studies unraveled biochemical mechanisms that provided the background for a theory of atherogenesis. This theory is centered on foam cells and on free radical–mediated modification of low density lipoprotein (LDL). Foam cells are the main cell type of atherosclerotic lesions and originate from monocytes migrated from blood and from smooth muscle cells of the arterial wall. Foam cells are engulfed of lipids taken from LDL. Paradoxically, accumulation of LDL in developing foam cells does not occur *via* the classic LDL receptor. Incubation of macrophages with even very high concentrations of LDL does not appreciably increase cholesterol content. Chemically modified LDL easily enter the cells of atherosclerotic plaque *via* an unregulated receptor, the scavenger receptor. The most studied chemical modification of LDL is that induced by free radicals.

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Half of the mortality in the Western population is due to disease of the cardiovascular system in which the main pathophysiologic factor is atherosclerosis. This process occurs at a young age in the arteries as accumulation of lipids in the subintimal area, known as fatty streaks (1). After years in localized areas of the arterial tree, the fatty streak undergoes changes to form an atherosclerotic plaque, which causes progressive narrowing of the lumen and eventually ischemia. The atherosclerotic plaque is composed of lipids in the extracellular space and within cells, cells, fibrosis and calcium deposits as the result of complex events of cell migration, cell growth, and apoptosis mediated by multiple cytokines and growth factors (2). Acute events associated with plaque rupture are local thrombosis and embolization, which are responsible for life-threatening events, i.e., myocardial infarction and stroke (3).

For a long time, only the manifestations of the disease and its consequences were known; the proposed biochemical mechanism of atherogenesis remained largely speculation. A central role in the atherogenic process is played by two components, i.e., cholesterol and macrophages, the cells that accumulate cholesterol in the plaque.

It has been known for many years that high levels of blood cholesterol are associated with premature death. The role of cholesterol was initially elucidated by studying familial hypercholesterolemia (FH), a clear-cut human model of premature atherosclerosis (4). FH is an inborn error of metabolism that causes very high blood cholesterol levels and heart attack in young people. Heterozygotes are frequent in the general population, accounting for 1:500 persons. They have twofold the normal cholesterol levels of low density lipoprotein (LDL)-cholesterol even before they are born, develop premature atherosclerosis, and frequently suffer heart attacks by the age of 35 yr. Homozygous individuals (1:100,000) can develop myocardial infarction even in the first 5 yr of life, and by the age of 20 yr, myocardial infarction is practically inevitable. However, there is considerable variation in the atherosclerotic disease at any given level of cholesterolemia, even in FH patients (5). On the other hand, atherosclerosis may also be present in persons with cholesterol levels <200 mg/dL; some patients with heterozygous FH and high cholesterol levels can survive until they are ≥70 yr old without signs of coronary heart disease (CHD).

Why and how cholesterol accumulates in the arteries remain largely unknown. Cholesterol is carried in plasma mainly in the LDL fraction, composed of a protein moiety, the apolipoprotein (apo) B100, an outer layer of phospholipids and free cholesterol, and a core composed of triglycerides and esterified cholesterol (6).

In 1974, Brown *et al.* (4) elucidated the receptor pathway of cholesterol influx into cells. Apo B100 binds to a specific cell membrane receptor, the complex is internalized, the protein component is degraded in lysosomes, and cholesterol is shifted into the cell cholesterol pool. Cells have fine-tuned mechanisms for cholesterol homeostasis. Intracellular cholesterol levels inhibit the activity of β-hydroxyl-β-methyl glutarate CoA reductase, with consequent inhibition of cholesterol synthesis, activate the acyl-CoA:cholesterol transferase enzyme to maintain low free cholesterol levels, and suppress the synthesis of the LDL receptor. The receptor has been shown to function in several cell types and is not present in an active form in skin fibroblasts from homozygous FH. Thus, understanding the LDL receptor pathway led to the explanation that the high plasma cholesterol of these patients was due to a reduction of LDL clearance. However, this discovery did not explain how these patients accumulated cholesterol in foam cells in the plaque. In other words, why did these patients with no B100 receptors have foam cells in plaque?

Goldstein *et al.* (7) demonstrated the absence of B100 receptor activity in macrophages that do not accumulate cholesterol

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Abbreviations: apo, apolipoprotein; CHD, coronary heart disease; FH, familial hypercholesterolemia; IP, isoprostane; LDL, low density lipoprotein; MDA, malondialdehyde.

if incubated with LDL. In contrast, it was shown that macrophages accumulated large amounts of cholesterol if incubated with LDL previously modified by acetylation. Thus, it was demonstrated that a second pathway of cholesterol influx can operate in macrophages *in vitro*, and is not present in fibroblasts. This receptor, the scavenger receptor, recognizes modified LDL and is not downregulated by cholesterol influx so that the macrophage continues to take up modified LDL until it is engulfed with lipids and becomes a foam cell (8). However, acetylation does not occur *in vivo*, and the physiologic importance of this receptor remained initially unknown until Fogelman and colleagues (9) reported on the LDL modification by malondialdehyde (MDA), which derivatizes lysines as occurs with acetylation. MDA-modified LDL induced cholesterol accumulation in human monocyte/macrophages *via* the scavenger receptor. The physiologic plausibility of the MDA-modification of LDL was suggested by generating MDA-LDL after incubation of LDL with platelets (9). In fact, MDA is formed during the arachidonic acid metabolism in platelets in nearly equimolar amounts with thromboxane $A_2$. Furthermore, Henriksen *et al.* (10) showed that LDL incubated with endothelial cells could be modified to bind to the scavenger receptor, in association with the peroxidation of LDL lipids (11,12).

In the last two decades, the discovery of the scavenger receptor pathway of cholesterol accumulation in monocyte/macrophages has stimulated an extensive investigation into the mechanism of LDL oxidation and its effect on atherosclerosis. In brief, peroxidation is initiated by free radicals that attack the very highly oxidizable polyunsaturated fatty acids, cholesterol, and other LDL lipids. The source of free radicals can be cells (endothelial cells, smooth muscle cells, activated monocytes, macrophages, and neutrophils), hemoglobin, and other metalloenzymes (6). The oxidation of LDL lipids induces formation of intermediates such as MDA and other aldehydes capable of derivatizing apo B100. The final result is modification of LDL into an analog with alterations of both the protein and lipid moiety that is recognized by the scavenger receptor; it is called oxidized LDL. The lipid peroxidation of LDL is important not only for protein modification and consequent scavenger receptor binding but also because lipid peroxidation intermediates have a broad range of biological activities, i.e., the induction of endothelial dysfunction, activation of endothelial adhesiveness, monocyte differentiation, cytotoxicity, monocyte chemotactic activity, and inhibition of macrophage migration with consequent sequestration of these cells in the atherosclerotic lesion (13,14). The importance of LDL-oxidation has also been suggested by studies with antioxidants. It was shown that vitamin E and butylated hydroxytoluene totally inhibited the LDL modification by cells (11,12). Native LDL carry several antioxidants with an important protective effect toward free radical attack (6).

On the basis of these findings, a new hypothesis was constructed on the mechanism of atherogenesis based on the oxidative modification of LDL (13,15,16). This hypothesis has been supported by much experimental evidence accumulated in the last two decades, which can be summarized as follows: (i) the scavenger receptor is the pathway of cholesterol influx in macrophages, the main cell type of the atherosclerotic lesion; (ii) the scavenger receptor recognizes only oxidized LDL and not native LDL; (iii) the scavenger receptor is not downregulated by the intracellular cholesterol content, and macrophages take up oxidized LDL until engorged with lipids; (iv) LDL oxidation has been produced *in vitro* by physiologically plausible mechanisms in both cell-free and cell systems. Importantly, the cell-mediated LDL oxidation has been obtained with monocyte/macrophages, endothelial cells, and smooth muscle cells, all of major importance in the development of atherosclerotic lesions; (v) by-products of peroxidation of LDL lipids have proatherogenic activity toward all of the cells involved in the atherosclerotic process (14); and (vi) antioxidants inhibit LDL oxidation and accumulation of cholesterol in macrophages (13,15–18). Several studies have shown inhibition of experimental atherosclerosis in animal models by natural and synthetic antioxidants. These studies included cholesterol-fed rabbits, LDL receptor–deficient rabbits, cholesterol-fed nonhuman primates, cholesterol-fed hamsters, and transgenic mice (16,19).

However, what is the importance of these findings in humans? Do we have additional information on the occurrence of oxidatively modified LDL? A first body of evidence comes from the immunohistochemical studies. A series of polyclonal and monoclonal antibodies to various forms of oxidized-LDL and aldehyde-modified LDL, used in Watanabe heritable hyperlipidemic (WHHL) rabbits, stained macrophages present in fatty streak areas (20–22). A monoclonal antibody raised against 8-epi-prostaglandin $F_{2\alpha}$, a specific free radical–generated oxidation product of arachidonic acid, stained macrophages of atherosclerotic plaque of patients undergoing carotid surgery (23). In addition, autoantibodies against epitopes of oxidized LDL have been demonstrated in the serum of both animals and humans (20).

Further evidence comes from isolation of specific markers of lipid peroxidation from the atherosclerotic lesion. As early as 1952, it was reported that chloroform extracts from human atherosclerotic lesions obtained *post mortem* contained lipid peroxide and correlated positively with the extension of lesions (24). In several studies, LDL extracted from human atherosclerotic plaque resulted in LDL similar to that oxidized *in vitro*, i.e., having increased electrophoretic mobility, increased lipid oxidation products, and being taken up by the macrophage receptor (25–28). Most of these studies used methodologies (the thiobarbituric acid and iodometric assays) whose specificity and sensitivity have been questioned. In addition, lipids both of isolated LDL and plaque are intrinsically unstable and represent potential sources of artifacts during handling. The main criticism was whether these altered LDL and lipid peroxidation products were actually present in the plasma/plaque or whether they were derived from spurious oxidation *in vitro* during processing.

Evidence for the presence of lipid peroxidation products in atheroma comes from studies that analyzed markers of lipid per-

oxidation by specific and sensitive methods based on gas chromatography coupled with mass spectrometry or mass spectrometry–negative ion chemical ionization. Chisolm *et al.* (29) found 7-α-OOH-cholesterol, a product of free radical–mediated cholesterol oxidation, and Praticò *et al.* (19) found two isoprostane (IP) isomers derived from free radical–mediated arachidonic acid oxidation in freshly excised human atherosclerotic lesions obtained at carotid endoarterectomy (23). Importantly, all of these lipid peroxidation products possess biological activity (19,29). In these studies, artifactual generation of 7-α-OOH-cholesterol, and $IPF_{2\alpha}$-III and $IPF_{2\alpha}$-VI, during sample processing was taken into account. These authors injected appropriate internal standards, [$^{14}$C]-cholesterol and [$^{2}H_{8}$]-arachidonic acid, into the lesion at the time of acquisition and no formation of labeled isomers was detected.

Recently, a study from this laboratory (30) provided strong support for the theory of LDL oxidation and scavenger pathway uptake *in vivo* by injecting radiolabeled native LDL in patients undergoing endoarterectomy for critical carotid stenosis (>70%). In the same clinical model, previous studies showed that both native and oxidized LDL labeled with $^{99m}$Tc can be detected at the level of carotid plaque by noninvasive gamma camera imaging (31). This study did not reveal the site of the plaque responsible for the uptake of radiolabeled LDL, but showed the uptake of native LDL at the site of atherosclerotic plaque, suggesting the occurrence of oxidation *in vivo*. Thus, a study was planned to specifically investigate the plaque uptake of native LDL by autoradiography in the clinical setting of carotid atherosclerosis (30). This human clinical model is relevant in the field of experimental atherosclerosis because patients with carotid stenosis >70% are eligible for surgery, and the plaque obtained by endoarterectomy can be used for analytical purposes. LDL separated from patients were labeled with $^{125}$I, the tracer currently used to measure the cellular uptake of LDL *in vitro*, and several tests were used to check for spurious oxidation during handling. On the basis of these postlabeling tests (thiobarbituric acid–reactive substances, lipid peroxides, electrophoresis, dienes, and protein fluorescence), radiolabeled LDL were comparable to prelabeled native LDL. Thus, native-$^{125}$I-LDL were injected 24–72 h before surgery in patients with carotid stenosis. Carotid specimens obtained at endoarterectomy were analyzed by autoradiography and immunohistochemistry.

Autoradiography revealed intense deposition of LDL primarily in the foam cells of atherosclerotic plaque and no accumulation in the lipid core. Immunohistochemistry revealed that foam cells that had accumulated radiolabeled LDL were mostly CD68 positive, and a small number were β-actin positive. This demonstrates that LDL uptake is specific; this was further supported by injecting radiolabeled human albumin that was not followed by specific accumulation of radioactivity in the plaque.

Thus, it seems logical that accumulation of LDL within the foam cells may be explained by assuming oxidative changes of LDL. In agreement, we observed that uptake of radiolabeled LDL by plaque-resident macrophages was almost completely suppressed in a group of patients under treatment with vitamin E, 900 mg/d, in the 4 wk preceding surgery (30). Taken together, these data indicate that oxidation of LDL is important also in advanced lesions and should be taken into account in the "vitamin E controversy" at the clinical level (32,33). Thus, in selected types of patients with atherosclerosis, new clinical trials with high-dose vitamin E may be warranted.

In conclusion, the last two decades have provided a wide experimental base for understanding the biochemical mechanisms of atherogenesis. LDL oxidation by free radicals and its consequences at the cellular and vascular levels have been thoroughly investigated with the result that it is now the main and most detailed mechanism in the network of the atherogenic process.

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# *In Vivo* Measurement of the Redox State

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**ABSTRACT:** As part of an aerobic life, we oxidize a large pool of biomolecules to obtain chemical energy. During this process, several intermediates are formed; some are chemically unstable and are referred to as free radicals (FR). FR tend to react quickly with their surrounding biological environment; depending on the nature of the molecule attacked, different reactions can occur, i.e., lipid peroxidation, protein oxidation, or DNA oxidation products. As aerobic life has evolved, antioxidant defense systems against FR have developed. When an imbalance between production of FR (oxidants) and defense systems against them (antioxidants) happens, a situation of oxidative stress occurs. This can lead to irreversible biochemical changes, with subsequent tissue damage and disease. Establishing the involvement of FR in the pathogenesis of a disease has been difficult because of the lack of sensitive and specific methodology to detect them. No ideal biomarkers for *in vivo* FR-induced damage are available as yet. However, some reliable indices of FR formation are now available, and in some pathologic conditions, evidence is accumulating to show that FR damage might play a functional role. The task for the near future will be to try to simplify the analytical methodology and elucidate the molecular mechanisms underlying the formation, disposition, and kinetics of FR marker molecules.

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During the last decade it has become more and more difficult to read any medical journal without having some report on the involvement of "free radicals" or "reactive oxygen species" in most human diseases. There are several definitions of free radicals (FR), but they are generally defined as *"any chemical species containing one or more unpaired electrons."* As part of aerobic life, we utilize (oxidize) a large pool of biomolecules to obtain chemical energy. This means that when we oxidize these substrates, oxygen itself can become reduced and originate intermediates (including FR), which we generally call reactive oxygen species (ROS). This is a collective name that includes not only the oxygen-centered ($O2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) radicals but also some nonradical species of oxygen such as hydrogen peroxide ($H_2O_2$). Nowadays, it is well accepted that FR are continuously produced *in vivo*, some accidentally (e.g., by autoxidation reactions), some deliberately (e.g., for phagocyte killing mechanisms and intra- or intercellular signaling). Once formed, they react with their surrounding in the following two main ways: (i) with another radical to join their impaired electrons and make a covalent bond; or (ii) with a nonradical. The latter is probably the more frequent type of reaction because the majority of the molecules *in vivo* are nonradicals and contain paired electrons. Depending on the nature of the nonradical attacked, different reactions can originate, e.g., lipid peroxidation, protein oxidation, or DNA oxidation (1).

As aerobic life forms, we have evolved and developed antioxidant defense systems to protect ourselves against FR. Cells have excellent defense mechanisms against oxidative damage. Enzymes such as superoxide dismutases, catalase, and glutathione peroxidase are highly active and expressed in most mammalian cells. Cell membranes have different molecules within their hydrophobic lipid layer that can act as antioxidants in this milieu, e.g., vitamin E and β-carotene. Finally, body extracellular fluids contain a long list of proteins and low-molecular-mass molecules that can act as antioxidants. These include transferrin, haptoglobin, albumin, ceruloplasmin, bilirubin, urate, and ascorbic acid (2). When an imbalance between production of FR (oxidants) and defense systems against them (antioxidants) occurs, a situation of oxidative stress arises. This can lead to irreversible biochemical changes and subsequent tissue damage. A growing list of diseases and syndromes, ranging from atherosclerosis to cancer, have been putatively linked to oxidative stress.

What is the exact role played by FR in human disease? It is important to emphasize that oxidative stress could be as much a cause of tissue damage as a consequence of it. Establishing the involvement of FR in the pathogenesis of a disease has been very difficult in the past for two major reasons, i.e., the evanescent nature of these chemical species and the lack of sensitive and specific technology to detect them in complex biological systems (3). In the next part of this article, I will review some of the most common techniques used to measure markers of oxidative stress *in vivo*.

## *Lipid Peroxidation (LP)*

Oxidation of lipids has been recognized since antiquity as a problem in the storage of fats and oils, but only in the 1950s was it considered to be relevant to biology and medicine. Many techniques are available to measure LP when such methods are applied in *in vitro* systems (liposomes, microsomes), but concerns arise when they are applied to more

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Abbreviations: FR, free radical; GC, gas chromatography; HNE, 4-hydroxynonenal; HPLC, high- performance liquid chromatography; iP, isoprostanes; LP, lipid peroxidation; MDA, malondialdehyde; MS, mass spectrometry; 8-OHdG, 8-hydroxy-2-deoxy-guanosine; 8-OHG, 8-hydroxyguanine; PG, prostaglandins; ROS, reactive oxygen species; TBARS, thiobarbituric acid reacting substances.

complex biological systems. As a general rule, it is always better to separate the product of interest before assaying the sample, in particular when complex mixtures are being studied. This can be done by high pressure liquid chromatography (HPLC) or by converting the analyte into a volatile derivative by gas chromatography (GC) and then unambiguously identifying it by mass spectrometry (MS). Although these more sophisticated analytical techniques require the most sample preparation and care, they should be the choice for investigations of LP in biological fluids (4).

### *Thiobarbituric Acid (TBA) Test and Conjugated Dienes*

Historically, these are the most commonly used tests to assess LP. They are both easy and inexpensive to perform. In the thiobarbituric acid reactive substances (TBARS) test, the material is heated with TBA at low pH and the formation of a pink chromogen is measured. The chromogen is formed by reaction of one molecule of malondialdehyde (MDA) with two of TBA. If applied to simple *in vitro* systems, this test can provide useful information, but problems emerge when it is used to assess LP in biological fluids. The specificity of the assay is improved by measurement of the TBA–MDA adducts after HPLC and GC/MS (5). Peroxidation of polyunsaturated fatty acids (PUFA) is accompanied by formation of conjugated diene structures (two double bonds separated by a single bond), which can be detected spectrophotometrically. However, many other compounds can have similar absorbance. The primary use of this test is in monitoring changes in absorbance in dynamic *in vitro* experiments of LP.

### *4-Hydroxynonenal (HNE)*

As breakdown products during LP, many aldehydes other than MDA are formed. Among them, the unsaturated aldehyde HNE has gained acceptance as an index of LP. It is volatile, very unstable, and tends to form adducts on lysine residues and the imidazole group on histidine residues of protein. It possesses several biological activities and can also be cytotoxic *in vitro*. It is generally assayed by HPLC or GC/MS (6).

### *Isoprostanes (iP)*

iP are members of a complex family of lipids, isomers of the conventional enzyme-derived prostaglandins (PG); they are produced primarily, if not exclusively, *in vivo* by a FR-catalyzed peroxidation of PUFA. Most of the work here has focused on a group of isomers of prostaglandin $F_{2\alpha}$, called $F_2$-isoprostanes ($F_2$-iP). Because of their mechanism of formation and chemical stability, they have potential as reliable, noninvasive indices of LP *in vivo*. Their analysis has been based for a long time on the highly sensitive GC/MS technique. A variation of this assay, based on an initial immunoaffinity column extraction, derivatization, and then analysis has been also described. More recently, enzyme-immunoassays have been developed for measuring a particular $F_2$-iP, 8-iso-$PGF_{2\alpha}$, now known as $iPF_{2\alpha}$-III. However, concerns have been expressed that the degree of cross-reactivity among $F_2$-iP may be high because they share the same basic ring structure (7).

### *DNA Oxidation*

FR can attack almost any cellular structure or molecule. Thus, they can cause DNA-protein cross-links; attack deoxyribose-phosphate backbone, causing base release and strand breaks; and finally, modify directly purine and pyridine bases, resulting in DNA mutations. Traditionally, direct damage to DNA by FR has been thought to contribute to aging and the development of cancer.

### *Oxidized Bases*

Oxidized bases represent one of the major classes of DNA lesions induced by FR. In the last decade, major efforts have been made to develop various assays aimed at measuring altered DNA bases. However, most of the available methods require an initial extraction from cells or tissues, followed by either chemical hydrolysis or enzymatic digestion before analysis. All of these steps could give artifactual oxidation of the normal purines and pyrimidines present in the specimen. In addition, acid hydrolysis may promote the decomposition of unstable compounds. Two major techniques have been widely applied to date, i.e., HPLC with electrochemical detection, restricted to a few electroactive oxidized bases and nucleosides, and GC/MS, which requires a HPLC prepurification step aimed at measuring a wide range of modified bases. There is also a more recent and promising HPLC–MS/MS technique (8).

In recent years, measurement of DNA hydroxylation base products has been widely used to obtain markers of *in vivo* DNA oxidation. Among these, the most popular base assayed is hydroxylated guanine, measured as the base 8-hydroxyguanine (8-OHG) or as the nucleoside 8-hydroxy-2-deoxyguanosine (8-OHdG). Urinary excretion of 8-OHdG is considered a measure of "whole-body" DNA oxidative damage. The applicability of 8-OHdG as a biomarker of DNA oxidative FR damage has been investigated in several *in vivo* studies. High levels have been described in patients subjected to whole-body irradiation, chemotherapy, or smoking. Diet can influence its levels and it may arise not only from the DNA base but also from DNA base precursors. In summary, analysis of a wide range of DNA base damage products is preferable to the analysis of a single base (9).

### *Protein Oxidation*

FR-mediated attack of protein has been studied since the beginning of the century, although the use of the products of these reactions as specific markers of oxidative damage *in vivo* has been developed only in the last few years. FR can react with proteins and modify (oxidize) both the backbone or their side chains (10).

### *Protein Backbone*

A number of mechanisms that give rise to cleavage of the protein backbone have been elucidated; it can be examined readily with isolated proteins (e.g.,SDS-PAGE or HPLC). However, its use as a marker of protein oxidation *in vivo* is very limited because of the quantity of other proteins present and the potential role of proteases. Thus, backbone fragmentation is rarely used to quantify protein oxidation in complex systems.

### *Protein Side Chains*

The reaction of FR with side-chain residues gives rise to a multitude of products that can, for example, attack amino acid residue side chains (particularly histidine, arginine, and lysine) to produce carbonyl functions (>C=O), which by reacting with 2,4-dinitrophenylhydralazine form a chromogen that can be measured spectrophotometrically. This is the base for the "carbonyl assay," which has been developed as a general method for measuring protein oxidation especially for *in vitro* systems. It has become widely used, but its use *in vivo* is problematic due to potential interference with aldehyde/ketones generated from sugar or lipids bound in the system when biological fluids are investigated (11).

In summary, three major methods have been developed for the measurement of specific amino acid side-chain oxidation products: a qualitative method based on specific antibodies that recognize episodes on tissue or isolated protein (e.g., 3-nitrotyrosine), and two quantitative methods based on GC/MS and HPLC with different detectors (fluorescence or electrochemical) depending on the oxidized molecules of interest. Both require isolation, purification, and hydrolysis of the protein under investigation before analysis of any particular oxidized amino acid side chain. Artifactual formation of oxidized amino acid during those procedures for sample preparation could be potentially serious; it is necessary to control for this using complementary approaches such as adding free parent amino acids to the sample before hydrolysis and determining their conversion in oxidized forms, or adding antioxidants to the specimen at the very beginning of the process. Much remains to be determined about the validity of such measurements, which markers most accurately reflect *in vivo* protein oxidation, and which ones are the least affected by metabolic or clearance pathways under different *in vivo* conditions.

## SUMMARY

In conclusion, from the data presented in this review, it is evident that no ideal biomarkers for *in vivo* FR damage are available to date. All of the biomarkers used so far have specific advantages and disadvantages. However, the major problem for most is that analytical methods are still quite laborious and expensive. At the same time, some reliable indices of FR-induced damage to specific cellular components are becoming available, and in some pathologic conditions, evidence is accumulating to show that FR damage might play a functional role. The task for the future will be to simplify the analytical methodology and elucidate the molecular mechanisms underlying the formation, disposition, and kinetics of marker molecules. Another important point will be the simultaneous measurement of markers representing damage to different cellular components (lipids, DNA, proteins) to evaluate any relationship among them. All of these considerations together will allow us to establish a causal relationship between the biomarker and the disease of interest.

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# The Role of Antioxidants in the Mediterranean Diet

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**ABSTRACT:** Traditional Mediterranean diets, as opposed to North European and American diets, include a significantly large amount of plant foods; this notable difference between the two eating styles, despite the similarities among other classic risk factors for coronary heart disease (CHD) such as high plasma cholesterol levels, has been associated with a lower risk of developing the CHD and certain cancers. The involvement of excessive free radical production and the great number of epidemiologic studies linking antioxidant intake with a reduced incidence of the above-mentioned diseases indicate that dietary antioxidants likely play a protective role. Because diets in the Mediterranean are (or better yet, were) characterized by abundant plant foods (fruits, vegetables, breads, nuts, seeds; wine and olive oil), this article includes a review of the potential activities of dietary antioxidants, which are plentiful in Mediterranean diets, and wine as related to human disease.

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Dietary intake of fruits and vegetables has a strong and inverse association with the risk of developing coronary heart disease (CHD) and cancer (1); low amounts of plant foods in the diet double the risk of most cancers and significantly increase the risk for developing CHD (2). Traditional Mediterranean diets, as opposed to North European and American diets, include a significantly large amount of plant foods; this notable difference between the two eating styles, despite the similarities among other classic risk factors for CHD such as high plasma cholesterol levels, has been associated with a lower risk of developing the above-mentioned diseases (3). The involvement of excessive free radical production and the great number of epidemiological studies linking antioxidant intake with a reduced incidence of the above-mentioned diseases indicate that dietary antioxidants likely play a protective role (4).

Because diets in the Mediterranean are (or better yet, were) characterized by abundant plant foods (fruits, vegetables, breads, nuts, seeds; wine and olive oil), this article will include a review of the potential activities of dietary antioxidants, which are plentiful in Mediterranean diets, and wine as related to human disease.

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Abbreviations: CHD, coronary heart disease; iNOS, nitric oxide synthase; LDL, low density lipoprotein; ROS, reactive oxygen species.

### *Reactive Oxygen Species (ROS) and Their Connection with Human Health*

The participation of excessive ROS production in the origination of several diseases is now supported by a wealth of experimental data. An illustrative example is the formation of oxidatively modified human low density lipoprotein (LDL), which renders these particles highly atherogenic (5). *In vivo* evidence of the formation of oxidized LDL includes their presence in human atherosclerotic plaques and in the bloodstream; further, their circulating levels have been positively correlated with the progression of carotid lesions (6). Moreover, many epidemiologic studies have correlated a high dietary intake of antioxidants (e.g., tocopherols, carotenoids, flavonoids, and polyphenols) with a lower incidence of cardiovascular disease (7,8). The features setting the Mediterranean diets apart from other diets are illustrated in various articles, but the abundance of fresh fruits and vegetables and the use of olive oil instead of hard fat are key factors for which the Mediterranean diets are renowned (9). Fruits, vegetables, wine, and olive oil (see below) are rich in antioxidants, which may therefore contribute to the observed protection from CHD.

Nevertheless, intervention studies performed with supplements have yielded mixed results. Descriptive, case-controlled, and prospective studies have shown a protection vs. cardiovascular events, whereas randomized trials have failed to confirm these results (10). Other trials are underway and may shed light on the contribution of antioxidant vitamins to a lower incidence of CHD.

## ANTIOXIDANTS IN THE MEDITERRANEAN DIET

Fruits and vegetables, in which the Mediterranean diet is very rich, represent the major source of antioxidants; they are rich in vitamins, minerals, and phytochemicals (mainly polyphenols). The relative contribution of individual compounds to human health remains unclear because of the concomitant presence of other beneficial components such as fiber and nonantioxidant molecules, which may mask or render extremely difficult the determination of the exact role of each compound. What follows is a brief overview of the biochemical aspects of each subclass.

### *Antioxidant Vitamins*

*Tocopherols.* The main dietary sources of tocopherols (notably α- and γ-tocopherols) include the following: nuts, wheat

germ, vegetable oils (i.e., seed oils), margarine, mayonnaise, butter, and eggs. It is noteworthy that although epidemiologic studies have clearly shown a correlation between tocopherol intake and protection from CHD and cancer, clinical trials have not confirmed such results (10).

*Carotenoids.* Carotenoids, the precursors of vitamin A, are found in heavily pigmented fruits and vegetables such as carrots, broccoli, tomatoes, red peppers, and pumpkins, all widely used components of a Mediterranean meal. Epidemiologic studies have found that plasma levels of carotenoids—lycopene in particular—are positively correlated with a lower incidence of CHD and lung cancer. Surprisingly, two large clinical trials (11,12) have reported a deleterious effect of β-carotene supplementation, indicating that the *in vivo* antioxidant actions of carotenoids are yet to be established. Indeed, one interpretation of the discrepancy between epidemiologic observations and clinical trials is that high circulating levels of carotenoids may simply reflect a diet rich in fruits and vegetables, which contain healthful components other than β-carotene that are responsible for the observed protective effects (13).

*Vitamin C.* Ascorbic acid in the Mediterranean area is provided by citrus fruits (orange, tangerine, grapefruit, lemon) and leafy vegetables. Vitamin C is the principal antioxidant of human plasma, and several reports add weight to the hypothesis that ascorbic acid plays a protective role toward development of CHD. For instance, Vitamin C has been shown to restore endothelial function (by plaque stabilization and enhanced vasorelaxation); thus, it may act directly on the arterial wall rather than exerting a direct action on the progression of atherosclerosis (14,15). It is noteworthy that most of the oxidative modifications of LDL take place in the aqueous phase and it is thus conceivable that water-soluble antioxidants such as ascorbate are more effective than those that are lipid-soluble. As for protection from cancer, it is conceivable that the protective role of vitamin C is due to removal, or neutralization, of exogenous noxious substances such as nitric dioxide and ozone.

*Nonvitamin antioxidants (phytochemicals).* Phytochemicals such as polyphenols are particularly abundant in the Mediterranean diet owing to its high proportion of fruits and vegetables and to the consumption of red wine and olive oil. Ubiquitous in plant foods, polyphenols include several classes of phenolic compounds such as simple phenols, phenolic acids, benzoquinones, hydroxycinnamates, and flavonoids (16). In the Mediterranean basin, because of the warm climate and the prolonged exposure of crops to sunlight radiation, some plant species such as olives and grapes are particularly abundant in polyphenolic compounds, which are synthesized by the plant in response to environmental stress. The presence of such secondary plant metabolites in the diet (the daily intake of polyphenols in the Mediterranean area is on the order of several hundreds of milligrams) has stimulated research on their "pharmacological" properties, including the possibility that the strong antioxidant activity exhibited *in vitro* by several polyphenols could explain in part the lower incidence of the diseases mentioned above.

*Wine.* The "alimentary" use of wine, i.e., wine in moderation as food, as in the case of the Mediterranean diets, very likely provides protection from coronary heart disease. In addition to the role played by ethanol itself, the protective effects of moderate wine consumption are possibly due to the healthful effects of the minor wine components, i.e., polyphenols (Table 1). For instance, the inhibition of platelet aggregation and therefore blood clotting by wine polyphenols, along with inhibition of LDL-oxidation, is likely to play an important role in CHD prevention (17). The most significant phenol in red wine, *trans*-resveratrol, has been experimentally demonstrated in mice to exert tumor-inhibiting properties (18). To date, it is difficult to distinguish between the protective effects of ethanol alone, which would imply that even beer and spirits are protective regardless of their composition, and those of minor wine components (19).

*Olive oil.* Peculiar to the Mediterranean area is the use of olive oil as the principal source of fat. As opposed to other vegetable oils, extra virgin olive oil (but not plain olive oil) contains phenolic compounds (50–800 mg/kg, depending on several factors such as the cultivar, soil, degree of drupe ripeness, and the manner in which the oil is produced and stored) that provide its unique aroma and taste. This is due to the fact that although most vegetable oils are extracted from seeds by organic solvents, olive oil is obtained from the whole fruit (drupe) by means of physical pressure, without the use of chemicals. From a nutritional point of view, it is noteworthy that the flavor of extra-virgin olive oil favors the consumption of raw vegetables, which are generally dressed with olive oil and vinegar (the latter ingredients also contain antioxidants); thus, the use of good quality olive oil likely provides indirect benefits such as an increased consumption of healthful foods.

The polyphenolic fraction amounts to 50–800 mg/kg, depending on several key factors such as the cultivar, the soil, the degree of ripeness of the drupes, and the way the oil is produced and stored. Many phenolic components have been identified and can be conveniently classified into two major subclasses, i.e., simple and complex (hydrolyzable). The former includes hydroxytyrosol [2(3,4-dihydroxyphenylethanol)], tyrosol, and phenolic acids such as vanillic and caffeic (20). The latter (complex) subclass comprises tyrosol and hydroxytyrosol esters, oleuropein and its aglycone, and several other molecules yet to be identified. Oleuropein is responsible for the bitter taste of olives and for the browning of the olive skin. Its levels slowly taper off during the late days of the ripening season, yielding

**TABLE 1**
**Biological Activities of Wine Phenolics**

- Inhibition of low density lipoprotein oxidation
- Phytoestrogenic activity
- Inhibition of tumorigenesis
- Vasorelaxation
- Inhibition of platelet aggregation
- Inhibition of cycloxygenase-2
- Inhibition of human polymorphonuclear leukocytes

several simpler molecules that build up the full, fruity taste of the oil.

Both hydroxytyrosol and oleuropein potently inhibit oxidation of LDL in a dose-dependent manner, when incubated from $10^{-6}$ to $10^{-4}$ M (21). Also, hydroxytyrosol was shown *in vitro* to inhibit platelets, the accumulation of the proaggregant agent thromboxane in human serum, the production of the proinflammatory leukotriene molecules by activated human leukocytes; and to inhibit arachidonate lipoxygenase (22,23). The potent (50% effective concentrations in the $10^{-5}$ M range) inhibitory effect of hydroxytyrosol toward all of these parameters discloses biological activities of olive oil phenolics that go beyond their antioxidant properties (Table 2).

Finally, when added to murine macrophages challenged with bacterial lipopolysaccharide, oleuropein increases the functional activity of these immune-competent cells, as evaluated by a significant increase (+58.7 ± 4.6% at a concentration of $10^{-4}$ M) in the production of the bactericidal and cytostatic factor nitric oxide. This effect is due to a direct effect of oleuropein on both the activity and expression of the inducible form of the enzyme nitric oxide synthase (iNOS), as demonstrated by Western blot analysis of cell homogenates and by the use of the iNOS inhibitor l-nitromethylarginine methylester (24).

The adoption of phenol-rich olive oil as the fat of choice, as a substitute for animal fat, contributes to the dietary intake of biologically active compounds in estimated quantities that have been correlated with a reduced risk of developing CHD (8).

### *Bioavailability of Phenolic Antioxidants*

The lack of a reliable method for measuring the polyphenolic content of foods (25), including olive oil and wine, renders the accurate estimation of the daily intake of such compounds difficult. Nevertheless, the calculated values of daily polyphenolic consumption in the Mediterranean area fluctuate from a few hundred milligrams up to 1.5 g (7,26).

One of the major issues in the antioxidant field today concerns the bioavailability of polyphenolic compounds, and several studies are under way to investigate the absorption and metabolism of these molecules. Depending on their structure, some classes of phenolics are absorbed as they are, others are metabolized in the gastrointestinal tract, and some polyphenols (i.e., the nonextractable phenolics) are not bioavailable. Recently, Visioli *et al.* (27) demonstrated that olive oil phenolics are absorbed in humans in a dose-dependent manner, and are excreted in the urine as glucuronide conjugates (Fig. 1). Interestingly, the increased availability of phenolic substrates increases their rate of conjugation with glucuronide.

Future development of an appropriate methodology, e.g., mass spectrometry, to determine quantitatively low levels of individual compounds in foods, human plasma, and urine, along with the availability of pure and even radiolabeled standards, will help to clarify this issue.

Finally, it should be noted that, in spite of the general consensus that calls for a protective role of polyphenols, two epidemiologic studies (28,29) failed to link a higher flavonoid intake with a lower mortality due to CHD, possibly due to imperfectly accounted for confounders, such as unhealthy lifestyles (e.g., tea consumption in the United Kingdom is currently predominant among blue-collar workers and is declining in the affluent class) or preexisting unidentified coronary risk factors. Also, *ex vivo* studies on the effects of tea and red wine on LDL oxidizability have again yielded controversial results (30). A plausible explanation for this apparent contradiction between epidemiologic observations and *ex vivo* studies is that strong antioxidant flavonoids such as flavanols and catechins probably do not accumulate in LDL or they are lost during LDL isolation; indeed, in humans, LDL oxidation is likely to take place in the aqueous phase of the subendothelial space.

In conclusion, antioxidant therapy, by means of supplementation of pure compounds such as vitamins, has yet to be proved beneficial. By contrast, a plethora of epidemiologic studies continue to link certain dietary habits, such as those in use in the Mediterranean area during the mid-1940s, with a lower incidence of cardiovascular events and certain cancers. Apparently, the key factor that affords protection from such pathologies might be the intake and the interaction of several "micronutrients" provided by a healthful diet, in which the

**TABLE 2**
**Biological Activities of Olive Oil Phenolics**

- Inhibition of low density lipoprotein oxidation, both *in vitro* and *ex vivo*
- Inhibition of apoprotein derivatization
- Inhibition of platelet aggregation
- Reduced thromboxane $B_2$ and leukotriene $B_4$ production by activated human leukocytes
- Scavenging of superoxide and other reactive oxygen species
- Inhibition of peroxynitrite-induced DNA damage
- Inhibition of peroxynitrite-induced tyrosine nitration
- Scavenging of hypochlorous acid
- Increased nitric oxide production by lipopolysaccharide-challenged macrophages
- Inhibition of neutrophil respiratory burst
- Inhibition of bacterial growth and activity
- Cytostasis
- Hypotensive action

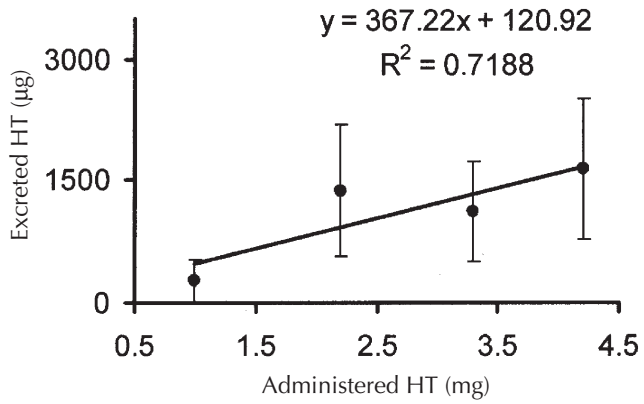


**FIG. 1.** Hydroxytyrosol (HT) is absorbed and excreted in urine as a glucuronide conjugate in a dose-dependent manner. *Source of data:* Reference 27.

abundance of bioactive phytochemical compounds provided by fruits, vegetables, wine, and olive oil grants a higher protection toward ROS-induced diseases.

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# Antioxidant Vitamins and Prevention of Cardiovascular Disease: Epidemiological and Clinical Trial Data

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**ABSTRACT:** Naturally occurring antioxidants such as vitamin E, β-carotene, and vitamin C can inhibit the oxidative modification of low density lipoproteins. This action could positively influence the atherosclerotic process and, as a consequence, the progression of coronary heart disease. A wealth of experimental studies provide a sound biological rationale for the mechanisms of action of antioxidants, whereas epidemiologic studies strongly sustain the "antioxidant hypothesis." To date, however, clinical trials with β-carotene supplements have been disappointing, and their use as a preventive intervention for cancer and coronary heart disease should be discouraged. Only scanty data from clinical trials are available for vitamin C. As to vitamin E, discrepant results have been obtained by the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study with a low-dose vitamin E supplementation (50 mg/d ) and the Cambridge Heart Antioxidant Study (400–800 mg/d). The results of the GISSI-Prevenzione (300 mg/d) and HOPE (400 mg/d) trials suggest the absence of relevant clinical effects of vitamin E on the risk of cardiovascular events. Currently ongoing are several large-scale clinical trials that will help in clarifying the role of vitamin E in association with other antioxidants in the prevention of atherosclerotic coronary disease.

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In the past few years, substantial interest has successfully focused on therapeutic strategies aimed at the treatment of acute coronary events. Availability of effective drugs [e.g., thrombolytics, aspirin, β-blockers, angiotensin-converting enzyme (ACE)-inhibitors, IIb/IIIa-glycoprotein antagonists as well as of emergency coronary revascularization procedures] has led to a clear reduction of intrahospital mortality rates (1). Out-of-hospital coronary heart disease (CHD) mortality, however, remains the major component of total cardiovascular disease (CVD) mortality (2,3). This figure clearly highlights the need of effective preventive strategies for at least two main issues: (i) lowering the risk of CHD events, and (ii) reducing the lag time between the onset of coronary symptoms and the admission to hospital to allow a prompt start of acute treatments.

Although the latter is mainly a public health issue concerning the degree of knowledge people have about CHD and the organization of health systems, the former is characterized by several questions that require more research. In this sense, further research on the use of antioxidants, particularly naturally occurring antioxidant vitamins such as vitamins E and C and β-carotene, seems to be a very promising field of investigation for the prevention of myocardial infarction (MI), progression of CHD, or stroke (4–12).

Several large observational epidemiologic studies done in the 1980s and 1990s suggested that persons with a high dietary intake of fruits and vegetables have a lower incidence of ischemic heart disease (IHD) events and strokes compared with those having a low intake of these nutrients. Higher intakes of β-carotene, vitamin C, and vitamin E have been implicated in this cardiovascular protective effect. These findings are supported by experimental data showing attenuation in the development and progression of atherosclerosis in animals, by higher resistance to radical-initiated oxidation in low density lipoprotein (LDL) isolated from vitamin-supplemented individuals, and by the observation of a lower event rates among persons with dietary intake or supplementation of these vitamins (13–23). The potential benefits of these vitamins are attributed mainly to their antioxidant activities on the oxidation of LDL, which is considered to be an important step in the development and progression of atherosclerosis (24–69). This article briefly summarizes epidemiologic studies as well as randomized clinical trials with regard to the potential role of antioxidant vitamin therapy in CVD prevention.

## MATERIALS AND METHODS

*Data sources.* A MEDLINE search of scientific literature was carried out to retrieve all relevant epidemiologic studies and randomized clinical trials of antioxidants (vitamin E, vitamin C, and β-carotene) in cardiovascular disease. To enhance the completeness of the literature search, we also examined bibliographies of review articles and original articles.

*Study selection.* Only studies specifically defining the type of intake or of supplements of antioxidants were included. To have a more reliable estimate, more stringent inclusion criteria

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Abbreviations: ATBC trial, Alpha-Tocopherol, Beta Carotene trial; CARET, β-Carotene and Retinol Efficacy cancer prevention Trial; CHAOS, Cambridge Heart Antioxidant Study; CHD, coronary heart disease; CI, confidence interval; CVD, cardiovascular disease; GISSI, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio; HOPE, Heart Outcomes Prevention Evaluation; IHD, ischemic heart disease; LDL, low density lipoprotein; MI, myocardial infarction; OR, odds ratio; PHS, Physicians' Health Study.

were used for clinical trials, i.e., patients had to be randomly assigned to experimental treatments, >100 patients had to have been included, and treatment duration had to be >1 yr.

*Statistical analysis.* The meta-analytic tool was used to depict more clearly the amount and quality of evidence furnished by epidemiologic studies. In this sense, the use of the meta-analytic technique should be viewed as exploratory and broadly indicating the existence of an association between antioxidant nutrients and CHD. For randomized clinical trials, meta-analysis can give a more reliable estimate of the benefit (if any) associated with the administration of vitamin supplements. Such estimates, however, should be carefully evaluated according to their quality as well as the amount of information currently available, i.e., the total number of patients included in each overview.

Data from different studies were combined by using the general variance-based method (70). This method requires only information on the odds ratio (OR) estimate and its confidence interval (95% CI) for each study (71). The 95% CI were used to assess the variance of each study effect measure. Adjusted OR and their CI, when these were provided by authors, were preferred. Crude OR and their 95% CI were used when an adjusted estimate was not provided. These estimates were used to carry out the overview for all studies as well as for the aforementioned subgroups.

## EPIDEMIOLOGIC STUDIES AND CLINICAL TRIALS OF ANTIOXIDANT VITAMINS AND CARDIOVASCULAR DISEASE

### *β-Carotene*

Figure 1 shows the results of main epidemiologic studies and clinical trials of β-carotene in cardiovascular disease (72–88).

*Epidemiologic studies.* Patients ($n$ = 84,331) were enrolled in observational, prospective studies. Overall, prospective studies had positive results, thus suggesting a relevant role of β-carotene as a cardioprotective nutrient (OR, 0.66; 95% CI, 0.57–0.78). Discrepant results, however, were obtained in the three main prospective studies. The Health Professional Study (which included >39,000 men, 40–75 yr old, free of CHD, and followed for an average of 4 yr) showed a 29% reduction (95% CI, –14 to –47%) of CHD events in the highest quintile of intake compared with the lowest (72). Conversely, no association was found between β-carotene intake and CVD risk in the Nurses' Health Study (which included 87,000 women followed for an average of 8 yr) once intake of vitamin E and C was accounted for (73). Similarly, no benefit as to CHD death was evident in the study of Kushi *et al.* (35,000 postmenopausal women followed for an average of 8 yr) (74). The Finnish study on 2,748 men and 2,385 women showed a reduction in the risk of CHD death (75). A smaller study of 1,299 nursing home residents suggested a ben-

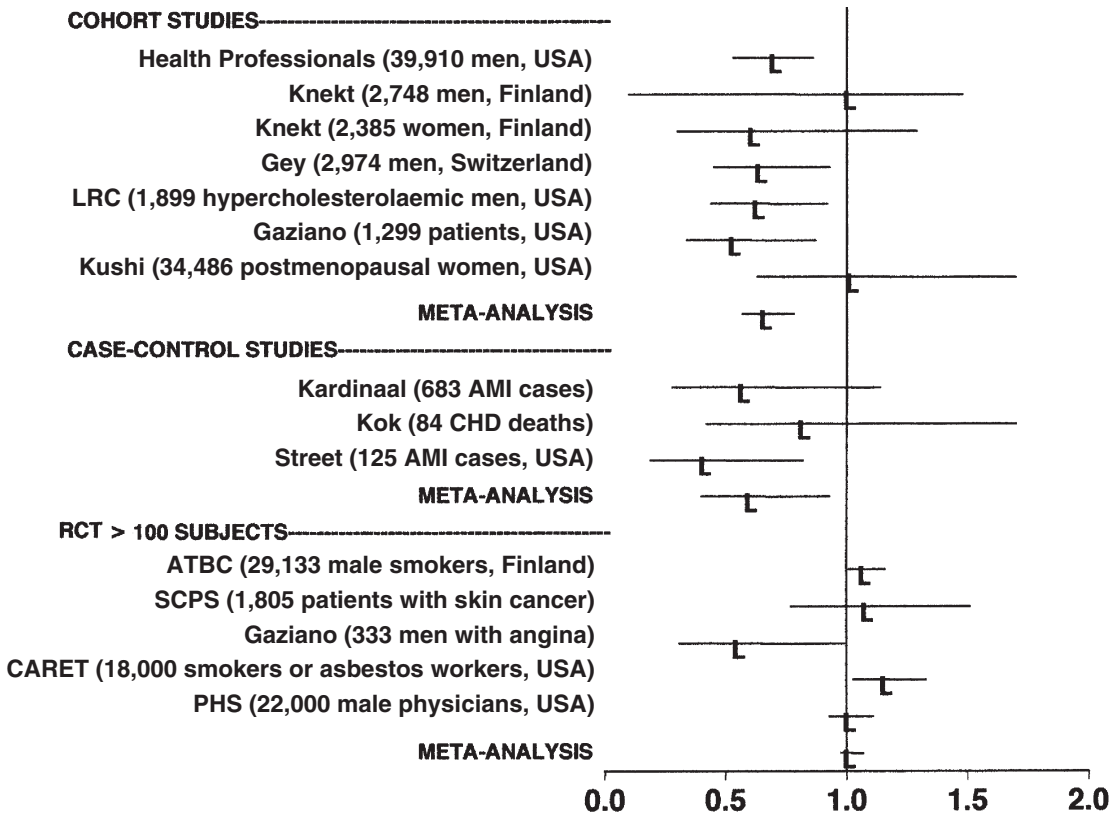


**FIG. 1.** Results of the main epidemiologic studies and clinical trials of β-carotene in cardiovascular disease. Abbreviations: LRC, Lipid Research Clinics; AMI, acute myocardial infarction; CHD, coronary heart disease; RCT, randomized clinical trials; ATBC, Alpha-Tocopherol, Beta Carotene trial; SCPS, Skin Cancer Prevention Study; CARET, β-Carotene and Retinol Efficacy cancer prevention Trial; PHS, Physicians' Health Study.

efit from high dietary intake of β-carotene (76). Various small prospective studies also found a protective effect associated with high intake of dietary β-carotene (77,78). In the case-control studies, two of three showed a significant association between high dietary intake of β-carotene and risk of CVD events (OR, 61; 95% CI, 0.40–0.93) (79–82).

*Clinical trials.* More than 71,000 subjects were included in randomized clinical trials with β-carotene. The results of trials of β-carotene have uniformly failed to show benefit in the prevention of CHD (OR, 1.02; 95% CI, 1.07) as well as of cancer. In addition, a significant increased risk of malignancy was apparent in individual studies and in the meta-analysis of their results. The Alpha-Tocopherol, Beta Carotene (ATBC) trial for the prevention of lung cancer in >29,000 Finnish male smokers tested the efficacy of 50 mg of α-tocopherol and 20 mg daily of β-carotene with a $2 \times 2$ factorial design (83). After 3 yr of study, serum levels of α-tocopherol were only modestly increased, whereas there was a 15-fold increase in the serum levels of β-carotene in subjects randomized to β-tocopherol and β-carotene, respectively. β-Carotene supplementation led to a significant increase in total mortality (+9%; 95% CI, +2 to +17%) and lung cancer (+18%; 95% CI, +3 to +36%), and a nonsignificant increase in mortality from CVD (+11%; –1 to +23%) after 6.5 yr of follow-up. The β-Carotene and Retinol Efficacy cancer prevention Trial (CARET) randomized 18,134 smokers, former smokers, and workers exposed to asbestos to supplements of 30 mg of β-carotene plus 25,000 IU of retinol or placebo (84). The CARET trial was stopped after 4 yr of followup (i.e., earlier than planned) due to a significant increase in lung cancer incidence in subjects randomized to active treatment (total mortality: +17%; 95% CI, +3 to +33%; lung cancer mortality: +28%; 95% CI, +4 to +57%; CVD mortality: +9%; 95% CI, –7 to +27%). The Physicians' Health Study (PHS) randomized 22,071 adult U.S. male physicians to receive 50 mg β-carotene on alternate days or placebo (85). No change of total mortality, cancer mortality, or CVD mortality was apparent after an average follow-up of 12 yr. Three additional smaller studies showed unclear benefit attributable to β-carotene supplementation (86–88). Interestingly, discrepant results between epidemiologic and experimental analyses became apparent in the ATBC trial as well as in the study by Greenberg and co-workers (88). In fact, the lack of association with or even the significant excess of deaths in patients allocated to β-carotene treatment was not paralleled by a similar association between plasmatic concentration of β-carotene and incidence of events in these two studies. On the contrary, a significant inverse association between increasing concentration of plasma levels of β-carotene and risk of death was apparent.

### *Vitamin C*

Figure 2 shows the results of main epidemiologic studies and clinical trials of vitamin C in cardiovascular disease (72–74, 89–94).

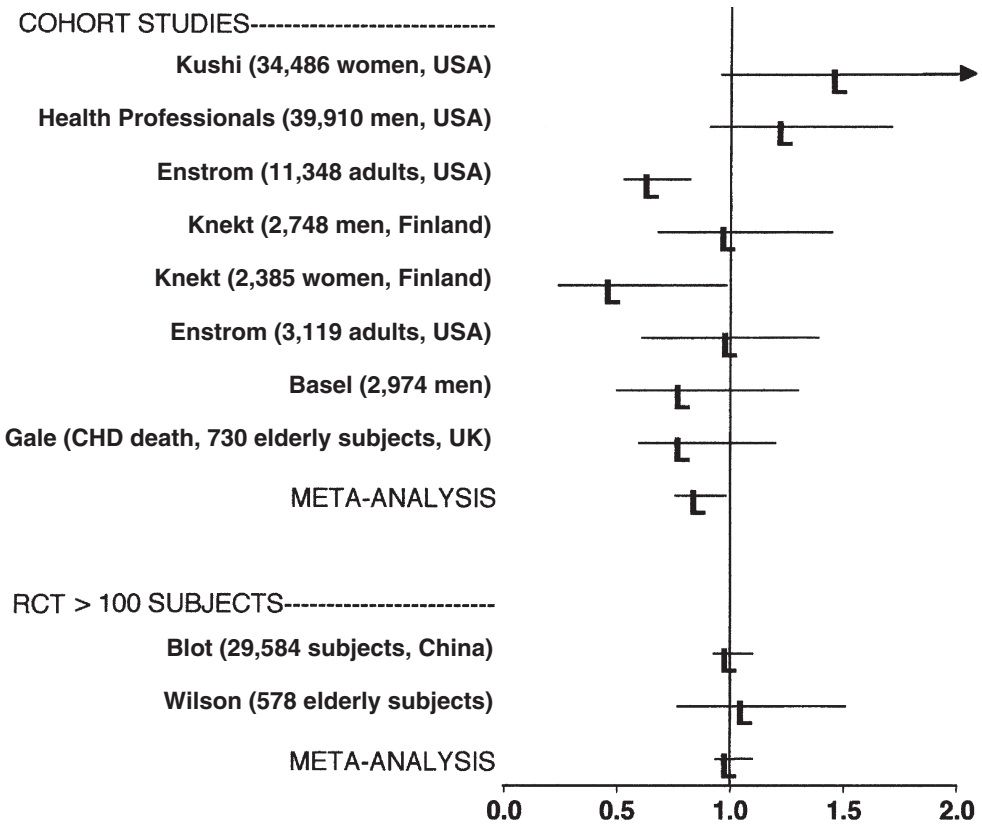


**FIG. 2.** Results of the main epidemiologic studies and clinical trials of vitamin C in cardiovascular disease. For abbreviations see Figure 1.

*Epidemiologic studies.* A number of epidemiologic studies conducted in England, Scotland, and the United States have suggested an inverse association between dietary intake of fresh fruit and vegetables, which are generally rich in antioxidant substances, and CVD mortality. Subjects ($n$ = 110,483) were enrolled in prospective studies on vitamin C; however, these had discordant results (OR, 0.93; 95% CI, 0.84–1.02). The three largest cohort studies (Kushi and co-workers, 73; Nurses' Health Study, 73; American Health Professional Health Study, 72), evaluating together more than 150,000 subjects, failed to find any association between vitamin C intake and CVD mortality. At variance with these results, the National Health and Nutrition Examination Survey, a large prospective, population-based study conducted in the United States among 11,348 adults followed for 10 yr found a 34% lower standardized mortality ratio (95% CI, –18.1 to –47.1%) among subjects consuming at least 50 mg of vitamin C per day by diet or supplements compared with those with lower intake (89). A number of epidemiologic studies found varied associations (in degree and direction) between vitamin C intake and CVD mortality and morbidity (74,75,89–92).

*Clinical trials.* Few randomized clinical trials of vitamin C (30,162 subjects recruited) have been conducted to date (OR, 1.01; 95% CI, 0.94–1.10). The Chinese Cancer Prevention trial was a large-scale randomized study of an undernourished population in Linxian County, China, designed to test the efficacy of various combinations of 10 antioxidant substances with a partial $2 \times 4$ factorial design in 29,854 subjects followed for 5 yr (94). In this study, supplementation of diet with vitamin C plus molybdenum was not associated with changes in CVD mortality. However, encouraging results were found for esophageal, gastric, and total cancer mortality. Supplementation of diet with 200 mg of vitamin C daily did not reduce mortality at 6 mon in a small trial of 578 geriatric patients (95).

### *Vitamin E*

Figure 3 shows the results of the main epidemiologic studies and clinical trials of vitamin E in cardiovascular disease (72–75, 94,96–103).

*Epidemiologic studies.* A number of large prospective epidemiologic studies have found a significant inverse correlation between vitamin E intake and risk of CVD. Overall, 166,774 subjects were studied, and there was a statistically significant reduction of deaths (OR, 0.64; 95% CI, 0.56–0.73). In the Nurses' Health Study, 87,000 women were followed for an average of 8 yr. Investigators compared the risk of heart disease among those in the highest intake category for vitamin E with those in the lowest (73). In the highest quintile of vitamin E intake group, there was a 34% reduction in risk of CHD events compared with women in the lowest quintile after adjustment for a number of potential confounding factors (including β-carotene and vitamin C). Reduced risk was seen only with vitamin E supplements (at least 100 IU daily) and not with multivitamin use. Presumably because of the low number of women with a long-term intake of vitamin E supplements, increasing duration of use did not show a dose-

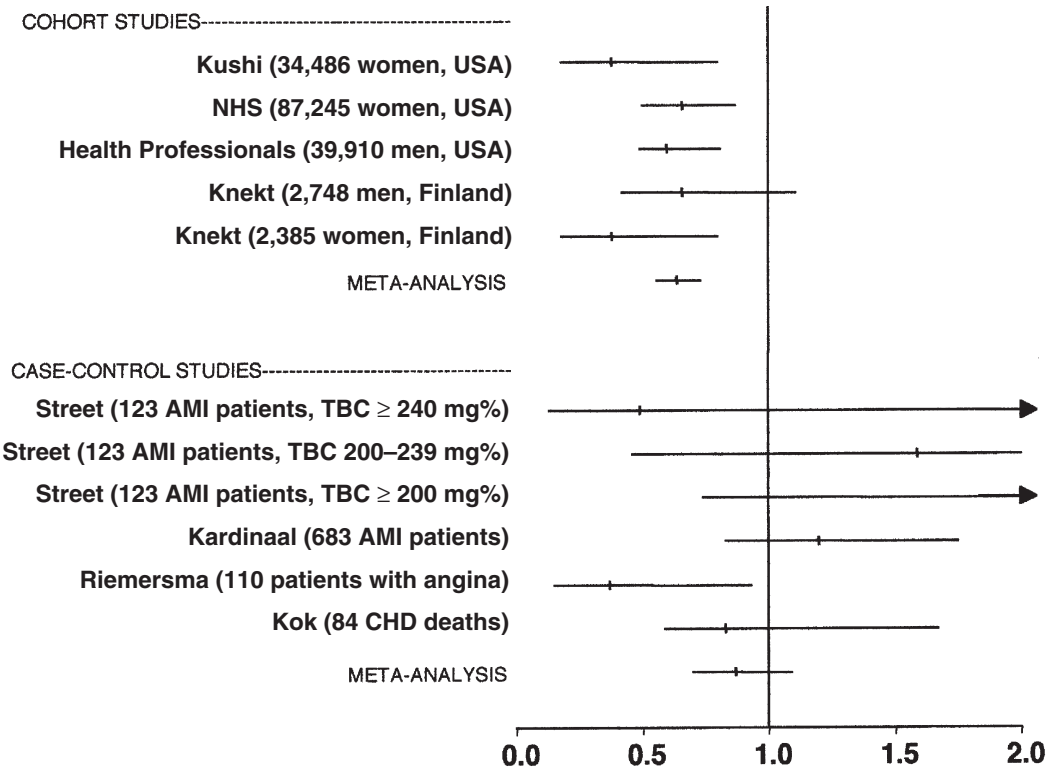


**FIG. 3.** Results of the main epidemiologic studies and clinical trials of vitamin E in cardiovascular disease. Abbreviations: NHS, Nurses' Health Study; AMI, acute myocardial infarction; for other abbreviation see Figure 1.

response relationship with the incidence of CHD events. Only use of vitamin E beyond 2 yr was significantly associated with better prognosis.

The Health Professionals' Study is a cohort of ~40,000 adult men who provided similar dietary data from a food-frequency questionnaire and were followed for an average of 4 yr (72). The Health Professionals' Study results were remarkably similar to those of the Nurses' Health Study. After controlling for age and several risk factors, there was a 39% reduction in risk of major coronary events in the highest intake category for vitamin E compared with the lowest. Short-term use of vitamin E supplements (≤2 yr) was not associated with a reduced risk for cardiovascular events. At variance with the results of the two aforementioned studies, a large epidemiologic study of ~35,000 postmenopausal women followed for an average of 7 yr found a 62% reduction (95%, CI, –0.18 to –0.80) in CVD deaths in those with higher vitamin E intake from food (74). No significant association was apparent when the intake of vitamin E from supplements as well as from supplements plus dietary sources was evaluated. In a Finnish epidemiologic prospective study, 2748 men and 2385 women were followed for an average of 14 yr. Investigators compared the risk of heart disease among those in the highest tertile of intake for vitamin E with those in the lowest (75). Men in the highest category of intake had a nonsignificant, although remarkable 34% reduction of risk of CHD death (95% CI, –58 to +11%). On the contrary, women in the highest category of intake had a significant 65% reduction of risk of CHD death (95% CI, –86 to –12%).

At least six case-control studies on vitamin E and CVD risk were published (79,81,82,96–99). They were small in size ($n$ = 1,846) and their results did not agree (OR, 0.88; 95% CI, 0.70 to 1.09). Such results are not surprising because these studies have major potential sources of error and short-term and long-term intake cannot be separated.

*Clinical trials.* Starting from the early 1950s, a number of small clinical trials in angina pectoris or intermittent claudication have been conducted (20). However, their size, the lack of a clear randomization procedure, and the use of surrogate end points (e.g., the use of nitrates, walking distance, or chest pain) hamper their usefulness. More recently, the results of two small- and five large-scale trials have been made available for a total of 80,000 subjects (83,94,100–103). Among the large-scale trials, the ATBC trial and the Chinese study were adequately sized, enrolling almost 30,000 patients each. In the ATBC trial, 50 IU/d of vitamin E showed a modest association with a lower risk of onset of angina, no reduction at all in CVD death, and slight, nonsignificant excess in intracerebral hemorrhages. In the substudy in men with previous MI, vitamin E was associated with a significant reduction of 38% in nonfatal MI but a nonsignificant increase in fatal MI (104). The Linxian trial was conducted in an unusual way in a malnourished population of China. The combination of β-carotene, selenium, and vitamin E (30 mg daily) had a marginally significant reduction in total mortality (–70%; 95% CI, –0 to –70%), which was due mainly to a reduced number of stomach cancers. A trend toward a reduction of CVD mortality, however, was also observed (–9%; 95% CI, –24 to +8%). The low vitamin E dose, the unusual population, and the complexity in study design, however, limit the transferability of this trial to other settings. The Cambridge Heart Antioxidant Study (CHAOS) is a relatively small trial in secondary prevention (101). It is a double-blind, placebo-controlled, randomized trial of 2002 patients with angiographically proven CHD. Patients allocated to the experimental arm received 400 or 800 IU of vitamin E daily and were followed for an average of 18 mon. Incidence of nonfatal MI was impressively reduced by 77% (95% CI, –53 to –9%). The combined end point of any major cardiovascular event was also significantly reduced in vitamin E recipients (–53%; 95% CI, –17 to –66%). Total and cardiovascular mortality, however, were increased in the experimental arm (total mortality +29%; 95% CI, –24 to +119%; CVD mortality, –10%; 95% CI, –39 to +96%). The short duration of the trial, the unbalanced nature of the randomized group at baseline, the unclear demonstration of follow-up completeness, the exceedingly high effect of vitamin E supplements, and the low number of events recorded in the study (i.e., 14 vs. 41 nonfatal myocardial infarctions) all suggest the need to wait for the publication of well-conducted, large-scale clinical trials before considering such promising evidence as the proof of the efficacy of vitamin E administration in patients at high CHD risk.

The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio (GISSI)-Prevenzione trial enrolled 11,323 men and women with a recent MI, who were allocated to vitamin E (300 mg/d) or no treatment in a four-arm trial over a 3.5-yr period (102). In the four-way analysis, the main study end points were not significantly reduced, although a significant reduction of fatal cardiovascular events was observed. In an additional analysis carried out according to the administration of vitamin E as a whole (two-way analysis), vitamin E intake was not associated with a reduction in cardiovascular events, although a nonsignificant trend toward lower fatal but not nonfatal cardiovascular events was observed.

Finally, the Heart Outcomes Prevention Evaluation (HOPE) trial randomized 9,541 high risk men and women according to their previous vascular event or diabetes associated with one risk factor (103). Vitamin E (400 IU) had no effect on death from CVD or IHD, nonfatal MI or stroke events. Tables 1–3 summarize the main findings in the four trials done in Europe and America on the effects of vitamin E on death from CVD and IHD and nonfatal MI.

*Comments on studies on vitamin E.* Large observational studies done in men and women mostly without CVD showed a significant lower rate of IHD events associated with vitamin E supplement or dietary intake. The different randomized trials on vitamin E can be divided according to the participants with and without CVD such as previous MI, defined coronary artery disease, stroke or peripheral artery disease. In the nearly 60,000 participants without CVD, vitamin E did not have a significant effect on IHD, total stroke events, or

**TABLE 1**
**Meta-Analysis from Large Randomized Clinical Trials on Vitamin E: Effects on the Risk of Cardiovascular Mortality[a]**

| Trial | Vitamin E: total *n* (%) | Placebo: total *n* (%) | Odds ratio | 95% Confidence interval |
|---|---|---|---|---|
| ATBC | 14,564 (5.9) | 14,569 (6.0) | 0.98 | 0.89–1.08 |
| CHAOS | 1,035 (2.6) | 967 (2.4) | 1.10 | 0.62–1.93 |
| GISSI | 2,830 (5.9) | 2,828 (7.2) | 0.81 | 0.66–0.99 |
| HOPE | 4,761 (7.1) | 4,780 (6.9) | 1.05 | 0.90–1.22 |
| Overall | 23,190 (6.0) | 23,144 (6.2) | 0.97 | 0.90–1.06 |

[a]Abbreviations: ATBC, Alpha-Tocopherol, Beta Carotene Trial; CHAOS, Cambridge Heart Antioxidant Study; GISSI, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio; HOPE, Heart Outcomes Prevention Evaluation.

peripheral artery disease. These results were consistent across a range of low or high doses of vitamin E used with other nutrients as in the Chinese Study (30 mg, β-carotene with selenium) and ATBC (50 mg alone or with β-carotene), and vitamin E alone in the participants with diabetes without CVD in the HOPE study (400 IU). In these studies, the vitamin was administered for a mean duration of 4.5–6.1 yr.

In the participants with CVD, vitamin E alone appeared to reduce the occurrence of nonfatal MI in the 1,035 patients with coronary disease in the CHAOS study (400 or 800 IU) and in the 466 patients with previous MI (50 mg) in the ATBC subgroup study (101,104). In the CHAOS study, the benefit of a 77% risk reduction in nonfatal MI within a relatively short period (1.4 yr) with no effect on fatal cardiovascular event is surprising. It is possible that the findings in this trial may be attributed at least in part to important imbalances in the baseline characteristics between the participants allocated to vitamin E and those to placebo, or to a type 1 error due to chance. The evidence for a benefit from the CHAOS study is not persuasive. In the subgroup analysis from the ATBC involving male smokers with a previous MI, there were only 40 nonfatal MI in the vitamin E group. In this subgroup, there was a nonsignificant increase in fatal IHD, and when associated with β-carotene, the decrease in nonfatal MI was no longer significant (101). Furthermore, these observations were not supported by larger trials such as the GISSI (300 mg) and the HOPE participants (400 IU) with CVD (102,103). Thus, in patients with a previous reported CVD, the overall evidence indicates that vitamin E administered for 3–6 yr does not improve prognosis in low- or high-risk persons. The latter large-scale clinical trials, however, were designed and powered to catch a 20% relative difference in risk of events, which was considered to be clinically relevant and worth pursuing with medical interventions. This means that a lower protective effect of vitamin E in the long term cannot be excluded.

**TABLE 2**
**Meta-Analysis from Large Randomized Clinical Trials on Vitamin E: Effects on the Risk of Fatal Ischemic Heart Disease[a]**

| Trial | Vitamin E: total *n* (%) | Placebo: total *n* (%) | Odds ratio | 95% Confidence interval |
|---|---|---|---|---|
| ATBC | 14,564 (4.1) | 14,569 (4.4) | 0.94 | 0.84–1.06 |
| CHAOS | 1,035 (2.3) | 967 (2.4) | 0.97 | 0.55–1.74 |
| GISSI | 2,830 (5.5) | 2,828 (5.2) | 0.76 | 0.60–0.98 |
| HOPE | 4,761 (7.1) | 4,780 (5.6) | 0.99 | 0.83–1.17 |
| Overall | 23,190 (4.3) | 23,144 (4.7) | 0.92 | 0.80–1.05 |

[a]For abbreviations see Table 1.

**TABLE 3**
**Meta-Analysis from Large Randomized Clinical Trials on Vitamin E: Effects on the Risk of Nonfatal Myocardial Infarction[a]**

| Trial | Vitamin E total n (%) | Placebo total n (%) | Odds ratio | 95% Confidence interval |
|---|---|---|---|---|
| ATBC | 14,564 (4.8) | 14,569 (4.8) | 0.99 | 0.89–1.10 |
| CHAOS | 1,035 (1.4) | 967 (4.2) | 0.23 | 0.11–0.47 |
| GISSI | 2,830 (4.2) | 2,828 (4.0) | 1.06 | 0.82–1.37 |
| HOPE | 4,761 (6.7) | 4,780 (7.0) | 1.03 | 0.88–1.21 |
| Overall | 23,190 (5.0) | 23,144 (5.1) | 0.97 | 0.88–1.07 |

[a]For abbreviations see Table 1.

In 1995, Steinberg (105) made the point that assessing the effect of antioxidants such as vitamin E over a 4- to 6-yr period of time, similar in duration to other intervention trials on lipid lowering or antiplatelets, was most likely not long enough. Vitamin E is hypothesized to reduce new plaque formation and early lesion progression in rabbits and nonhuman primates, instead of having an effect on reduction of the atherosclerotic lesions or on the stability of the plaque (14,106). For this reason in part, the HOPE trial is continuing the followup with the vitamin E arm for another 3 yr. However, it should be noted that trials such as the PHS conducted over a 12-yr period involving β-carotene failed to show any effect (85). Nevertheless, longer trials with vitamin E are warranted. A second issue is whether the current trials enroll the best population with which to assess the effects of vitamin E. It is possible that the participants in most of these randomized trials already had well-established atherosclerosis, which may require a longer duration of observation to detect the effect of vitamin E, if it is attenuating only the development of new lesions but not the progression of established lesions.

In the different randomized trials, the doses of vitamin E used were different. However, in all trials, the plasma levels of vitamin E were significantly increased from the baseline; however, despite the various doses, the levels were not markedly different. The possibility that use of a high dose of vitamin E for a long period of time may induce some prooxidant effects as demonstrated *in vitro* cannot be excluded. It is also possible that in the presence of other antioxidants such as vitamin C and ubiquinol, vitamin E may not have a prooxidant function (107). As discussed for β-carotene, it is possible that vitamin E requires some co-factor(s) or other vitamin(s) (e.g., vitamin C) to have a prolonged antioxidant effect. Trials currently in progress are assessing such associations (108,109).

## DISCUSSION

According to randomized clinical trials, which have taken place mostly in men, β-carotene does not reduce cardiovas-

cular events. In fact, in men who smoke or had smoked or had been exposed to asbestos, β-carotene increased the risk of mortality and, in those who had a previous MI, the risk of death from IHD. Furthermore, in male smokers, β-carotene may increase the risk of intracerebral hemorrhage. In women, most large observational studies did not show any cardiovascular effect. Thus, there is no evidence to recommend β-carotene to adults to prevent IHD or stroke. According to the results of trials in subjects at high risk of lung cancer, it is likely that the use of β-carotene supplements in such subjects could cause real harm, i.e., produce at least 1 excess lung cancer per 1000 high-risk subjects treated per 1 yr. To explain such unexpected results, it has been suggested that high-dose supplements of β-carotene could have prooxidant effects *in vivo*; for instance, it is now known that reactive epoxides and other oxidation products of carotenoids can be formed *in vivo*. Such substances could in turn exert negative biologic effects [e.g., oxidized β-carotene could increase the formation of benzo(α)pyrene-DNA adducts]. The discrepant results of epidemiologic studies and clinical trials with β-carotene are disappointing and highlight the need for basing firm conclusions on the efficacy of antioxidant supplements only on the results of large-scale, well-designed, randomized clinical trials because observational studies are flawed by several limitations. Differences in study design, characteristics of populations, assessment of intake of antioxidants, end points, and length of followup hamper a precise estimate of the magnitude of the association between antioxidant levels (measured as dietary intake or blood levels) and the risk of CVD (Table 4).

The observational studies and randomized trials on vitamin C and cardiovascular events are limited. Nevertheless, there is no evidence now to recommend the administration of this vitamin to reduce cardiovascular events.

Vitamin E administered in persons without or with IHD during a period of 4 yr does not reduce the risk of IHD and all stroke events, and does not have a significant deleterious effect. In male smokers, vitamin E has no beneficial effect on the incidence or progression of intermittent claudication. There is no justification to prescribe this vitamin to prevent CVD until the results of the current trials in progress are known. It is possible that vitamin E associated with a co-factor or another vitamin such as vitamin C may reduce cardiovascular events. However, such recommendations must be confirmed by randomized trials before any recommendation is made. Major discrepancies exist between the findings of observational studies and randomized trials. Perhaps, in randomized trials, the limited (5–6 yr) allocation of vitamin E or the absence of administration of other cofactors found in fruits and vegetables with the vitamins may explain the discrepancies. Randomized trials in progress are addressing some of these issues.

The efficacy of antioxidants is a major issue for CVD prevention. The great improvement in the treatment of acute coronary events has impressively reduced in-hospital mortality (1). Recent estimates of international CHD mortality trends suggest that out-of-hospital mortality is the major component of CHD death rates (almost two-thirds), and it is apparent that only thoughtful preventive strategies can lower such a burden (3).

On the other hand, nutrition is a very complex research topic in CVD, and only recently has the interest of researchers shifted from the evaluation of the effects of diet on blood lipids to other components of diet. Various epidemiologic studies suggest an inverse association between dietary intake of fruit and vegetables and CVD (110,111). However, it is not clear whether an individual component of the diet (antioxidant vitamins, low intake of saturated fatty acids, high intake of unsaturated fatty acids) or a particular combination of nutrients or dietary habits might exert full cardioprotective effects (20,23,24,112–117).

It is important to underline that CHD risk could be lowered either by reducing the level of selected risk factors (e.g.,

**TABLE 4**
**Limitations of Epidemiologic Studies**

| Case-control studies |
|---|
| 1. Sample size, quality of information on exposures, biases in cases and control selection, differential misclassification, and recall bias. |
| 2. Blood levels of vitamins assessed with a single measurement some time after the index event, thus not allowing a distinction between short- and long-term exposures to usual blood levels. |
| 3. Time from blood collection to laboratory dosages, i.e., long-term storage of blood samples tends to decrease vitamin levels. |
| **Cohort studies** |
| 1. Existence of various confounders that could lead to underestimation as well as overestimation of results (the discrepant results of cohort studies and randomized clinical trials on carotene are paradigmatic). |
| 2. Existence of confounding factors that were either not measured or controlled for in multivariate analyses. |
| 3. Subjects taking vitamin supplements may have been more health conscious than nonusers. As such, they had more regular physical activity, did not smoke or were light smokers, had healthier dietary habits, and were more often users of acetylsalicylic acid or estrogen replacement therapy. |
| 4. The precision and reliability of nutrient intake ascertained by means of food-frequency questionnaires is questionable. |
| 5. The intake of antioxidant vitamins as dietary supplements could be viewed as an indicator of other protective health or life style habits. Statistical adjustment in multivariate models cannot eliminate the effects of such confounders if they were poorly measured or not measured. |
| 6. Intake of vitamin supplements could be only a piece of a more complex pattern of healthy habits of life that cannot be separated into their individual component parts. |
| **Randomized clinical trials** |
| 1. Supplementation of diet with individual antioxidants could not exert its optimal effects because of the concerted mechanism action of the antioxidant system, which could require the availability of "harmonized" levels of various antioxidants. |
| 2. Choice of the right antioxidant, i.e., the intake of vitamin E, C, and β-carotene could be a marker for the intake of another not yet identified or measured antioxidant substance, which is in turn responsible for the protective effect attributed to the aforementioned vitamins (e.g., intake of other carotenods such as lutein and zeaxanthin contained in dark green, leafy vegetables, flavonoids such as quercetin, phytoestrogens, and phenol-derived substances). |

blood lipids) or by increasing the level of "protective" factors, such as antioxidants or unsaturated fatty acids (118). In addition, the efficacy of dietary interventions aimed at reducing the level of blood lipids is somewhat unclear. A meta-analysis of metabolic ward studies on the cholesterol-lowering effects of replacing 5% of energy as dietary saturated fatty acids with polyunsaturated or monounsaturated fatty acids suggests that total blood cholesterol can be reduced up to 39 and 24%, respectively. Similarly the reduction of dietary cholesterol intake by 200 mg can lower total blood cholesterol by 13%. All of the aforementioned changes could lower total blood cholesterol up to 76% (119).

However, the results of multifactorial studies aimed at improving life style habits and reducing risk factor levels in the population at large suggest that in free-living subjects, total blood cholesterol can be reduced by 0.14 mM (5 mg/dL), smoking prevalence by 4.2%, and systolic and diastolic blood pressure by 4.2 and 2.7 mm Hg, respectively (120). Studies evaluating the cholesterol-lowering efficacy of low-fat diets, such as the American Heart Association Step 1 and 2 diets, can decrease total blood cholesterol by 3.0 and 5.8%, respectively. Dietary intervention studies aimed at increasing the polyunsaturated/ saturated fatty acids showed better results, with decreases of total blood cholesterol up to 7.6%. Even though these results suggest a limited efficacy of cholesterol-lowering diets in free-living subjects, the potential protective effect of correct dietary habits cannot be discarded. The decrease of saturated fatty acids, in fact, is accompanied by an increase in the intake of unsaturated fatty acids as well as of antioxidant substances, i.e., nutrients that could have cardioprotective effects by themselves. An exploratory analysis conducted in the Nurses' Health Study database, for example, suggests that the substitution of mono- or polyunsaturated fatty acids for saturated fatty acids or *trans* fatty acids (equivalent to 5% of the total energy intake) could be associated with a 30–50% reduction in the risk of CHD events (120).

Finally, it should also considered that not only the quality of diet but also the quantity of food can be associated with different quality and duration of life. For instance, experimental studies in animals suggest that the amount of food eaten lifelong could be associated with a prolonged life, i.e., thinner animals live longer than fatter ones (121). In this sense, the ever-increasing prevalence of obesity in Western countries is disturbing.

In conclusion, no positive suggestion can be given at present concerning the use of vitamin supplements. The best possible advice is extremely close to common sense derived from clinical experience, i.e., eat less; increase the intake of fruit and vegetables, vegetable oils, and unsaturated fats; and lower the intake of animal fats. Better life style habits, including regular physical exercise and avoidance of cigarette smoke, could significantly reduce the burden of CVD. It would be advisable that agricultural and food industries, with the help of government agencies, agree to make healthy foods available at reasonable prices.

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# Marine n-3 Fatty Acids: Basic Features and Background

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**ABSTRACT:** There is some evidence from epidemiology that intake of n-3 polyunsaturated fatty acids (PUFA) from seafood may protect against coronary artery disease (CAD). This hypothesis is further supported from animal data showing a beneficial effect of n-3 PUFA on thrombosis and atherosclerosis in animals fed fish oils in most, but not all, studies. There are several mechanisms by which an increased intake of marine n-3 PUFA may protect against CAD; the most universal finding is a reduction of plasma triglycerides. It is puzzling, however, that a very low amount of n-3 PUFA, with no known beneficial biochemical effects, seems to be cardioprotective. It has therefore been of paramount interest to perform clinical trials. Such evidence and trials are discussed in later chapters, and the results have been very encouraging.

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This article will first review basic features about the composition of different types of fatty acids. We will primarily deal with n-3 polyunsaturated fatty acids (PUFA) derived from seafood and their content in various types of seafood. We will then address the background for the interest in n-3 PUFA as a way to reduce coronary artery disease (CAD) and comment on their potential beneficial and detrimental biochemical effects in relation to CAD. Other chapters will deal specifically with the effect of n-3 PUFA in atherogenesis, thrombogenesis, and in particular, their effect in clinical trials with hard endpoints—the cornerstone of evidence-based medicine.

### *n-3 PUFA—An Introduction*

Fatty acids are separated into saturated (no double bonds), monounsaturated (one double bond), and PUFA (more than one double bond) with important examples of PUFA shown in Figure 1.

The n-3 PUFA are members of an essential fatty acid family characterized by having their first double bond at carbon atom number 3, as opposed to the other essential fatty acid family, n-6 PUFA, whose members have their first double bond at carbon atom number 6, counted from the methyl end of the carbon chain constituting the backbone of fatty acids (1).

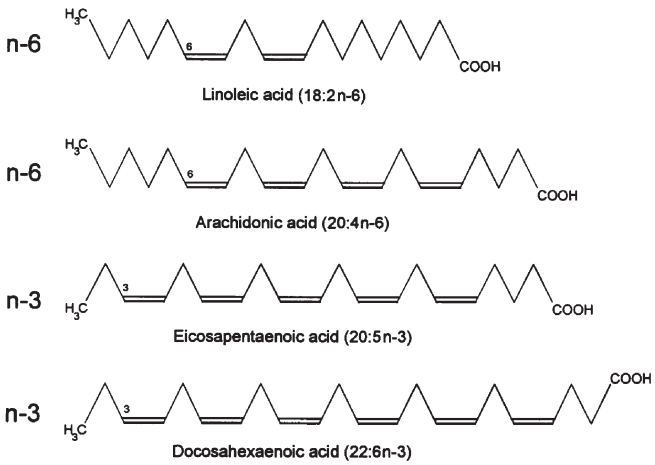


**FIG. 1.** Major n-3 and n-6 PUFA. The number of carbon atoms is given before and the number of double bonds after the colon. The position of the first double bond counted from the methyl end of the fatty acid separates PUFA into n-3 or n-6 PUFA.

There are two subgroups of n-3 PUFA. One is α-linolenic acid derived from plant oils (canola oil, rapeseed oil, and linseed oil) composed of 18 carbon atoms and 3 double bonds (nomenclature 18:3n-3). The daily intake of this fatty acid in Denmark is ~2 g/d. The other group of n-3 PUFA is derived from seafood; the major marine n-3 PUFA are eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Because of their larger number of carbon atoms (20 and 22, respectively), these are also sometimes called long-chain n-3 PUFA. α-Linolenic acid can to some (disputed) degree be elongated and desaturated in humans to EPA and DHA; otherwise, EPA and DHA are acquired only from seafood.

The content of n-3 PUFA varies among fish species. It is high in fatty fish such as mackerel, herring, and salmon and low in lean fish such as flounder and cod (Table 1, adapted from Ref. 2). It is worth noting that the content of n-3 PUFA in seafood varies considerably in relation to the location and the time of year of capture (2,3). Furthermore, the way the fish is prepared is also important. It is therefore very difficult to estimate the amount of n-3 PUFA ingested in populations and also in (long-term) clinical trials with fish as the source of n-3 PUFA.

The intake of long-chain marine n-3 PUFA varies considerably among populations; intake is very high in traditionally living Greenland Eskimos (10-14 g/d), low in Western populations (<0.5 g/d), and intermediate in countries such as Japan

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Abbreviations: CAD, coronary artery disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acids.

**TABLE 1**
**The Approximate Content of n-3 Polyunsaturated Fatty Acids (PUFA) in Seafood[a]**

| Seafood | g n-3 PUFA/100 g |
|---|---|
| Mackerel | 1.8–5.3 |
| Herring | 1.2–3.1 |
| Salmon | 1.0–2.0 |
| Trout | 0.5–1.6 |
| Tuna | 0.5–1.6 |
| Halibut | 0.5–1.0 |
| Shrimp | 0.2–0.4 |
| Cod, plaice, flounder | ~0.2 |

[a]Depends on variables such as season and place of capture.

and Norway (1-3 g/d). The content of n-3 PUFA in cod liver oil is ~20%, and can be up to 90% (30–60% in many preparations) in fish oil capsules.

## *Epidemiology*

There is some evidence from epidemiologic data that populations with a high intake of marine n-3 PUFA have a low risk of CAD. This has been shown most clearly in Greenland Eskimos (4), but a low occurrence of CAD has also been reported in Alaskan Eskimos (5). In studies from Japan, the incidence of CAD was also lower in fishing villages compared with farming villages with a lower intake of fish (6). The possible association between consumption of seafood and CAD has also been studied in Western populations with an average intake of n-3 PUFA <0.5 g/d. In Zutphen, a Dutch area included in the Seven Countries Study, an inverse correlation between fish consumption and CAD was reported in middle-aged men during 20 yr of follow-up (7). A similar inverse correlation between heart disease and fish consumption in Caucasian populations was reported by some, but not by all investigators (for review see Refs. 1 and 8). Importantly, only in the study from Finland (9) was there a positive correlation between fish consumption and CAD, which the authors attributed to a high content of mercury in the fish consumed. In the Health Professionals Study, there was no correlation between fish consumption and CAD, but an inverse correlation between dietary α-linolenic acid intake and CAD was noted (10). In a recent review of the literature, Marckmann and Grønbœk (8) concluded that fish consumption at 40–60 g daily markedly reduced CAD mortality in populations at high, but not low risk for CAD.

Finally, it is of interest that fish consumption in the Physicians Health Study had no protective effect against cardiovascular mortality, but total mortality and the risk of sudden cardiac death were indeed reduced in those who ate fish (11).

It is puzzling why an intake of fish (and n-3 PUFA) of this order of magnitude seems to offer protection against CAD, because the beneficial biochemical effects of n-3 PUFA (see below) have not been shown at this low dosage (1). The possibility that components in fish other than n-3 PUFA might contribute to this effect therefore must be kept in mind, and it may be that fish consumption is associated with a healthier lifestyle including more prudent dietary habits.

## *Marine n-3 PUFA and Risk Factors for CAD*

The risk of CAD is determined in part by inherited genetic traits, age, and gender, all risk factors that cannot be modified. Known modifiable risk factors for CAD include smoking, hypertension, adiposity, high plasma levels of low density lipoprotein (LDL) cholesterol, and low levels of high density lipoprotein (HDL) cholesterol; the effect of hypertriglyceridemia on cardiac risk remains controversial (12,13). High levels of plasma homocysteine, fibrinogen, and coagulation factor VII as well as impaired fibrinolysis are also associated with an increased risk of CAD (12,13). Several other risk factors for CAD have been proposed and the list of risk factors grows steadily with new research (14).

The effect of n-3 PUFA on risk factors for CAD has been investigated in many studies (1). Although dietary n-3 PUFA in practical doses (in contrast to common public belief) have no effect on LDL cholesterol levels, they slightly increase the antiatherogenic $HDL_2$ cholesterol, and substantially decrease plasma triglycerides (15,16). n-3 PUFA may also decrease blood pressure by 2–5 mm Hg, in particular in patients with high blood pressure (1,17). Most studies have shown no effect of n-3 PUFA on plasma fibrinogen and coagulant factor VII levels (1), but n-3 PUFA do slightly reduce platelet reactivity and may impair fibrinolysis (1). Other potential effects of n-3 PUFA with respect to preventing atherosclerosis and thrombosis by modifying risk factors for CAD include a reduction in leukocyte reactivity, an improvement of vessel wall function, and a beneficial effect on blood rheology (1,18). The biochemical effects of n-3 PUFA are commonly seen after daily doses between 2 and 5 g and are dose dependent; the most favorable effects are achieved with the higher doses of n-3 PUFA (1).

The concept of assessing the individual risk of CAD as the sum of risk factors, instead of focusing on single risk factors, e.g., high plasma cholesterol, is very important as stressed in the recently published guidelines on prevention of CAD (12,13). n-3 PUFA induce several beneficial changes in risk factors (Table 2), which in our view make them very attractive in the prophylaxis and treatment of the multifactorial disease, CAD (19).

**TABLE 2**
**Suggested Beneficial Effects of n-3 Polyunsaturated Fatty Acids (PUFA) in Coronary Artery Disease (CAD)[a]**

| |
|---|
| Triglycerides ↓ |
| $HDL_2$-cholesterol ↑ |
| Platelet reactivity ↓ |
| Monocyte reactivity ↓ |
| Neutrophil reactivity ↓ |
| Blood pressure ↓ |
| Improvement of vasoreactivity |
| Antiarrhythmic properties |

[a]↑ represents an increase; ↓ represents a decrease.

### *n-3 PUFA: Types and Safety*

Lean fish provide low amounts of n-3 PUFA but contain little saturated fat and cholesterol and can be recommended for reducing the risk of CAD. Fatty fish have a lower content of saturated fat than most meat servings and may for this reason, independent of likely beneficial effects of n-3 PUFA, be a better food source.

The concept of substituting fish for other food sources and not supplementing fish oils to the habitual diet must be emphasized. However, if fish oils are considered, products of high quality with declared amounts of EPA and DHA, and with antioxidants added, should be chosen.

Concern has been expressed about increased consumption of fish due to intake of heavy metals and pesticides from contaminated fish (9). The risk is low but must not be neglected, and pollution of the sea and its fish should be monitored by international organizations. During industrial processing, it is possible to remove toxic substances from fish oil concentrates.

PUFA (including n-3 PUFA) are susceptible to oxidation due to their high number of double bonds. This may be of importance because of the suggested pivotal role of oxidation of LDL in atherogenesis (20). However, epidemiologic data do not suggest increased atherosclerosis in fish consumers. Whether increased intake of n-3 PUFA leads to clinically relevant enhanced *in vivo* oxidation of LDL continues to be debated (21). Nevertheless, oxidation of n-3 PUFA in fish oil concentrates should be minimized before their intake, which can be achieved by the addition of antioxidants (vitamin E), proper storage, and encapsulation.

There has been concern about an increased risk of bleeding, especially after consumption of larger doses of fish oil concentrates, but there is very little clinical evidence in support of this (22). Furthermore, there is no indication that n-3 PUFA are contraindicated in patients treated with aspirin or anticoagulation, but this has been studied only to a very limited extent (1).

It has been claimed, on the basis of limited data, that n-3 PUFA are detrimental to glycemic control in patients with overt diabetes or impaired glucose intolerance, but recent data have discarded this hypothesis (17,23,24). Although immune responses may be reduced by n-3 PUFA, there is no evidence that intake of n-3 PUFA is associated with an increased risk of cancer or serious infections (1,22). Overall, we conclude that dietary fish and n-3 PUFA are unlikely to promote health problems (25).

In summary, there is some evidence from epidemiology that intake of n-3 PUFA from seafood may protect against CAD. There are several mechanisms by which an increased intake of marine n-3 PUFA may protect against CAD. It is puzzling, however, that a very low amount of n-3 PUFA, with no known beneficial biochemical effects, seems to be cardioprotective. On the other hand, it is reassuring that there is little indication that increased consumption of fish is harmful.

On the bases of epidemiology and the biochemical effects reported, it has therefore been of paramount interest to perform clinical trials, because properly conducted clinical trials remain the cornerstone for a decision to increase the intake of marine n-3 PUFA in the prevention and treatment of CAD. Such evidence and trials are discussed in later chapters, and it should be noted here that the results have indeed been encouraging.

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# n-3 Fatty Acids: Antiatherosclerotic Effects

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**ABSTRACT:** The low incidence of cardiovascular disease associated epidemiologically with high consumption of food rich in n-3 fatty acids suggests the possibility that part of the beneficial cardiovascular effects of these natural substances may be due to a reduction of atherosclerosis. This has been recently confirmed in autoptic data and in at least one prospective trial evaluating the progression of coronary atherosclerosis in humans. This paper reviews published literature on n-3 fatty acids and atherosclerosis in animal models and in humans and *in vitro* experimental data yielding suport to the hypothesis of antiatherosclerotic effects of these substances.

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The n-3 (omega-3), polyunsaturated fatty acids, particularly eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), are thought to display a variety of beneficial effects in areas ranging from fetal development to cancer prevention (1). The occurrence of apparently protective effects on cardiovascular disease provided the stimulus for the early biomedical interest in such compounds and is well documented. Historically, the epidemiologic association between dietary consumption of n-3 fatty acids and cardiovascular protection was first suggested by Bang and Dyerberg (2,3). These authors identified the high consumption of fish, whale, and seal, and thus of n-3 fatty acids, as the likely explanation for the strikingly low rate of coronary heart disease events reported in the Inuit population (2,3). Since their initial reports, research has proceeded along several lines to provide further evidence for this cardioprotection and to understand its underlying mechanisms. Decreased atherogenesis is currently thought to be a part of the cardiovascular protection by n-3 fatty acids. This chapter summarizes the evidence for such a claim and mechanisms putatively involved.

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Abbreviations: AA, arachidonic acid; AGE, advanced glycosylation endproducts; ALA, α-linolenic acid; apo, apolipoprotein; DHA, docosahexaenoic acid; ELAM, endothelium-leukocyte adhesion molecules; EPA, eicosapentaenoic acid; HDL, high density lipoprotein; ICAM, intercellular adhesion molecule; IL, interleukin; LDL, low density lipoprotein; LDLR–/–, LDR receptor deficient; LPS, lipopolysaccharide; MCP-1, macrophage chemoattractant protein-1; NF, nuclear factor; PEG, polyethylene glycol; P/S, polyunsaturated/saturated fatty acid ratio; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule; VLDL, very low density lipoprotein; WHHL, Watanabe heritable hyperlipidemic rabbits.

## *Evidence for Antiatherogenic Effects of n-3 Fatty Acids*

*Effects of n-3 fatty acid in animal models of atherosclerosis.* In animal models, the hypotriglyceridemic action of n-3 fatty acids, which is well documented in humans, is not observed consistently, whereas a reduction in high density lipoprotein (HDL)-cholesterol has been reported consistently (4). Putative antiatherogenic effects of n-3 fatty acids are therefore not attributable to the favorable effects on serum lipids that occur in humans (see below). The n-3 fatty acids have been reported to lessen the development of atherosclerotic lesions in several animal models. However, results have not been unequivocal, due to species differences and variations in study design. The n-3 fatty acids were usually supplemented as fish oils. Studies aimed at determining their effect on atherogenesis or on lesion regression have been carried out in nonhuman primates, pigs, rabbits, and mice. The design of most such studies has been criticized for a variety of reasons including the extremely high levels of supplementation with fish oils, i.e., between 10 and 40% of total energy intake as n-3 fatty acids in most studies, whereas "realistic" dietary values for these species should be 1–2% (4). Another reason is the frequent lack of an appropriate control. In some cases, fish oils were simply substituted for saturated fats; in others, the control oil had a different polyunsaturated/saturated (P/S) ratio, a variable that might *per se* have an influence on lesion development (5,6). Also, the choice of the end point parameters and relative methods in the evaluation of atherosclerosis have been quite variable. The choice of the experimental model is also relevant to explain the different outcome, i.e., in some cases atherogenesis has been induced by drastic dietary changes, quite different from the pathophysiologic conditions occurring in humans. Ideally, animal models showing similarities with the inception and progression of human atherosclerosis should be used, and the control oil should be carefully chosen (4). The majority of experimental studies available in the literature do not meet these criteria.

*Studies in nonhuman primates.* Monkeys have been used to determine the effects of n-3 fatty acid on lipoprotein cholesterol and triglyceride plasma levels (7–10) and on atherosclerosis development (11–13). Such studies are difficult to compare to one another due to metabolic differences among species and to different study designs. Usually n-3 fatty acids were substituted for fat in high amounts, so that the experimental results can be either attributed to n-3 fatty acids or to the removal of hyperlipidemic (saturated) fatty acids. A consistent finding in monkey studies has been the decrease of plasma HDL-cholesterol, an effect often observed in other

species (4). The development of atherosclerosis in general appears to be retarded or diminished (11,12). These results were obtained with the concomitant removal of saturated fats from the diet; these were replaced by high amounts of fish oil without balancing the saturated/unsaturated ratio. Studies aimed at demonstrating the effect of α-linolenic acid (ALA) on atherogenesis support a protective effect also for this specific n-3 fatty acid (14,15). In one regression study, dietary supplementation with a relatively low dose of fish oil (2.5% of total energy intake) added to both an atherogenic and a therapeutic diet with sunflower oil as a control for both, caused an increase in cholesterol and phospholipid content in the aortic intima and no changes in atherosclerosis (13). In an *in vivo* baboon model, high doses of fish oil (vs. an olive oil control) prevented platelet deposition on a plastic vascular shunt and vascular lesion formation in response to mechanical vascular injury in endarterectomized carotids as well as in uninjured aortae (16). Recently, in a different macaque model, the effect of fish oil on the long-term occlusive tendency of aorto-coronary vein bypass grafts was evaluated and found no more effective than olive oil (17).

*Studies in swine.* Pigs have been widely used in studies of lipoprotein metabolism, which appears to be similar to that of humans, and for the study of effects on atherosclerosis, because spontaneous atherogenesis occurs in this species with an early spontaneous beginning. However, native lesion development in pigs is slow and has to be accelerated by high-fat diet, cholesterol, and bile acid supplementation. Fish oils have usually decreased triglycerides and HDL-cholesterol (4). The effects on atherosclerosis are, again, conflicting. A significant dose-dependent reduction in the extent of aortic and coronary luminal enchroachment has sometimes been observed when the vessel is mechanically abraded, whereas atherosclerosis progression is prevented in nonabraded arteries (18–21). The aortic lesion area was, however, not reduced in one set of studies (18–21). The question of lesion regression was also addressed specifically. A favorable effect of fish oil supplementation after a period of atherosclerosis induction by an atherogenic diet was observed in one set of experiments, and regression appeared to be more evident with low levels of plasma cholesterol. In these studies, however, there was no control oil or a control oil was used with a P/S ratio different from that of fish oil (22,23). When a control oil with a matching polyunsaturated/saturated fatty acid ratio was used in one lesion regression study, fish oil had no effect on lesion regression (24). This was accompanied by an unfavorable increase in low density lipoproteins (LDL), a decrease in HDL, and increased susceptibility of LDL to oxidation (25).

*Studies in rabbits.* Rabbits provide a convenient model for atherosclerosis because atherosclerosis can be easily and quickly induced in this species. This is usually obtained by feeding either a cholesterol-rich or a casein-based, fat-free diet. Also, Watanabe heritable hyperlipidemic rabbits (WHHL), lacking functional native LDL receptors and thereby promoting atherosclerosis due to a very high increase in circulating atherogenic lipoproteins, are a well-established model for atherosclerosis, resembling one type of human inheritable type of atherosclerosis. Lipoprotein metabolism, however, is different in rabbits and humans. In rabbits, an increase in dietary cholesterol does not result in LDL but in β-very low density lipoprotein (β-VLDL) elevation, so that any change in lipoprotein levels is not necessarily pertinent to most situations occurring in humans. Studies aimed at verifying the effect of n-3 fatty acids in the rabbit atherosclerosis model are inconsistent with regard to both lipoprotein and triglyceride/cholesterol concentrations (4). In addition, results of n-3 fatty acid supplementation on atherosclerosis are conflicting in this species, and most studies lack an adequate control. Fish oils were reported to inhibit atherosclerosis development in cholesterol-fed rabbits (26–28), to enhance lesion formation (29,30), or to have no effect (31,32). Fish oils, however, reduced intimal proliferation in arteries after balloon injury (33,34). This effect was inversely related to serum cholesterol values, in agreement with data obtained in porcine models (22,35). Also, the efficacy of fish oil was apparently enhanced by vitamin E supplementation (28). In WHHL rabbits, n-3 fatty acids were initially reported to have either no effect on plasma lipids and aortic lesion size (36), or to lower triglycerides, total lipoproteins, and cholesterol in female rabbits, but to be ineffective on lesion size of treated vs. untreated controls (37). With a similar experimental protocol, but with different criteria for lesion evaluation, fish oil reduced triglyceride and cholesterol levels and aortic lesions (38). Recently, a direct comparison of fish oil against olive oil treatment confirmed the occurrence of a hypolipidemic effect of fish oil in this species, and these findings were associated with a retardation of atherosclerosis development in young WHHL rabbits (39).

*Studies in mouse models.* Studies in mouse models have been scarce. Reiner *et al.* evaluated the effect of n-3 fatty acid on the development of atherosclerosis and on the secretory activity of peritoneal macrophages in the atherosclerosis-susceptible strain C57BL/6J (40). These authors compared a saturated fatty acid and a fish oil–supplemented diet. Lesion size was diminished by fish oils. Macrophages displayed a decreased ability to produce basal tumor necrosis factor (TNF)-α and lipopolysaccharide (LPS)-elicited TNF-α and interleukin (IL)-1β production, reduced lipoprotein lipase expression, and an enhanced nitrate synthesis (used as an index of nitric oxide production). In a recent study on murine macrophages, fish oil reduced the expression of intercellular adhesion molecule (ICAM)-1 and of scavenger receptor A type I and II (41). These findings suggest an effect of n-3 fatty acid on macrophage phenotype and on their role in lesion formation.

Recently, transgenic mouse models of atherosclerosis have been introduced (42). The LDL receptor-deficient (LDLR–/–) mouse develops atherosclerosis when fed a Western-type, high-fat diet (43). In this model, LDL are not efficiently cleared from plasma, and hypercholesterolemia and atherogenesis proceed with a pattern similar to the human situation. The apolipoprotein (apo)E-deficient mouse is another model of atherogenesis (44,45). In this model there is no need to

modify the diet because atherosclerosis proceeds spontaneously and very rapidly. The serum lipid profile is quite different from the human condition, however, because apoE is a constituent of all lipoproteins except LDL, and serves as a ligand for receptors involved in the clearance of chylomicrons and VLDL remnants. The only human counterpart for this situation is the genetic defect of apoE, a rare clinical condition that leads to severe atherosclerosis. In both models, atherosclerotic lesions start to spread from the abdominal aorta to the whole aorta and to its main branches, predominantly at bifurcation sites. The pattern of lesion formation has been characterized and found to be similar in both of the above models (46). The influence of gender is controversial, nonetheless the presence of an active production of estrogens seems to be protective.

Although the effect of n-3 fatty acid in the LDLR–/– model has not yet been the object of any published work, studies of n-3 fatty acid in the apoE–/– mice have been done. In the work of Calleja *et al.* (47), apoE–/– mice were fed diets enriched with different oils commonly used in human nutrition, without cholesterol addition. In this model, after the evaluation of lesion area, male mice appeared to respond to sunflower oil, whereas females responded to palm oil and elevated amounts of dietary olive oil. This model therefore seems suitable for the evaluation of dietary oil supplementation. In the recent work of Adan *et al.* (48), 7-wk-old apoE-deficient mice were fed an atherogenic diet in the presence or absence of DHA supplementation (1% final concentration) for 8 wk. No effect of DHA on atherosclerosis was reported, i.e., the size and extent of lesions in the aortic arch, and the thoracic and abdominal aorta did not differ in the two experimental groups. These results might have been influenced by the use of cholesterol and cholate in the diet.

In summary, animal experimental studies with n-3 fatty acid are pointing mainly to the existence of an antiatherogenic effect, but are hampered by differences in study design and species. No definite claim on the existence of true antiatherogenic effects of n-3 fatty acid can be made to date on this basis.

*Studies in humans.* Nutritional intake of n-3 fatty acids is highly likely to lead to cardioprotection. This was confirmed recently by a 30-yr follow-up in men who were free of overt cardiovascular disease at baseline and who consumed up to 35 g of fish per day (49). However, because of the multifactorial nature of ischemic heart disease, in which atherosclerosis is one important component, but not the only one, evidence about the occurrence of a true antiatherogenic effect of n-3 fatty acid in humans is not easy to gather. Autopsy studies in Alaskan natives (consuming high amounts of fish-derived products) and nonnatives consuming mainly Western-type diets provide circumstantial evidence about a lesser extent of atherosclerosis in populations exposed to high nutritional intake of n-3 fatty acids. Newman and co-workers (50) reported a decreased percentage of intima covering with fatty streaks and raised lesion in Alaskan natives, with a high n-3 fatty acid dietary intake (51), vs. nonnatives. In their study, the magnitude of difference in fatty streak development appears larger in younger age groups (Fig. 1), suggesting an effect of diet mainly in the early events leading to fully developed atherosclerotic lesions. Prospective studies in humans are few, but point mainly to the true occurrence of such effects. A study of high-dose n-3 fatty acid supplementation on coronary artery disease regression, evaluated by angiography, was negative (52), but a subsequent well-controlled study [the Study on prevention of Coronary atherosclerosis by Intervention with Marine Omega-3 fatty acids (SCIMO)] showed a slower progression in subjects supplemented with lower doses (1.65 g/d EPA + DHA) (53). Interestingly, the same authors have recently reported no effect of the treatment on carotid intima-media thickness evaluated by carotid ultrasound in the very same subjects [see von Schacky *et al.*, pp. S99–S102 of this volume], indicating some regiospecificity for the fish oil effect. It has been speculated that 0.5–2.0 g of n-3 fatty acids per day is effective in reducing clinical end points (54). By contrast, higher doses have been suggested to yield no effect (52). This contention is based, however, on very few studies examining the effects of these substances on true atherogenesis and not on a mixed end point. One study conducted after coronary by-pass surgery indicates that n-3 fatty acids significantly reduce vein graft stenosis (55), a process that may be regarded as an accelerated form of atherosclerosis. Studies on restenosis after percutaneous coronary angioplasty have been contradictory and, in the end, largely negative when studies of sufficient size and power were performed (56,67). Issues of study design still leave open the door in the minds of some investigators to the possibility that n-3 fatty acids can have some efficacy on restenosis (68). Restenosis after percutaneous interventions, however, is the result of a mechanical injury to an already diseased vessel wall, and its relevance to native atherosclerosis is very questionable.

In summary, evidence for an antiatherogenic effect of n-3 fatty acids is perhaps more persuasive in human than in animal studies because it is now based on at least one placebo-

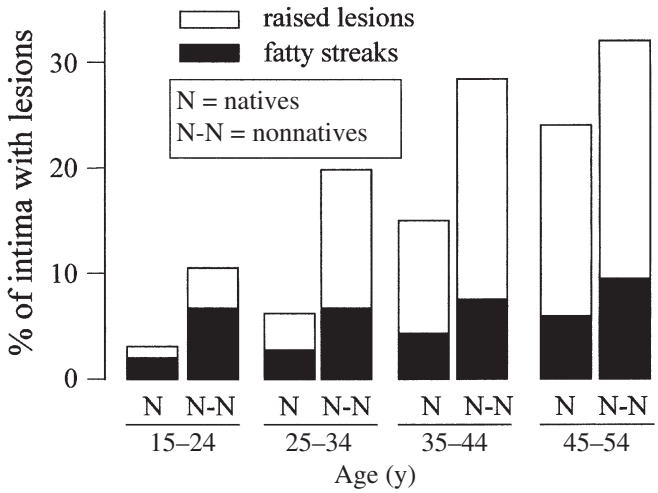


**FIG. 1.** The percentage of coverage of the aorta with fatty streaks (solid areas) and raised plaques (open areas) in Alaskan natives (N) vs. nonnatives (NN), according to age. Notice the larger difference, attributable to the prevalence of fatty streaks, in the younger age groups. Redrawn, with modifications, from Reference 51.

controlled prospective study in native atherosclerosis in the coronary arteries and a placebo-controlled prospective study in coronary bypass surgery grafts. These studies indicate a reduced atherogenesis in the coronary arteries and probably the aorta.

### *Putative Mechanisms by Which n-3 Fatty Acids Interfere with Atherogenesis*

*Molecular mechanisms in atherogenesis.* The initial event in the development of atherosclerosis is a condition of endothelial dysfunction, which precedes any morphologic evidence of endothelial damage (69). Endothelial dysfunction can be triggered by a number of different stimuli (e.g., toxins, shear stress, cigarette smoking, or high cholesterol levels). One type of endothelial dysfunction is termed endothelial "activation" in which the endothelium modifies its phenotype in a proadhesive direction, triggering an increased adhesion of circulating monocytes. Their subsequent infiltration into the arterial intima is one of the first visible findings in atherosclerosis. In the intima, monocytes become activated and begin to incorporate circulating LDL that have become oxidized through the exposure to reactive oxygen species of both endothelial and macrophagic origin, thus initiating the formation of the fatty streak (70) (Fig. 2).

Atherosclerosis and inflammation share similar basic mechanisms, involving the adhesion of leukocytes to the vascular endothelium in their early phases. Multiple protein families, each with a distinct function, provide "traffic signals" for leukocytes. These include the following: (i) the "selectin" family of adhesion molecules; (ii) the chemoattractants, some of which ("classical" chemoattractants, such as *N*-formyl peptides, complement components, leukotriene $B_4$, and platelet-activating factor), act broadly on neutrophils, eosinophils, basophils, and monocytes, whereas more recently described "chemokines," such as monocyte chemoattractant protein-1 (MCP-1) and IL-8, have selectivity for leukocyte subsets; and (iii) the immunoglobulin superfamily members on the endothelium ICAM-1, ICAM-2, ICAM-3, and vascular cell adhesion molecule (VCAM)-1, recognizing "integrin" ligands on the leukocyte surface. For neutrophil (and likely lymphocyte) adhesion, selectins mediate initial tethering of the circulating leukocyte over the endothelium, allowing it to roll over the endothelium, considerably slowing down its speed, and allowing leukocytes to "sense" the presence of chemotactic gradients. Final firm attachment of leukocytes to the endothelium requires the interaction of integrin ligands on the leukocyte surface with immunoglobulin superfamily members, expressed on the endothelium, such as ICAM-1, ICAM-2, and VCAM-1. The multiple molecular choices available for each of these ligand–ligand interactions provide great combinatorial diversity in signals, allowing the selective responses of different leukocyte classes to inflammatory agents, the preferential recirculation patterns of lymphocyte subpopulations, or the selective binding of monocytes to arterial endothelium during early phases of atherogenesis.

Monocyte recruitment into the intima of large arteries is specific for atherosclerosis compared with other forms of leukocyte-endothelial interactions. It was therefore hypothesized that these localized monocyte-endothelium interactions reflect specific molecular changes in the adhesive properties of the endothelial surface, leading to surface expression of

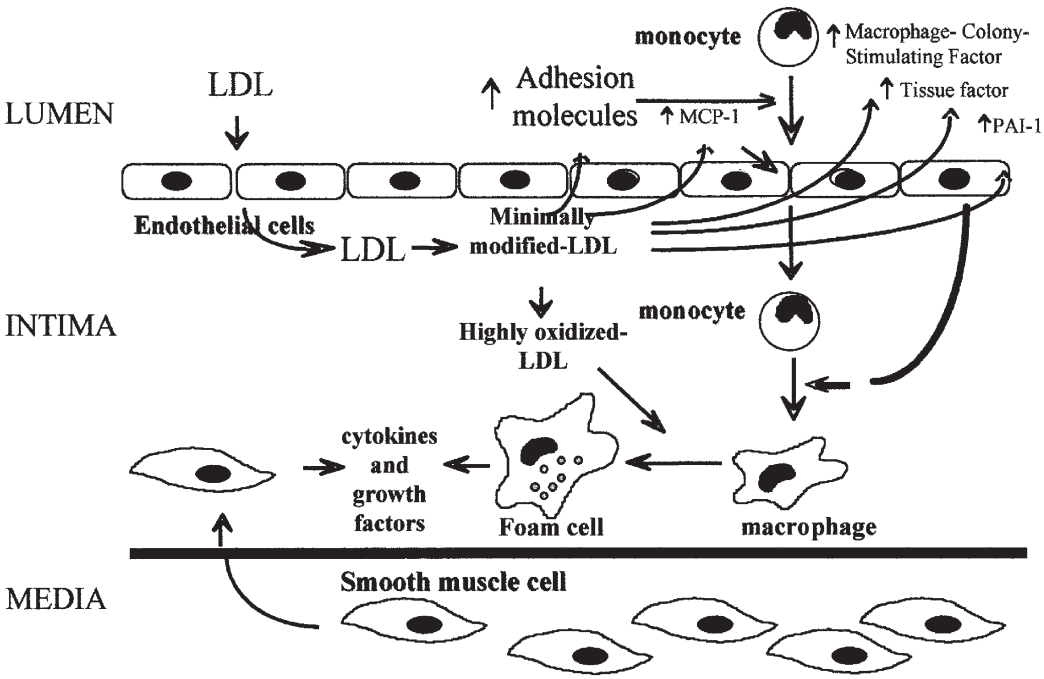


**FIG. 2.** A scheme of the modifications of the arterial intima occurring in early atherogenesis. Abbreviations: MCP-1, macrophage chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1; LDL, low density lipoprotein. From Reference 90, with permission.

"athero-ELAM," i.e., endothelium-leukocyte adhesion molecules (ELAM) expressed in the early phases of atherosclerosis. The first such protein, originally identified in the rabbit hypercholesterolemic model, is VCAM-1, a member of the immunoglobulin superfamily, expressed on human vascular endothelium in at least two molecular forms. Both forms are able to bind a heterodimeric integrin receptor, very late antigen (VLA)-4, whose leukocyte selectivity of expression, on monocytes and lymphocytes, but not on neutrophils, can explain the selectivity of monocyte recruitment in early atherogenesis (71). Endothelial cells express VCAM-1 early during cholesterol feeding in rabbits, before the appearance of macrophages/foam cells in the intima of developing fatty streak, in a temporal pattern consistent with its pathogenetic role in lesion development. Pathophysiologically relevant stimuli for VCAM-1 expression in atherogenesis could include minimally oxidized LDL or β-VLDL, the advanced glycosylation end-products (AGE) associated with diabetes, lipoprotein (a), or perhaps homocysteine, elevated in homocysteinuria and in subtler forms of congenital or acquired enzyme defects in its biosynthetic pathway. In addition to these humoral stimuli, VCAM-1 endothelial gene expression also responds to hemodynamic forces, thus potentially explaining the localization of atherosclerosis in particular points of the arterial vasculature. For a general review on these issues, see Reference 71.

The progression from fatty streak to atheroma is driven by the production of cytokines and chemoattractants that determine the intimal accumulation of leukocytes, smooth muscle cells, and fibroblasts, as well as platelet adhesion. The thin-capped, lipid-rich atheromatous plaques have a strong tendency to rupture and are at risk of complicating with thrombosis, which is usually the ultimate event leading to unstable angina and myocardial infarction (72).There are, therefore, multiple potential points of action of n-3 fatty acids on atherogenesis. The best-characterized ones are reviewed briefly.

*Effects of n-3 fatty acids on plasma lipids.* In humans, n-3 fatty acids decrease serum triglycerides, an effect that is pronounced in marked hypertriglyceridemia. VLDL-cholesterol is reduced, whereas LDL-cholesterol tends to be either elevated or unchanged (73,74). In fact, in patients with mixed hyperlipidemia and in marked hypertriglyceridemia, n-3 fatty acids are a highly effective means for reducing both triglyceride and VLDL. Thus, n-3 fatty acids appear to reduce one of the atherogenic triggers.

*Effects of n-3 fatty acids on cellular responses to atherogenic triggers.* Dietary intake of n-3 fatty acids, such as EPA and DHA, allows their incorporation into the phospholipids of cell membranes, replacing arachidonic acid (AA). Originally, the beneficial effects of n-3 fatty acids on the cardiovascular system were attributed to their substitution of AA. Metabolites that are enzymatically derived from n-3 fatty acids (through cyclooxygenase, lipoxygenase and cytochrome P-450 monoxygenase) are less prothrombotic and vasoconstrictive than the corresponding AA derivatives. Many vascular effects of n-3 fatty acids are equally shared by DHA and EPA, or possibly even more prominently shown by DHA than EPA. Because DHA, at variance with EPA, is a poor substrate for metabolization into eicosanoids, effects of n-3 fatty acids other than generation of eicosanoids are likely to play a greater role in preventing atherogenesis. In recent years, direct effects of n-3 PUFA on endothelial activation have been demonstrated. These include the following: (i) reduced production of cytokines such as IL-1 and TNF in LPS-stimulated monocytes (75); reduced production of the mitogen and smooth muscle cell attractant platelet-derived growth factor (PDGF A and B protein and mRNA) (76,77); reduced expression of tissue factor by monocytes (78); increased endothelial nitric oxide bioavailability (79); the specific downregulation of gene expression for MCP-1 (80); and reduced expression of endothelial adhesion molecules, essential for monocyte adhesion to sites of inflammation and dysfunctional endothelium (81). Research on this last aspect will be now highlighted in greater detail because it provides a potentially comprehensive explanation of the behavior of these agents as modulators of gene expression.

*Modulation of endothelial-leukocyte interactions by n-3 fatty acids.* We used human adult saphenous vein endothelial cells activated by cytokines, as an *in vitro* model of these early steps in atherogenesis, first assessing the effects of various fatty acids on the surface expression of endothelial leukocyte adhesion molecules, and subsequently characterizing mechanisms and functional relevance of such effects. One n-3 fatty acid, DHA, when added to cultured endothelial cells hours to days before the stimulation with cytokines, early enough to allow a significant incorporation of this fatty acid in cell membrane phospholipids, significantly inhibited events connected with endothelial activation. These included the expression of adhesion molecules such as VCAM-1, E-selectin and, to a lesser extent, ICAM-1, after stimulation with virtually any stimulus able to elicit the coordinated expression of such genes (81,82). Thus, this inhibition could be demonstrated with IL-1α and IL-1β, TNF-α, IL-4, and LPS (Fig. 3). Inhibition of adhesion molecule expression occurred in a range of DHA concentrations compatible with nutritional

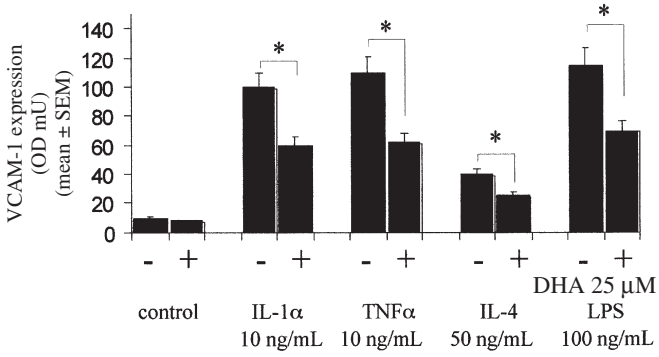


**FIG. 3.** The inhibition of vascular cell adhesion molecule (VCAM) expression by docosahexaenoic acid (DHA), occurring with diverse stimuli, including interleukin (IL)-1α and IL-1β, tumor necrosis factor (TNF)-α, IL-4 and lipopolysaccharide (LPS). Asterisks denote significant differences, $P < 0.01$. From De Caterina, R., unpublished data.

supplementation of this fatty acid to normal Western diets. Also, this inhibition occurred at any time point after the appearance of the cytokine effects, modifying the specific kinetics of surface expression of adhesion molecules, and was strictly related in its magnitude to the extent of incorporation into total cell lipids. Indeed, the extent of VCAM-1 inhibitory effect paralleled the incorporation of DHA and the overall increase in incorporation of n-3 fatty acids, and was inversely related to the content of n-6 fatty acids. Following the fate of $^{14}$C-labeled DHA into cell phospholipids, we could show a significant incorporation of DHA into the phosphatidylethanolamine pool. This is a specific and second most abundant phospholipid pool, with the majority of molecules in the inner plasma membrane, and therefore possibly in a strategic position to alter intracellular signal transduction pathways. This effect was not limited to the expression of transmembrane molecules involved in leukocyte recruitment. It also appeared to occur for other cytokine-activated products, such as the soluble proteins IL-6 and IL-8, involved in either the amplification of the inflammatory response (IL-6) or in the specific chemoattraction for granulocytes (IL-8). The effect was also accompanied by a functional counterpart, i.e., a reduced monocyte or monocytoid cell adhesion to cytokine-activated endothelium. Compared with DHA, EPA was a weaker inhibitor of the expression of these molecules and of monocyte adhesion, although still more potent than other fatty acids. We also showed that the effects of DHA on VCAM-1 expression are accompanied by parallel reductions in VCAM-1 mRNA steady-state levels, as assessed by Northern analysis (81,82). Similar results, in experiments with remarkably similar design, were later reported by Weber *et al.* (83). These authors also carried these investigations one step further, demonstrating an inhibition by DHA of the activation of the nuclear factor (NF)-κB system of transcription factors (83), which controls the coordinated expression of adhesion molecules and of leukocyte-specific chemoattractants upon cytokine stimulation (84,85).

We further analyzed endothelial effects of various fatty acids differing in chain length, number, position (n-3 vs. n-6 vs. n-9), and *cis/trans* configuration of the double bonds. Using VCAM-1 surface expression as a marker, we concluded the following marker: (i) saturated fatty acids are inactive; (ii) the potency of polyunsaturated fatty acids increases with the number of unsaturations; (iii) potency does not depend on chain length; (iv) the single double bond present in the monounsaturated fatty acid oleic acid is indeed sufficient to produce all of the effects obtainable with higher unsaturated fatty acids, albeit at higher concentrations; and (v) for such an effect to occur, even the configuration (*cis* vs. *trans*) of the double bond does not really matter because oleic acid (19:1n-9 *cis*) and its *trans* stereoisomer elaidic acid are of equal potency (86). Indeed, inhibition of NF-κB activation could also be reproduced upon incubation of endothelial cells with oleic acid (87).

*Possible molecular mechanisms by which unsaturated fatty acids inhibit endothelial activation.* To ascertain mechanisms for these effects, we demonstrated inhibition of NF-κB activation by DHA (the most potent fatty acid inhibitor of endothelial activation) in parallel with measurements of production of hydrogen peroxide by cultured endothelial cells. This reactive oxygen species (or one or more of its downstream unstable products) appears to be a critical mediator of NF-κB activation (Fig. 4). Indeed, we had previously shown that treatment of endothelial cells with polyethylene glycol (PEG)-complexed superoxide dismutase (a cell membrane–permeable form of this enzyme, which catalyzes the conversion of superoxide anion to hydrogen peroxide) does not greatly affect VCAM-1 mRNA production. On the contrary, a treatment with PEG-catalase, which acts by accelerating the degradation of hydrogen peroxide, quenches endothelial activation (88). This suggests that hydrogen peroxide (or some of its downstream products) is more relevant than upstream products (i.e., superoxide anion) in the activation of NF-κB. We also assessed the production of intracellular hydrogen peroxide (and/or its downstream products) by dichlorofluorescein before or after stimulation with IL-1 or TNF-α. In both these experimental systems, we could document (De Catarina, R., preliminary results) a decrease in baseline production of reactive oxygen species after cell membrane enrichment with DHA, and an even more pronounced dampen-

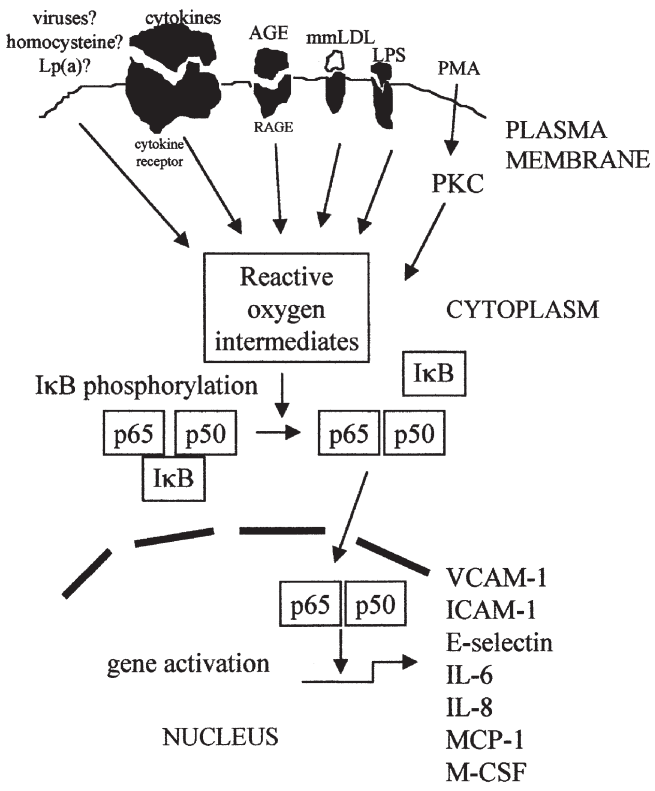


**FIG. 4.** A scheme of the intracellular signal transduction pathways leading to increased gene expression of target genes upon endothelial cell exposure to atherogenic triggers. Abbreviations: Lp(a), lipoprotein (a); AGE, advanced glycosylation end-products; RAGE, receptor for AGE; LDL, low density lipoprotein; LPS, lipopolysaccharide; PMA, phorbol myristic acid; PKC, protein kinase C; IκB, Inhibitor of NF-κ-B; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; IL, interleukin; MCP, macrophage chemoattractant protein; M-CSF, microphage colony stimulating factor. Redrawn, with modifications, from Reference 84.

ing of the increase produced by stimulation with cytokines. Saturated fatty acids served as a negative control in these experiments. Therefore, our current understanding of these phenomena is that a property related to fatty acid peroxidability (the presence of multiple double bonds), usually regarded as a detrimental consequence of polyunsaturated fatty acid enrichment of cell membranes, is indeed also directly related to this putatively favorable outcome (Fig. 5).

These results have led to a reappraisal of how fatty acids may act on endothelial cells in modulating general phenomena such as atherogenesis (investigated mainly by our experimental systems), but also, potentially, inflammation or some immune responses. Because all of these effects could be confirmed to occur even in the presence of inhibitors of metabolic conversion of fatty acids to eicosanoids, they provide a novel explanation for the modulating effect of n-3 fatty acids in atherogenesis, distinct from the original hypothesis of substrate substitution (89). The results with oleic acid might also be an explanation for at least some of the beneficial effects of olive oil–rich ("Mediterranean") diets on atherogenesis. It is noteworthy in this regard that oleic acid appeared to be incorporated mainly at the expense of saturated fatty acids, thus disclosing the possibility of additive effects with n-3 fatty acids, which substitute mainly less unsaturated fatty acids in the membrane phospholipid pools. If extended to cell types different from endothelial cells, such as the monocyte-macrophage, also undergoing "activation" phenomena upon cytokine or LPS stimulation, our results may provide a coherent explanation for a number of previous observations such as the inhibition of cytokine formation from LPS-activated macrophages (75). As to the mechanism(s) involved, these effects might be closely linked to polyunsaturated fatty acid peroxidability.

Future research will have to further elucidate the molecular basis for these phenomena as well as expanding the scope of this research line into explaining many biological effects of unsaturated fatty acids as modulators of the biological responses to cytokines.

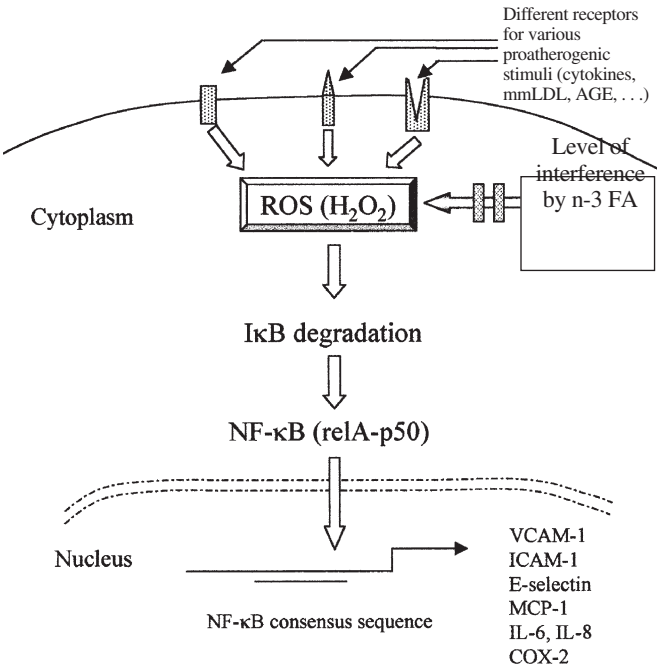


**FIG. 5.** A scheme of the putative site of action of n-3 fatty acids (FA) on endothelial activation. Abbreviations: ROS, reactive oxygen species; NF-κB, nuclear factor-κB; COX, cyclooxygenase. For other abbreviations see Figure 4.

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# n-3 Polyunsaturated Fatty Acids and Coronary Thrombosis

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**ABSTRACT:** Studies of Greenland Eskimos showed that a very high intake of marine n-3 fatty acids markedly inhibited platelet reactivity and suggested that intake of these fatty acids might prevent coronary thrombosis. Later studies with lower, more practical doses of n-3 fatty acids also have shown a platelet inhibitory effect of n-3 fatty acids, albeit fairly marginal. Furthermore, n-3 fatty acids have little effect on measures of blood coagulability and may slightly decrease fibrinolysis. In animal models, n-3 fatty acids often have been shown to inhibit thrombosis, but again the doses have tended to be very high. Finally, there has been little effect of (low-dose) n-3 fatty acids in clinical trials in humans on the incidence of myocardial infarction. Overall, there is little evidence for a major antithrombotic effect of practical doses of n-3 fatty acids on coronary thrombosis. This does not exclude a beneficial effect of n-3 fatty acids on coronary heart disease as suggested from clinical trials, but the major effect may be antiarrhythmic rather than antithrombotic.

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Dyerberg and Bang suggested that the low incidence of acute myocardial infarction (MI) observed in Greenland Eskimos could be due to their high intake of n-3 polyunsaturated fatty acids (PUFA). Their hypothesis, published in *The Lancet* in 1978 and 1979, was that the major effect of n-3 PUFA was a shift in the balance between pro- and antiaggregatory eicosanoids changing the platelet–vessel wall interaction in an antithrombotic direction (1,2). We shall briefly review the evidence of an antithrombotic effect of n-3 PUFA and discuss whether such an effect might be of clinical importance in patients with ischemic heart disease (IHD).

## *Epidemiology*

Epidemiologic studies to some extent support the notion that intake of n-3 PUFA is associated with a low incidence of acute MI and mortality from cardiovascular disease. Kromann and Green (3) studied the disease pattern from 1950 to 1974 from files at the Upernavik Hospital, Greenland, and found only three cases of MI when 40 were expected according to Danish data. In the period from 1979 to 1983, when 92% of deaths were registered by death certificates, the age-standardized incidence of deaths from IHD in Greenland was approximately half that in Denmark (4,5). The incidence of atherosclerosis and IHD has also been reported to be low in Alaskan Eskimos (6,7), and the incidence of IHD in Japan was reduced in fishing villages with a high consumption of fish compared with farming villages with a lower fish intake (8–10). In the Zutphen study, a negative correlation between IHD and fish intake was reported (11). Although the results from Western countries are not entirely consistent, most studies have shown a beneficial effect of fish consumption on IHD, in particular, in high-risk populations (for review see Ref. 12). In a recent review it was concluded that fish consumption of 40–60 g/d was associated with a markedly reduced risk of IHD in high-risk, but not in low-risk, populations (12).

## *Experimental Studies*

Hornstra (13) investigated the effect of PUFA on experimental arterial thrombosis in rats and showed that n-3 PUFA in combination with a low intake of saturated fat reduces thrombosis. In an extensive study in baboons, a very high dose of fish oil markedly reduced thrombus formation in arteriovenous shunts and segments of endarterectomized aortae, impaired smooth muscle cell proliferation, and virtually abolished vascular lesion formation at sites of carotid endarterectomy (14).

Microvascular perfusion after ischemia and reperfusion were improved in hamsters given fish oil, probably because leukocyte adhesion and obstruction of capillaries were inhibited by n-3 PUFA (15). Dietary fish oil improved the microcirculation secondary to an ischemic insult in an experimental rat model (16). We recently reported that n-3 PUFA decreased microvascular thrombosis after ischemia–reperfusion injury in a porcine experimental model (17). Finally, cod liver oil has been shown to reduce platelet deposition and injury-related vasoconstriction in pigs (18).

In conclusion, there is evidence from different animal models that n-3 PUFA may have beneficial effects on arterial thrombosis and may improve the microcirculation. However, the dose of n-3 PUFA in most of these experiments was considerably higher than what can be consumed by humans.

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Abbreviations: AA, arachidonic acid; DART, diet and reinfarction trial; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IHD, ischemic heart disease; MI, myocardial infarction; PG, prostaglandin; PUFA, polyunsaturated fatty acids; TX, thromboxane.

### *n-3 PUFA and Platelet Reactivity*

Platelets enriched with n-3 PUFA generate thromboxane (TX) $A_3$, and often less arachidonic acid (AA) is available for the formation of $TXA_2$. Competition between AA and eicosapentaenoic acid (EPA) for the cyclooxygenase enzyme and, possibly, an inhibitory effect of docosahexaenoic acid (DHA) on cyclooxygenase may further reduce platelet formation of $TXA_2$ (13). Furthermore, both EPA and DHA may antagonize AA and $TXA_2$ at receptor levels (19,20) and may be metabolized *via* lipoxygenase pathways in platelets to products that have been suggested to inhibit platelet reactivity (21,22).

The effect of n-3 PUFA on human platelet function has been studied extensively (see Refs. 23 and 24 for reviews). In summary, n-3 PUFA inhibit $TXB_2$ production, platelet aggregation, and platelet adhesion and cause a minor prolongation of the bleeding time. However, these effects are probably marginal, and the inhibitory effect of n-3 PUFA on the production of proaggregatory thromboxanes is well below that obtained after intake of a low dose of aspirin (25). Some studies have shown that n-3 PUFA and aspirin have synergistic inhibitory effects on measures of platelet reactivity (26,27). Clearly, such an effect would be of importance in patients with IHD for whom aspirin treatment is now mandatory.

### *n-3 PUFA and the Vessel Wall*

Dietary n-3 PUFA lead to endothelial formation of prostaglandin $I_3$ $PGI_3$ with effects believed to be similar to those of $PGI_2$ (13); the sum of $PGI_2$ and $PGI_3$ increases rather than decreases after intake of n-3 PUFA (28,29), whereas n-3 PUFA may augment the release and effects of NO (30–33). Interestingly, dietary n-3 PUFA have been reported to reduce vasoconstriction and improve vasodilatory responses in healthy humans (32). In conclusion, n-3 PUFA have effects on endothelial cells and vessel wall function likely to be beneficial in the prevention of IHD.

### *n-3 PUFA and Coagulation*

Numerous studies have evaluated the effect of n-3 PUFA on various coagulation factors (reviewed in Ref. 24). Early uncontrolled studies tended to indicate that n-3 PUFA lowered plasma fibrinogen, a well-described risk factor for IHD, but recent studies have not been able to confirm this. Data are also conflicting for other measures of coagulability but overall coagulability is probably not substantially altered by n-3 PUFA.

### *n-3 PUFA and Fibrinolysis*

Impaired fibrinolysis is linked to an increased risk of IHD (34). Initially, intake of n-3 PUFA was reported to enhance fibrinolysis (35), but a number of studies investigating this issue were unable to confirm this finding. In fact, several studies have reported that plasminogen activator inhibitor may increase after dietary n-3 PUFA, suggesting that n-3 PUFA may depress fibrinolysis (24). Again, results are conflicting, but n-3 PUFA likely have little effect on fibrinolysis.

### *Clinical Studies*

What is the evidence that n-3 PUFA prevent coronary thrombosis in humans? There are no data from healthy persons, but two large and one small secondary prevention, randomized studies in patients surviving an acute MI have been published.

The Diet And Reinfarction Trial (DART) study (36) included 2,033 men surviving a recent MI. The patients were randomized to different kinds of dietary advice, i.e., reduction in fat intake, increase in dietary fiber, or increase in fish consumption. The patients given fish advice were asked to eat at least two meals of fatty fish per week [or in case this was not possible, to take fish oil capsules containing ~1 g n-3 PUFA (taken by 22% of the patients)]. The groups were followed for 2 yr. The patients receiving fish advice had a significant (29%) reduction in total death and a significant (33%) reduction in cardiovascular death. However, the number of total coronary events was not significantly reduced because the number of nonfatal MI was higher in the fish advice group. The results from DART therefore do not support an antithrombotic effect of dietary n-3 PUFA.

These results were confirmed in the Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto Miocardio (GISSI)-Prevenzione trial (37). In this large-scale, Italian multicenter trial, 11,324 patients with recent (<3 mon) MI were randomized to four treatment groups as follows: n-3 PUFA (0.85 g), vitamin E (300 mg), the combination of n-3 PUFA (0.85 g) and vitamin E (300 mg), or no supplement. Most patients received recommended preventive treatments (including aspirin, 92%). n-3 PUFA supplementation reduced long-term complications of MI to a clinically important extent. The combined primary end point of death, nonfatal MI, and nonfatal stroke was significantly reduced by n-3 PUFA supplementation; relative risk reduction was 15%. All of the benefit seen in the combined end point was due to the reduction in total mortality (relative risk reduction, 20%); the rate of nonfatal cardiovascular events (MI and stroke) was unchanged. n-3 PUFA significantly reduced the risk of cardiovascular death, coronary death, and sudden death. Again, there was no decrease in MI in the group receiving n-3 PUFA, arguing against a significant antithrombotic effect of n-3 PUFA in this trial.

In a study from India (38), 360 patients with suspected MI were randomized to treatment with 1.9 g of EPA + DHA, 2.9 g of α-linolenic acid, or placebo. After 1 yr, there was a significant reduction in cardiac death, nonfatal MI, and total cardiac events in patients randomized to EPA + DHA. This may indicate an antithrombotic effect of n-3 PUFA. This is supported by the recent finding from a Finnish epidemiologic study (39). In that trial, there was a negative association between the serum level of DHA + docosapentaenoic acid and the risk of acute coronary events, often thrombotic in origin, over 10 yr and follow-up.

Experimental studies suggest that intake of n-3 PUFA inhibits thrombus formation and has beneficial effects on microcirculation, especially after ischemia–reperfusion injury.

Intake of n-3 PUFA inhibits human platelet reactivity and has beneficial effects on the arterial wall. Changes in fibrinolysis after intake of n-3 PUFA have not been established, whereas routine clinical indices of coagulation are definitely unchanged. In large-scale clinical trials, intake of n-3 PUFA has been shown to improve survival in patients with recent MI. However, n-3 PUFA intake did not significantly protect against reinfarction in these trials; therefore, it is unlikely that n-3 PUFA exert a major antithrombotic effect in patients with recent MI.

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# n-3 Fatty Acids and Human Health: Defining Strategies for Public Policy

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**ABSTRACT:** The last quarter of the 20th century was characterized by an increase in the consumer's interest in the nutritional aspects of health. As a result, governments began to develop dietary guidelines in addition to the traditional recommended dietary allowances, which have been superseded now by dietary reference intakes. In addition to governments, various scientific societies and nongovernmental organizations have issued their dietary advice to combat chronic diseases and obesity. Human beings evolved on a diet that was balanced in n-6 and n-3 essential fatty acid intake, whereas Western diets have a ratio of n-6/n-3 of 16.74. The scientific evidence is strong for decreasing the n-6 and increasing the n-3 intake to improve health throughout the life cycle. This paper discusses the reasons for this change and recommends the establishment of a *Nutrition and Food Policy*, instead of a *Food and Nutrition Policy*, because the latter subordinates the nutritional aspects to the food policy aspects. Nutrition and food planning comprise a tool of nutrition and food policy, whose objectives are the achievement of the adequate nutrition of the population as defined by nutritional science. The scientific basis for the development of a public policy to develop dietary recommendations for essential fatty acids, including a balanced n-6/n-3 ratio is robust. What is needed is a scientific consensus, education of professionals and the public, the establishment of an agency on nutrition and food policy at the national level, and willingness of governments to institute changes. Education of the public is essential to demand changes in the food supply.

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The last quarter of the 20th century was characterized by an increase in the consumer's interest in the nutritional aspects of health (1). As a result, governments began to develop dietary guidelines in addition to the traditional recommended dietary allowances (RDA), which have been superseded by dietary reference intakes (DRI) (Table 1) (1,2). In addition to governments, various scientific societies and nongovernmental organizations have issued their dietary advice to combat chronic diseases and obesity (3–5).

Obesity has increased exponentially over the past 20 yr. In industrialized societies, a number of factors have contributed to a large proportion of the population being overweight and obese. Among them, an increase in energy intake owing to a palatable food supply and a decrease in energy expenditure, the latter due to sedentary lifestyle, are most important. The advent of the automobile and mechanical devices doing the work previously accomplished by walking and muscular work have compounded the problem. In the beginning of the 20th century, 30% of energy came from muscular work, whereas now it is only 1% (6). Therefore, recommendations on the type and amount of physical activity must be included in addition to dietary recommendations to overcome the enormous increases in obesity in both developed and developing countries (5,7–11).

**TABLE 1**
**Dietary Reference Intakes (DRI)[a,b]**

| |
|---|
| EAR (Estimated Average Requirement): the intake that meets the estimated nutrient need of 50% of the individuals in that group. |
| RDA (Recommended Dietary Allowance): the intake that meets the nutrient need of almost all (97–98%) individuals in that group. |
| AI (Adequate Intake): average observed or experimentally derived intake by a defined population or subgroup that appears to sustain a defined nutritional state, such as normal circulating nutrient values, growth, or other functional indicators of health. |
| UL (Tolerable Upper Intake Level): the maximum intake by an individual that is unlikely to pose risks of adverse health effects in almost all (97–98%) individuals. |

[a]Refers to daily intakes, averaged over time.
[b]*Source:* Reference 2.

Consumer interest in the nutritional aspects of health has led to the development of health messages by governments for some food components, i.e., fiber, calcium, sodium, and to the use of new terminology—nutraceuticals, functional or designer foods—despite the fact that these particular foods have been part of the diet of humans for thousands of years. But what is the situation with the nutrients of interest to us, essential fatty acids and particularly n-3 fatty acids?

Although essential fatty acids (EFA) were recognized as such by Burr and Burr in 1929 and 1930 (12,13), major interest in n-3 fatty acids followed the work of Bang and Dyerberg in the 1970s (14–18). Since then, there has been major expansion in our knowledge on the role of the various n-3 fatty acids in growth and development and in health and disease (19–26), particularly cardiovascular disease (27–34). Studies on the composition of the diet at various periods of time reveal a continuous decrease of n-3 fatty acids in the food supply, but an enormous increase in n-6 fatty acids (35,36). Evidence on

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DRI, dietary reference intakes; EFA, essential fatty acids; LA, linoleic acid; RDA, recommended dietary allowances.

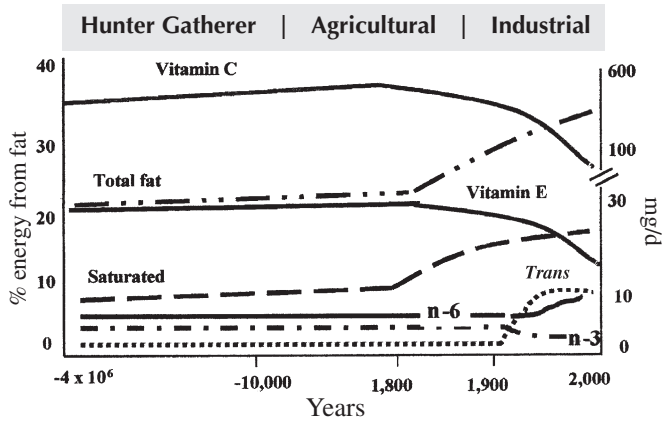


**FIG. 1.** Hypothetical scheme of fat, fatty acid (n-6, n-3, *trans,* and total) intake (as a percentage of energy from fat) and intake of vitamins E and C (mg/d). Data were extrapolated from cross-sectional analyses of contemporary hunter-gatherer populations and from longitudinal observations and their putative changes during the preceding 100 yr. *Source:* Reference 39.

the composition of the diet from the Paleolithic period ~40,000–15,000 yr ago suggests that the Paleolithic diet was characterized by a lower fat and lower saturated fat intake than Western diets, a balanced intake of n-6 and n-3 EFA, small amounts of *trans* fatty acids, contributing <2% of dietary energy, large amounts of green leafy vegetables and fruits providing higher levels of vitamin E and vitamin C and other antioxidants than today's diet, and higher amounts of calcium and potassium but lower sodium intake (36–39) (Fig. 1). Grains were not part of the Paleolithic diet. In terms of their entry into the diet 10,000 yr ago during the agricultural revolution, grains are in essence "newcomers" (40). It is estimated that during the late Paleolithic period, 35% of energy came from animal sources and 65% from vegetable sources (38). Although major changes have taken place in our diet over the past 10,000 yr since the beginning of the agricultural revolution, our genes have not changed. Information from archeological findings and studies from modern day hunter-gatherers suggest that the Paleolithic diet is the diet on which we evolved, and for which our genetic profile was programmed. The spontaneous mutation rate for nuclear DNA is estimated at 0.5% per million years. Therefore, over the past 10,000 yr, there has been time for very little change in our genes, perhaps 0.005%, but major changes in our food supply have occurred, particularly during the last 150 yr.

### *The Status of n-3 Fatty Acids in the Food Supply*

Today, the ratio of n-6/n-3 is between 15:1 and 20:1 in Western Europe and the United States, whereas during our evolution it was 1:1 or even less (Tables 2–4) (19,35–39). The change in the EFA balance came about because of the recommendation in the 1960s to substitute vegetable oils, i.e., corn oil, safflower, sunflower, and cottonseed oils, for saturated fat. These vegetable oils are very high in n-6 fatty acids and very low in n-3 fatty acids. Corn oil has a ratio of n-6/n-3 of 60:1 and safflower oil 77:1 (Table 5). In addition, because farm animals are grain-fed, their carcasses contain small amounts of n-3 fatty acids, but high amounts of saturated fat and n-6 fatty acids, unlike the composition of meat from animals in the wild (41). Similarly, eggs and poultry (42,43), fish from aquaculture (44), and cultivated plants (45,46) contain lower amounts of n-3 fatty acids than eggs and meat from free-ranging chickens, fish in the wild, and uncultivated plants (42–48).

Table 6 shows the ratio of n-6/n-3 in various countries (35,36,49,50). Only the traditional diet of Greece has a ratio similar to that of the Paleolithic diet. The Greek diet, as exemplified by the diet of Crete, is associated with the longest life expectancy and lowest rate of cardiovascular disease (51,52). A modified diet of Crete was used in the Lyon Heart study, which

**TABLE 2**
**Estimated n-3 and n-6 Fatty Acid Intake in the Late Paleolithic Period[a,b]**

| | (g/d) |
|---|---|
| Plants | |
| LA | 4.28 |
| ALA | 11.40 |
| Animals | |
| LA | 4.56 |
| ALA | 1.21 |
| Total | |
| LA | 8.84 |
| ALA | 12.60 |
| Plants and animals | |
| AA (n-6) | 1.81 |
| DTA (n-6) | 0.12 |
| EPA (n-3) | 0.39 |
| DPA (n-3) | 0.42 |
| DHA (n-3) | 0.27 |
| Ratios of n-6/n-3 | |
| LA/ALA | 0.70 |
| (AA + DTA)/(EPA + DPA + DHA) | 1.79 |
| Total n-6/n-3 | 0.79[c] |

[a]*Source:* Reference 36.
[b]Abbreviations: LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DTA, docosatetranoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.
[c]Assuming an energy intake of 35:65 of animal/plant sources.

**TABLE 3**
**Estimated n-3 and n-6 Fatty Acid Intake in Current Western Diets[a,b]**

| | (g/d) |
|---|---|
| LA | 22.5 |
| ALA | 1.2 |
| AA | 0.6 |
| EPA | 0.05 |
| DTA | — |
| DPA | 0.05 |
| DHA | 0.08 |
| Ratios of n-6/n-3 | |
| LA/ALA | 18.75 |
| (AA + DTA)/(EPA + DPA + DHA) | 3.33 |
| Total n-6/n-3 | 16.74[c] |

[a]*Source:* Reference 36.
[b]See Table 2 for abbreviations.
[c]Assuming an energy intake of 35:65 of animal/plant sources.

**TABLE 4**
**Ratios of Dietary n-6/n-3 Fatty Acids in the Late Paleolithic Period and in Current Western Dietary Patterns (g/d)[a,b]**

| | Paleolithic | Western |
|---|---|---|
| LA/ALA | 0.70 | 18.75 |
| (AA + DTA)/(EPA + DPA + DHA) | 1.79 | 3.33 |
| Total | 0.79 | 16.74[c] |

[a]*Source:* Reference 36.
[b]See Table 2 for abbreviations.
[c]Assuming an energy intake of 35:65 of animal/plant sources.

showed a 70% decrease in mortality in 2 yr (27). The Lyon Heart study (27–29), the Singh study (31,32), and Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto Miocardico (GISSI)-Prevenzione trial (34) were characterized by a dietary pattern similar to that of Crete with the following differences: (i) the addition of 850–882 g of n-3 fatty acids on top of a Mediterranean diet in the GISSI trial; (ii) an increase in fish or mustard oil along with fruits and vegetables in the Singh study; and (iii) the addition of canola oil as a source of α-linolenic acid (ALA) in the Lyon Heart study. Similarly, the addition of canola oil along with other changes—lower saturated fat intake, more fruits and vegetables—brought about a desirable decrease in the death rate from cardiovascular disease in Finland (53).

### *EFA: Dietary Recommendations*

A number of organizations have already made dietary recommendations (54–56). In April 1999, an international group consisting of scientists from academia, government, and industry held a Workshop on the Essentiality of and Recommended Dietary Intakes (RDI) for Omega-6 and Omega-3 Fatty Acids. The group's recommendations are shown in Tables 7 and 8 (57). The scientific evidence is indeed sufficiently robust to permit a definition of an Adequate Intake (AI) for n-6 and n-3 fatty acids.

In addition to these recommendations, industry has already begun to return n-3 fatty acids to the food supply (58–59) (Table 9), which has also raised a number of issues (Table 10) that must be considered carefully. It is desirable that nutrition science drive food science and production, particularly in the development of novel foods.

It is now necessary to continue discussions on these issues with government officials at the level of the scientific community and with consumer organizations to return n-3 fatty acids to the food supply, decrease n-6 intake, and balance the n-6/n-3 fatty acid ratio. Because the reformulation of products is expensive, one could first begin to change the cooking and salad oils. Corn, safflower, sunflower, and cottonseed oils have high concentrations of n-6 fatty acids (Table 5). These oils have been part of the food supply only for the last 150 yr, i.e., since the advent of the oil press (19). Never before in the history of human beings has there been such a mass dietary change. Even soybean oil and canola oil, because they are hydrogenated, are lower in ALA than in their natural state. In the Lyon Heart study the ratio of linoleic acid (LA)/ALA was 4:1 and AA/eicosapentaenoic acid (EPA) was 1:1. We should aim for a ratio of LA/ALA of 4:1 or less (60), a goal already achieved in Japan (49) (Table 6).

### *Defining Strategies for Public Policy: A Nutrition and Food Sciences Agency Is Needed*

The League of Nations proposed that it was a collective duty of society to assume responsibility for a Nutrition and Food Policy. Until 1950, the main challenges for the policy makers were to provide enough food for all of the people. After the

**TABLE 5**
**Comparison of Dietary Fats[a,b]**

| | | Polyunsaturated fat | | | | |
|---|---|---|---|---|---|---|
| | | LA | ALA | LA/ALA | Monounsaturated | |
| Dietary fat | Saturated fat | Fatty acid content normalized to 100% | | | fat | Cholesterol |
| Flaxseed oil | 10 | 16 | 53 | (0.3) | 20 | 0 |
| Canola oil | 6 | 22 | 10 | (2.2) | 62 | 0 |
| Walnut oil | 12 | 58 | 12 | (4.8) | 18 | 0 |
| Safflower oil | 10 | 77 | Trace | (77) | 13 | 0 |
| Sunflower oil | 11 | 69 | — | (69) | 20 | 0 |
| Corn oil | 13 | 61 | 1 | (61) | 25 | 0 |
| Olive oil | 14 | 8 | 1 | (8.0) | 77 | 0 |
| Soybean oil | 15 | 54 | 7 | (7.7) | 24 | 0 |
| Margarine | 17 | 32 | 2 | (16) | 49 | 0 |
| Peanut oil | 18 | 33 | — | (33) | 49 | 0 |
| Palm oil[c] | 51 | 9 | 0.3 | (30) | 39 | 0 |
| Coconut oil[c] | 92 | 2 | 0 | (2.0) | 7 | 0 |
| Chicken fat | 31 | 21 | 1 | (21) | 47 | 11 |
| Lard | 41 | 11 | 1 | (11) | 47 | 12 |
| Beef fat | 52 | 3 | 1 | (3.0) | 44 | 14 |
| Butterfat | 66 | 2 | 2 | (1.0) | 30 | 33 |

[a]*Sources:* Canola oil, data on file, Procter & Gamble. For all others, see Reference 64.
[b]See Table 2 for Abbreviations.
[c]Palm oil and coconut oil have arachidic acid contents of 0.2 and 0.1%, respectively.

**TABLE 6**
**n-6/n-3 Ratios in Various Populations[a,b]**

| Population | n-6/n-3 | Reference |
|---|---|---|
| Paleolithic | 0.79 | 36 |
| Greece before 1960 | 1.00–2.00 | 34 |
| Current United States | 16.74 | 36 |
| United Kingdom and northern Europe | 15.00 | 50 |
| Japan | 4.00 | 49 |

[a]*Source:* Reference 36
[b]Assuming an energy intake of 35:65 of animal/plant sources.

1950s, the dietary problems changed. The economy improved markedly, and the variety of foods within markets proliferated. With energy-rich foods in abundance, people could now eat according to the longstanding recommendation to avoid malnutrition (undernutrition). No one foresaw that this could create health problems, and soon the effects of overnutrition and a diet unbalanced in EFA revealed a substantial increase in mortality from coronary heart disease, an increase that was associated with changes in food habits and lifestyle.

**TABLE 7**
**Adequate Intakes (AI) for Adults[a,b]**

| Fatty acid | Amount (2000 kcal diet) (g/d) | % Energy |
|---|---|---|
| LA | 4.44 | 2.0 |
| Upper limit[1] | 6.67 | 3.0 |
| ALA | 2.22 | 1.0 |
| DHA + EPA | 0.65 | 0.3 |
| DHA to be at least[2] | 0.22 | 0.1 |
| EPA to be at least | 0.22 | 0.1 |
| *Trans*-FA | | |
| Upper limit[3] | 2.00 | 1.0 |
| SAT | | |
| Upper limit[4] | — | <8.0 |
| MUFA[5] | — | — |

1. Although the recommendation is for AI, the Working Group felt that there was enough scientific evidence also to state an upper limit (UL) for LA of 6.67 g/d based on a 2000 kcal diet (3.0% of energy).
2. For pregnant and lactating women, ensure 300 mg/d of DHA.
3. Except for dairy products, other foods under natural conditions do not contain *trans*-FA. Therefore, the Working Group does not recommend *trans*-FA to be in the food supply as a result of hydrogenation of unsaturated fatty acids or high-temperature cooking (reused frying oils).
4. Saturated fats should not comprise >8% of energy.
5. The Working Group recommended that the majority of fatty acids be obtained from monounsaturates. The total amount of fat in the diet is determined by the culture and dietary habits of people around the world (total fat ranges from 15 to 40% of energy) but with special attention to the importance of weight control and reduction of obesity.

[a]If sufficient scientific evidence is not available to calculate an Estimated Average Requirement, a reference intake called an Adequate Intake is used instead of a Recommended Dietary Allowance. The AI is a value based on experimentally derived intake levels or approximations of observed mean nutrient intakes by a group (or groups) of healthy people. The AI for children and adults is expected to meet or exceed the amount needed to maintain a defined nutritional state or criterion of adequacy in essentially all members of a specific healthy population.
[b]Abbreviations: *trans*-FA, *trans* fatty acids; SAT, saturated fatty acids; MUFA, monounsaturated fatty acids. For other abbreviations see Table 2.

**TABLE 8**
**AI for Infant Formula/Diet[a]**

| Fatty acid | (%) |
|---|---|
| LA[1] | 10.00 |
| ALA | 1.50 |
| AA[2] | 0.50 |
| DHA | 0.35 |
| EPA[3] | |
| Upper limit | <0.10 |

1. The Working Group recognizes that in countries such as Japan, the breast milk content of LA is 6–10% of fatty acids and the DHA is higher, ~0.6%. The formula/diet composition described here is patterned on infant formula studies in Western countries.
2. The Working Group endorsed the addition of the principal long-chain polyunsaturates, AA and DHA, to all infant formulas.
3. EPA is a natural constituent of breast milk, but in amounts >0.1% in infant formula, it may antagonize AA and interfere with infant growth.

[a]See Table 7 for explanation of AI and abbreviations.

**TABLE 9**
**New Products on the Market Enriched with n-3 Fatty Acids[a]**

| Product | Description |
|---|---|
| Oils | Oils rich in ALA, such as canola, flaxseed, perilla, and soybean. In addition, avoidance of vegetable oils rich in n-6 and the use of oils lower in LA such as olive oil, canola oil, and new vegetable oils rich in monounsaturated oils help bring about an improvement in the LA/ALA ratio. |
| Bakery products | Flaxseed flour and encapsulated fish oils are used in bakery products, including breads. |
| Eggs | Changes in chicken feeds lead to enrichment of n-3 fatty acids in eggs; n-3–enriched eggs are found in many markets around the world. Chicken feeds are enriched with fish-meal, flaxseed, or DHA from algae. These eggs have a lower n-6/n-3 ratio and contain significant amounts of AA and DHA and are modeled after the "natural egg" (i.e., the egg from the Ampelistra farm in Greece), which is the egg obtained under completely natural conditions. |
| Infant formula | Human milk contains AA, DHA, and EPA, whereas infant formula based on cow's milk does not. In Europe and the Far East, infant formula is now enriched with AA and DHA from various sources. However, in the United States, infant formula does not yet contain AA and DHA. |
| Milk | Research shows promising results in increasing DHA in cow's milk. |
| Mayonnaises, margarines, and salad dressings | Hydrogenated fish oils and canola oil are used in the preparation of these products. |
| Meat/poultry | Research on how best to titrate the amount of fish oils and other sources of n-3 fatty acids (i.e., flax, DHA from algae) in animal feeds without affecting the stability and organoleptic properties is advancing in many parts of the world. Poultry, cattle, and pigs are being studied, and the consumption of n-3–enriched meats is not far into the future. |
| Farmed fish | There is a need to further improve the fatty acid composition in aquaculture. |

[a]See Table 2 for fatty acid abbreviations.

**TABLE 10**
**Issues Related to the Return of n-3 Fatty Acids to the Food Supply[a]**

→How much n-3 fatty acids should be in each serving?
→How much ALA, EPA, DPA, and DHA?
→What should be the ratio of total n-6/n-3?
→What should be the ratio of LA/ALA and (AA + DTA)/(EPA + DPA + DHA)?
→What should be the proportions of EPA, DPA, and DHA?
→What are the best models, i.e.,
land animals in the wild?
fish in the wild?
mother's milk from women consuming a Paleolithic diet?
mother's milk from women consuming a Greek/Mediterranean diet?
other?
→What can we learn from the composition of edible wild plants?
→Should we incorporate n-3 fatty acids into foods rather than supplement?

[a]See Table 2 for fatty acid abbreviations

In 1974, the FAO/WHO World Conference in Rome proposed that each nation should have a Nutrition and Food Policy. It is important to use the term "Nutrition and Food Policy" because the term "Food and Nutrition Policy" subordinates the nutritional aspects to the food policy aspects. Nutrition and food planning is a tool of a Nutrition and Food Policy. The goal of a Nutrition and Food Policy is the satisfaction of nutritional needs of the population as defined by nutritional science. The primary Nutrition and Food Policy objective is "The achievement of the adequate nutrition of the population." Adequate nutrition is also determined by physiologic requirements, and levels can be stated within a certain range. It is therefore necessary to define the nutritional requirements for the EFA. The prerequisites for a Nutrition and Food Policy are based on the following:

1. Nutritional needs become the central element, and are expressed as dietary allowances.
2. Establishment of a Department or Agency relating to the nutrition of the population.
3. Nutrition education for the public.
4. Nutrition education for professionals.

Public policy should be based on scientific evidence. Education of the public is essential to induce changes in the food supply. In many Western countries, including the United States and the United Kingdom, nutrition research, food inspection, and regulation is either shared by the Departments of Health and Agriculture (USA) or is carried out by the Department of Agriculture (UK). Neither of these situations is satisfactory, and having the Department of Agriculture in charge represents a conflict of interest as shown by the problems in "mad cow" disease. The solution to "mad cow" disease included among other things the recommendation for the establishment of a new food standards agency (61). The health department would assume responsibility for food policy in Britain (62) and would create an independent agency to protect consumers' interests in every aspect of food safety as well as nutrition. The loss of public confidence in meat after the experience of bovine spongiform encephalopathy and *Escherichia coli* contributed to this action. In the 1980s, the United States and other countries began to develop dietary recommendations (1). In 1989, I wrote a paper giving the reasons for the establishment of a Nutrition and Food Sciences Agency in the United States, separate and independent of the Departments of both Health and Agriculture (63). As we begin the new century, scientists and particularly physicians must work together with consumer organizations, industry, and governments to establish a Nutrition and Food Sciences Agency in every country.

Because scientific change is more rapid than changes in the law, continuing education of professionals and the public will be an essential part of progress in bringing about change and improving the health and well-being of people worldwide. The development of a Dietary Reference Intake (DRI) for essential fatty acids is urgently needed (Table 1). The science is strong; what is needed is collaboration among government, scientists, regulators, and industry. Finally, there is a need for the establishment of one agency in the European Community that will take responsibility for advancing the following:

- Nutrition research
- Food sciences research
- Education for professionals
- Education for the public
- Development of dietary guidelines for health
- Food regulation (implementation of nutrition and food policy)

Nutrition and genetics have a strong influence on health and disease. Early in 2001, sequencing of the human genome was complete in rough draft form. Eventually the function of all genes will be known, and diets will be targeted to individuals for the prevention and management of chronic diseases. For the prevention and treatment of chronic diseases, universal recommendations are inappropriate because of genetic variation and the differences in frequency of polymorphisms in various parts of the world. Therefore, nations cannot adopt each other's dietary recommendations. Furthermore, traditional diets must be studied carefully and serve as a model for foods to be developed. Physicians must take an active part because they are the only profession responsible for the diagnosis and treatment of the chronically ill. An academic consensus is considered vital when developing goals and proposing strategies for implementing a nutrition policy. Once the nutrition policy is established, an effective administrative body is required to supervise, on a daily basis, a nutrition policy and its implementation. Nongovernment organizations and other consumer groups should be linked in a cooperative process.

In summary, the scientific basis for the development of a public policy to develop dietary recommendations for EFA, including an n-6/n-3 ratio, exists. Necessary conditions include a scientific consensus, education of professionals and the public, the establishment of an agency on nutrition and food policy at the national level, and willingness of governments to institute changes. In fact, I would suggest that in bringing about a decrease in dietary n-6 fatty acid intake and

an increase the n-3 fatty acids, government should require proof from the opponents (to a change in policy) that such changes will do harm and proof that continuation of existing dietary patterns do not.

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# The Effect of n-3 Fatty Acids on Low Density Lipoprotein Subfractions

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**ABSTRACT:** A predominance of small, dense low density lipoprotein (LDL) represents a significant source of increased risk for the development of coronary heart disease in Westernized countries. Dietary long-chain n-3 polyunsaturated fatty acids exert a potent triglyceride-lowering effect that redistributes LDL subfractions toward larger and lighter particles. These dietary fatty acids thus have a key role to play in providing protection against this particularly atherogenic type of LDL.

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Conclusive evidence now exists to support the benefits of dietary long-chain n-3 fatty acids in fish oil in individuals with existing coronary heart disease (CHD) (1). Although this quality of evidence is still lacking in normal, healthy populations, we can now identify with confidence individuals who are predisposed to increased coronary disease by a collection of risk factors that are responsive to these dietary fatty acids. These susceptible individuals stand to gain the greatest benefit from increased consumption of n-3 polyunsaturated fatty acids (PUFA) and are thus the most appropriate population to target and treat.

## *Plasma Triacylglycerols (TAG) and Cardiovascular Risk*

Interest in the cardioprotective effects of dietary n-3 PUFA stems from early observations of the native Eskimo who, in spite of a high-fat diet, suffer low rates of cardiovascular disease as a consequence of a high intake of fish (2). Sadly, the subsequent application of this knowledge in the primary and secondary prevention of CHD was hindered, largely because fish oil exerted minimal effects on serum cholesterol, and in patients with raised serum triacylglycerols (TAG), actually increased low density lipoprotein (LDL). Large epidemiologic studies in the 1970s such as MRFIT (3) helped to establish the "absolute" coronary risk associated with increased serum cholesterol and the cholesterol hypothesis, which has since been tested and impressively confirmed in cholesterol-lowering drug trials (4,5). However, for free-living individuals within populations, serum cholesterol is actually a poor discriminator of disease status (6) and provides an inadequate basis by which to explain the relationship between diet and CHD. There are other more important risk factors that confer significantly greater "attributable" risk within populations and that are more amenable to dietary modification. One such factor is raised serum TAG. Despite its early exclusion as a coronary risk factor on statistical grounds, there has been a resurgence of interest in the role of serum TAG in relation to CHD. A meta-analysis of existing clinical studies (7) and intensive study of the mechanisms by which TAG-rich lipoproteins influence the disease process (8) have together provided incontrovertible evidence to implicate raised TAG in the development of atherosclerosis and also in the more acute manifestations of heart disease such as coronary thrombosis.

There is now convincing evidence to show that even moderately raised plasma TAG confers increased cardiovascular risk in otherwise normal, healthy individuals. Moderately raised plasma TAG (>1.5 mM) has a predicted frequency of between 25 and 30% in middle-aged men and postmenopausal women. It is a major progenitor of abnormalities in high and low density lipoprotein subfractions, which are collectively known as an atherogenic lipoprotein phenotype (ALP) (9). In its most frequent form of presentation, an ALP represents a subclinical dyslipidemia and thus silent risk factor for CHD. Its metabolic origins lie in insulin resistance and, as such, it is likely to represent the most common source of lipid-mediated CHD risk in free-living populations (8).

## *Small, Dense "Atherogenic" LDL-3*

LDL shows structural heterogeneity and exists in the plasma of all humans as a small number of discrete subfractions (8) (Fig. 1). These subfractions express variable metabolic properties and a gradient of atherogenic potential. In clinical studies, LDL subfractions have been traditionally grouped into two patterns or phenotypes, i.e., a normal pattern "A" consisting of predominantly large and less dense LDL particles, and an abnormal pattern "B" of high risk in which small, dense LDL particles predominate. The latter pattern is strongly associated with low high density lipoprotein (HDL; <1 mM) and increased plasma TAG. Moreover, a concentration for plasma TAG in excess of 1.5 mM has been identified as a critical threshold for the development of small, dense

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Abbreviations: ALP, atherogenic lipoprotein phenotype; apo, apolipoprotein; AUC, area under the curve; C, cholesterol; CETP, cholesteryl ester transfer protein; CHD, coronary heart disease; CM, chylomicrons; CMR, chylomicron remnant; HDL, high density lipoprotein; HTG, hypertriglyceridemia: IAUC, incremental area under the curve; LDL, low density lipoprotein; LPL, lipoprotein lipase; NEFA, nonesterified fatty acids; PUFA, polyunsaturated fatty acids; TAG, triacylglycerols; VLDL, very low density lipoprotein.

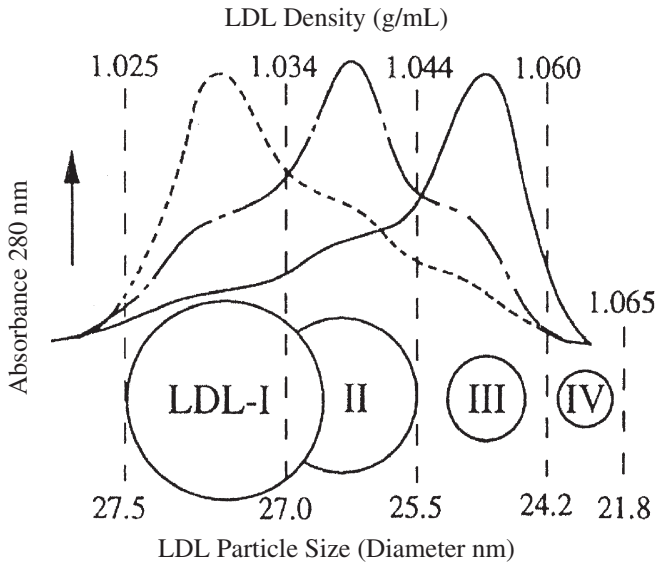


**FIG. 1.** Plasma low density lipoprotein (LDL) shows structural heterogeneity. LDL subclass profiles from three different individuals as measured by density gradient centrifugation are shown. Predominance of large LDL-I (1) e.g., normal, healthy premenopausal female (--------); predominance of intermediate LDL-II (2) e.g., normal, healthy male (— - —); predominance of small, dense LDL-III (3) e.g., pattern found in an atherogenic lipoprotein phenotype (———).

LDL-3 (Fig. 2) (10). Case-control studies have shown that small dense LDL carries at least a threefold increase in risk of myocardial infarction (11), whereas prospective trials have shown small LDL size to be predictive of future coronary events, with relative risk comparable to that of smoking or hypertension (12,13). The properties that render small, dense LDL-3 more atherogenic include rapid penetration of the endothelial barrier lining the arterial wall by virtue of its small size (14), its selective binding to arterial proteoglycans in the subintimal space (15), and its relatively greater susceptibility to oxidative modification compared with its larger and lighter counterparts (16).

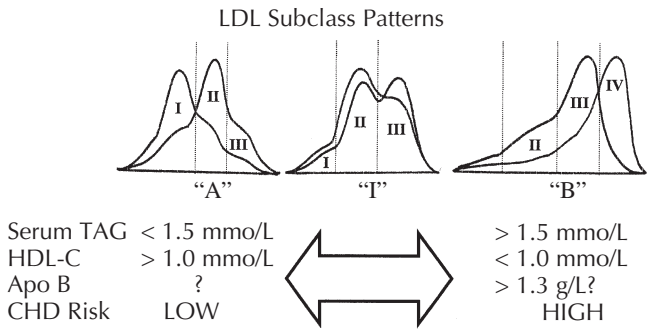


**FIG. 2.** LDL subclass patterns or phenotypes. Pattern "A" represents the normal pattern of low coronary heart disease (CHD) risk and is associated with low serum triacylglycerols (TAG; <1.5 mM) and raised high density lipoprotein cholesterol (HDL-C) (>1 mM). Pattern "B"represents the abnormal pattern of high CHD risk and is associated with moderately raised serum TAG (>1.5 mM) and low HDL (<1 mM). Pattern "I" is intermediate between "A" and "B." Apo B, apolipoprotein B. For abbreviation see Figure 1.

### *Small, Dense LDL-3: Relevance of Relative Abundance (%) (Pattern "B") vs. Increased Particle Number (Hyperapo B)*

Because each LDL particle carries a single molecule of apolipoprotein B (apoB), the measurement of apo B in LDL provides a surrogate marker for LDL particle number. It has been known for many years that an increased number of LDL particles as measured by apo B (hyperapo B) represents a major source of CHD risk (17). Hyperapo B is always associated with a predominance of small, dense LDL but, conversely, a predominance of small, dense LDL-3, as found in an ALP, is not always associated with hyperapo B (18). This raises the following question: Are individuals with a predominance of small, dense LDL-3 but without a raised particle number still at increased risk? This issue is of relevance because dietary n-3 PUFA may positively influence the distribution of LDL subfractions without altering the number of particles and consequently have minimal effect on CHD risk.

The mechanisms by which moderately raised TAG generate small, dense LDL are complex but relevant in the present context because dietary long-chain n-3 PUFA are thought to specifically target and correct the metabolic aberrations that underlie this condition. They almost certainly involve the overproduction of TAG in the liver which, through the production of TAG-enriched very low density lipoproteins (VLDL), perturbs the clearance of TAG-rich lipoproteins in the postprandial period (19).

It is over 30 yr since Zilversmit (20) linked enhanced postprandial lipemia with CHD and proposed atherosclerosis to be a postprandial phenomenon (Fig. 3). In the intervening period, postprandial TAG-rich lipoproteins, which, in addition to the chylomicrons (CM) and chylomicron remnants (CMR) carrying dietary fat, will include substantial quantities of VLDL, have been ascribed direct and indirect atherogenic potential (21,22). Although CMR have been shown to be the only native (nonoxidized) lipoproteins capable of inducing foam cell formation in arterial lesions, it is the indirect effects of these postprandial lipoproteins in modulating the structure and function of LDL and HDL that may be of even greater relevance to the atherogenic process.

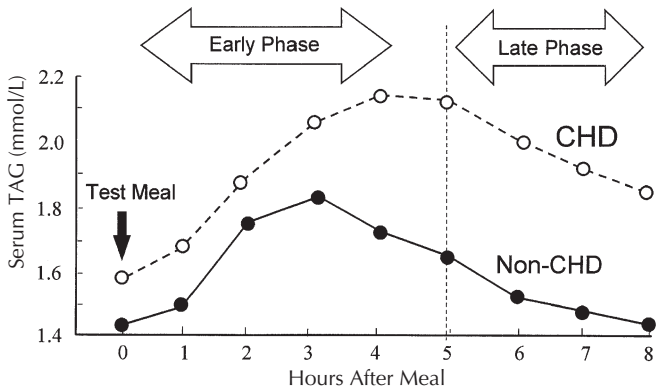


**FIG. 3.** Typical postprandial responses to fat-containing meal in subjects with and without CHD. Patient with CHD shows enhanced postprandial lipemia and an increase in the duration and magnitude of serum TAG concentration. For abbreviations see Figure 2.

Postabsorptive (fasting) serum TAG is a major determinant of variation in the distribution of LDL subfractions, accounting for ~40% of variation in small, dense LDL-3 in nearly all studies. However, because most people spend most of the day in a postprandial state, it could be argued that the fasting state is unphysiologic in this situation and that greater emphasis should be placed on repeated bouts of lipemia throughout the day. The latter would be responsible for maintaining plasma TAG levels above the critical threshold value for the development of small, dense LDL-3 of 1.5 mM. This is again relevant to the effects of long-chain n-3 PUFA, which have been shown to reduce the magnitude and duration of these lipemic bouts.

The explanation for the existence of a threshold for plasma TAG lies in the complex transfer and exchange of neutral lipids between circulating TAG-rich lipoproteins and cholesterol-rich lipoproteins (LDL and HDL). At subthreshold TAG levels, TAG-rich lipoproteins donate TAG to LDL and HDL in equimolar exchange for cholesteryl esters (CE), a process facilitated by cholesteryl ester transfer protein (CETP) (19). For HDL, this process forms an essential part of the centripetal transport of cholesterol from the peripheral tissues, including arterial lesions, back to the liver for excretion. As plasma TAG increases, a point is reached at 1.5–1.6 mM at which this equimolar exchange is replaced by a net transfer of TAG into LDL; the particle becomes transiently TAG rich and a favored substrate for hepatic lipase. The result is the generation of smaller and denser LDL particles (Fig. 4).

The concentration of TAG in plasma at any given time is a variable function of the rate of production and clearance of TAG-rich lipoproteins. The rate of production and secretion of VLDL from the liver is controlled to a major extent by the supply of substrates for the synthesis of TAG [remnant lipoproteins and nonesterified fatty acids (NEFA) chiefly], whereas CM production is determined mainly by the size and fat content of each meal (21). Both of these lipoproteins share a common saturable pathway for the hydrolysis of their TAG in the circulation *via* the endothelial lipase lipoprotein lipase (LPL) (23). The overproduction of TAG and large, TAG-rich VLDL in the liver has been implicated in the generation of small, dense LDL-3 in insulin-resistant states such as obesity, type II diabetes, and combined hyperlipidemia (24). Nevertheless, TAG clearance is also frequently impaired in these conditions (25,26), implicating the failure of LPL as an additional contributory factor. It is important to appreciate that raised VLDL levels make a significant contribution to enhanced postprandial lipemia by competing, relatively ineffectively, with CM for LPL-mediated clearance (27). VLDL particles are also in greater abundance than CM throughout the postprandial period (28). Competition will be more pronounced when the VLDL particle is larger and is heavily enriched with TAG as in the insulin-resistant states described above. In summary, factors that influence the production and clearance TAG-rich lipoproteins are of key importance in conferring protection against the development of small, dense LDL-3.

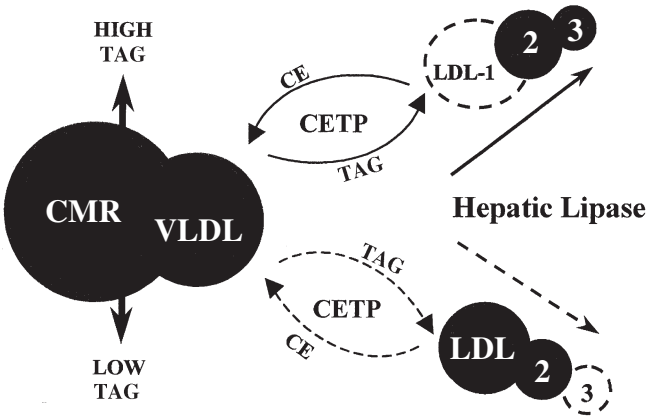


**FIG. 4.** Neutral lipid exchange. Relationship between serum TAG is explained in part by a mechanism of exchange and transfer of neutral lipids. At low concentrations of serum TAG, there is an equimolar exchange of cholesteryl esters (CE) from LDL to TAG-rich lipoproteins and TAG from TAG-rich lipoproteins back to LDL. In contrast, at high concentrations of serum TAG, there is a net transfer of TAG into LDL. This latter process produces a transient enrichment of LDL with TAG, which is rapidly hydrolyzed in the liver by hepatic lipase producing smaller and denser LDL particles (LDL-3). CETP, cholesteryl ester transfer protein; CMR, chylomicron remnant; VLDL, very low density lipoprotein; for other abbreviations see Figures 1 and 2.

### *Effects of n-3 Fatty Acids on Plasma TAG, Lipoproteins and LDL Subfractions*

Review of the influence of fish oils on plasma cholesterol and lipoproteins reveals their effects to be highly dependent on the initial lipid phenotype (29); in all cases, however, they significantly decrease plasma TAG in the fasting state and, when introduced into the habitual diet, produce a marked attenuation of enhanced postprandial lipemia (Fig. 5) (30). In view of the central role of TAG in regulating LDL subfractions, it is predictable that fish oils will redistribute LDL particle size toward larger and less dense particles. Baumstark *et al.* (31) were among the first to demonstrate this effect on both LDL and HDL subfractions.

A well-accepted explanation for this TAG-lowering action lies in the capacity of long-chain n-3 PUFA to suppress the production of TAG in the liver (32) by inhibiting the activity

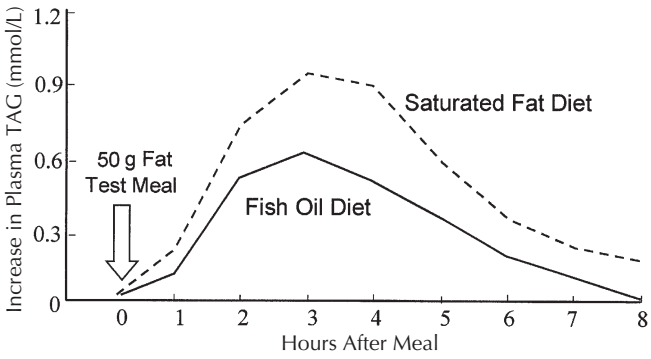


**FIG. 5.** A fish oil diet rich in long-chain n-3 polyunsaturated fatty acids (PUFA) will attenuate the postprandial response to dietary fat relative to a diet. For other abbreviation see Figure 2.

of lipogenic enzymes responsible for the synthesis of TAG, most notably diacylglycerol acyltransferase (33). This limits the amount of TAG available for packaging into VLDL, which results in the liver producing a smaller VLDL particle that contains relatively less TAG. There may well be additional contributory factors for the TAG-lowering effects on n-3 PUFA; these include limiting the supply of lipid substrates for TAG synthesis, essentially circulating NEFA and lipoprotein remnants circulating in the postprandial period. Failure to suppress the release of NEFA from adipose tissue and catabolize TAG-rich lipoproteins results from dysregulation of the insulin-sensitive enzyme's hormone-sensitive lipase and LPL, respectively (34). There is evidence to suggest that dietary long-chain n-3 PUFA can increase the sensitivity of certain tissues to the action of insulin (35); by doing so, they may upregulate the expression of these enzymes at pre- and post-transcriptional levels of control.

Whatever the underlying metabolic mechanisms, fish oil–induced changes in the structure and composition of VLDL will have major consequences for the production of LDL and remodeling LDL subfractions. It reduces the net exchange of neutral lipids between VLDL and LDL, which will counteract the production of small, dense LDL. Small VLDL is a weaker competitor for LPL in the postprandial phase and will contribute to the attenuation of postprandial lipemia. Furthermore, trace-labeling studies have shown that small VLDL is more likely to be converted into LDL, which implicates the former in the increases in LDL cholesterol (LDL-C) that frequently accompany reductions in plasma TAG in patients with hypertriacylglycerolemia (HTG). There are alternative explanations for this effect on LDL-C involving LDL subfractions and a common polymorphism in the apo E gene as discussed below.

### *Intervention with Dietary Long-Chain n-3 PUFA in ALP Subjects*

A study was designed in collaboration with the Department of Food Biosciences at the University of Reading, to examine the influence of n-3 PUFA on LDL subfractions in subjects expressing an ALP, and to elucidate the molecular basis by which these dietary fatty acids reverse this high-risk lipoprotein phenotype (36). The study was unique in screening potential volunteers from the free-living population for the presence of an ALP and selecting these individuals as the most responsive and therefore most appropriate target group for this form of intervention. Initial selection of subjects was based on existing data to show a fasting plasma TAG in excess of 1.5 mM and HDL-C < 1.1 mM. Those with acceptable lipid values were screened for the predominance of small, dense LDL-3 (>50% or LDL subclass pattern B). With this method of recruitment, 60% of those screened were shown to express all three features of the dyslipidemia known as an ALP and were selected for the intervention trial (18). The trial consisted of a double-blind crossover design with two 6-wk intervention periods with consumption of either a long-chain n-3 PUFA supplement (6 g/d 50% fish oil concentrate, equivalent to 3 g eicosapentaenoic acid and docosahexaenoic acid; 'Pikasol,' EPAX 5500 TG, Pronova, Norway) or a placebo of olive oil separated by a 12-wk washout period. Both supplements were superimposed on the subjects' habitual diets. At the end of each intervention period, subjects were given two standard test meals, a relatively high-fat breakfast (49 g fat) at baseline (0 min), and a lower-fat lunch (30 g fat) after 330 min. Blood samples for the analysis of LDL subfractions were taken at the beginning and end of each intervention period.

### *Effects on Lipids, Lipoproteins and LDL Subfractions*

Supplementation with dietary long-chain n-3 PUFA produced marked decreases in fasting plasma TAG ($P < 0.05$) and attenuation of postprandial lipemia; the latter was measured by the area under the curve (AUC; $P < 0.05$) and the incremental area under the curve (IAUC = TAG values – baseline TAG; $P < 0.05$) (Fig. 6). The decrease in plasma TAG in both fasting and postprandial plasma was inversely associated with the initial plasma TAG values. The group as a whole showed no significant changes in total plasma cholesterol or HDL-C but a small, nonsignificant increase in LDL-C (+7%) (36). There was no change in LDL apo B but a highly significant decrease in both the relative abundance (%) and particle number (apo B) of small, dense LDL-3 (–26%, $P < 0.01$), as measured by quantitative density gradient centrifugation (37). Of the group ($n = 12$), 25% showed a change in LDL subfraction phenotype from pattern "B" to pattern "A" (Fig. 7). Of the remaining 75%, 50% ($n = 24$) showed decreases in LDL-3, whereas 25% showed either no response or a slight increase in this LDL subfraction.

### *Determinants of Change in LDL Subfractions*

The change in the distribution of LDL subfractions was highly correlated with fasting TAG, which explained ~40% of the variation in small, dense LDL-3 as seen previously. The TAG AUC

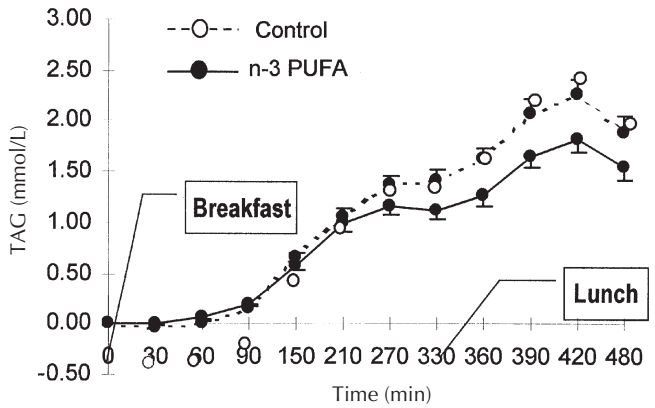


**FIG. 6.** Supplementation with long-chain n-3 PUFA (3 g/d for 6 wk) attenuates the postprandial response to fat-containing meals in subjects with an atherogenic lipoprotein phenotype. Graph shows the incremental postprandial TAG responses (serum TAG – baseline TAG) (36). Data represent mean ± SEM. For abbreviations see Figures 2 and 5.

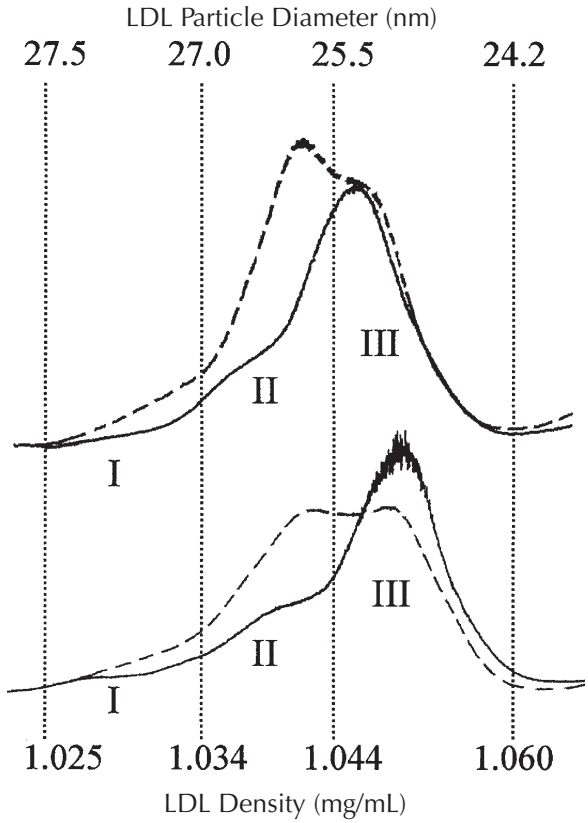


**FIG. 7.** Effect of dietary long-chain n-3 PUFA on LDL subclass profile. n-3 PUFA induce a redistribution of LDL subclasses toward larger and less-dense particles (LDL-1,-2). For abbreviations see Figures 1 and 5.

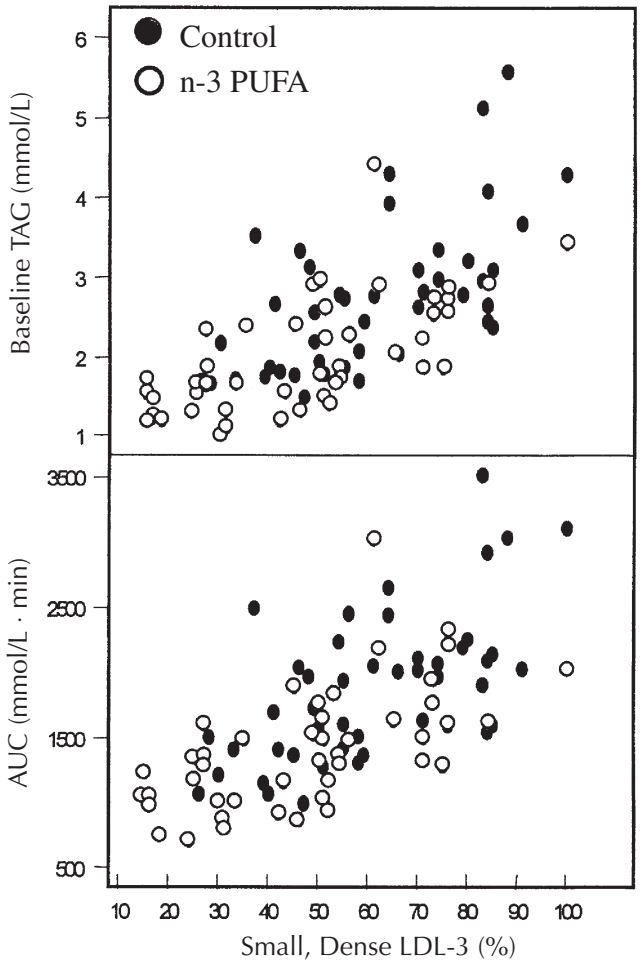


**FIG. 8.** Relationship between small, dense LDL-3 and postabsorptive and postprandial TAG levels. LDL-3 shows significant association with postabsorptive TAG levels in control subjects, $r = 0.62$, $P < 0.001$) and subjects supplemented with n-3 PUFA, $r = 0.66$, $P < 0.001$); and postprandial TAG as measured by area under the curve (AUC) (control $r = 0.61$, $P < 0.001$; n-3 PUFA $r = 0.63$, $P < 0.001$) but not with postprandial TAG as measured by the incremental AUC. For abbreviations see Figures 1, 2, and 5.

was again highly correlated with LDL-3 but provided no additional information (Fig. 8). In contrast, the IAUC showed no relationship with these changes in LDL subfractions. Because the IAUC reflects postprandial clearance of TAG-rich lipoproteins and particularly CM from the influx of dietary fat from the second meal, this finding suggests that fish oil–induced decreases in VLDL are of greater importance to the observed changes in LDL subfractions than effects on CM clearance. Note that reduced VLDL levels would be expected to contribute to the attenuation of postprandial lipemia by allowing CM greater access to LPL, i.e., through reduced competition between these TAG-rich lipoproteins. Of the 25% of subjects who demonstrated a change in LDL phenotype, all achieved reductions in plasma TAG of between 25 and 45%, with 6 of 12 subjects achieving subthreshold values for post–fish oil plasma TAG (<1.5 mM). There were subjects who achieved greater absolute and subthreshold reductions in plasma TAG who showed decreases in LDL-3 but no change in overall phenotype. These subjects generally were more extreme in their phenotypic expression with higher levels of small, dense LDL-3 at baseline.

### *Effects on LDL Cholesterol Explained by Shift in LDL Subfractions*

Dietary long-chain n-3 PUFA in fish oil have frequently been associated with increases in LDL-C, most notably in patients with HTG. One explanation for this effect is that n-3 PUFA generate smaller VLDL particles, which are a favored precursor of LDL. From our data, it was apparent that LDL-C increased in subjects who showed greater reductions in small, dense LDL-3. (This is also likely to be the case in subjects with HTG because of the strong metabolic links between initial plasma TAG, change in plasma TAG, and a decrease in small, dense LDL-3.) Because larger LDL-2 particles contain more molecules of cholesterol ester per LDL particle, both in normal and HTG subjects (38) (Fig. 9), an increase in LDL particle size will almost certainly be accompanied by an increase in LDL-C. The intermediate LDL-2 subfraction has also been shown to have a greater binding affinity to the LDL receptor than its larger (LDL-1) and smaller (LDL-3) relatives (39). Furthermore, there is evidence to show that LDL-1 and LDL-3 are converted into LDL-2 as a means of facilitating the removal of LDL-C from the circulation. Thus, n-3 PUFA–induced increases in LDL-C may reflect changes in

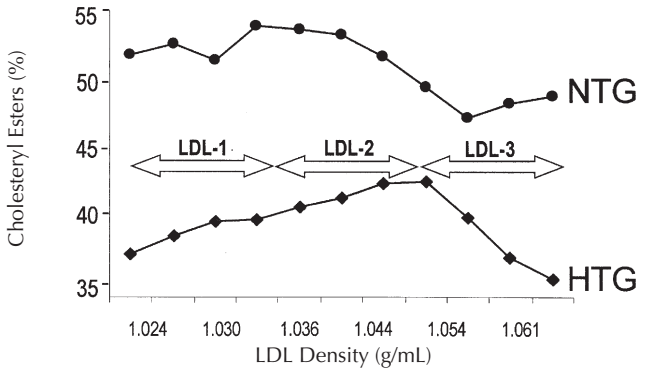


**FIG. 9.** Cholesteryl ester content in LDL subfractions in normolipidemic (NTG) and hypertriacylglycerolemic (HTG) subjects. Data for plots taken from Reference 38. For abbreviation see Figure 1.

LDL composition that, in the presence of an efficient removal of LDL-C, makes physiologic sense and should not necessarily be viewed as an adverse effect of n-3 PUFA. Interestingly, the increases in LDL-C were confined mainly to carriers of a common polymorphism in the gene for apolipoprotein E, apo E4.

### *Effects of Apo E Polymorphism on Responsiveness to n-3 PUFA*

A common genetic polymorphism in the apo E gene gives rise to three structural variants in apo E (apo E2, E3, and E4). These different isoforms of apo E have a profound influence on the metabolism of TAG-rich lipoproteins and LDL and consequently on cholesterol levels and the postprandial response to dietary fat. Their effect on metabolism arises from their variable affinities for the LDL (B/E receptor) and remnant receptor (E receptor) in the order of apo E2 > apo E3 > apo E4. As a result of this difference, carriers of the E2 allele tend to have lower serum cholesterol levels and carriers of E4 higher serum cholesterol through the classic feedback mechanism whereby increasing intracellular cholesterol suppresses the production and thus activity of LDL receptor activity (40). The latter group suffers from a higher risk of CHD and also shows greater responsiveness to low-fat, high-carbohydrate diets in terms of LDL-C lowering (41), largely because of dramatic decreases in LDL particle size, the exact opposite effect to that of dietary n-3 PUFA. The frequency of the E4 allele in Northern Europeans is ~15–20%, whereas in our ALP, 40% were carriers. These subjects showed the greatest decreases in fasting plasma TAG and small, dense LDL-3, and small increases in LDL-C. Because the E4 isoform associates preferentially with VLDL particles, its greater binding affinity for the LDL (B/E) receptor may accelerate the direct clearance of large, TAG-rich VLDL and in turn limit the net transfer of TAG into LDL and thus LDL-3 production. Although this would help to explain the accentuated fall in fasting plasma TAG and LDL-3 in E4 carriers, the down-regulation of LDL receptor activity in this group will almost certainly contribute to the increase in LDL-C.

In summary, dietary long-chain n-3 PUFA exert powerful effects on LDL subfractions by decreasing the concentration of small, dense LDL-3 and, in certain cases, totally correct the abnormal and high-risk LDL pattern "B." This effect is mediated through significant reductions in plasma TAG, chiefly VLDL, and is more pronounced in carriers of the apo E4 polymorphism. Synthetic events in liver seem to be central to the effects of n-3 PUFA on VLDL. However, changes in VLDL will almost certainly impinge on the capacity to clear TAG-rich lipoproteins from the circulation in the postprandial period. As such, factors that determine the rate of TAG removal such as LPL may be equally important in this equation.

## ACKNOWLEDGMENTS

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# The Effect of n-3 Fatty Acids on Coronary Atherosclerosis: Results from SCIMO, an Angiographic Study, Background and Implications

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**ABSTRACT:** According to the model of "response to injury," the arterial endothelium is occasionally injured in hyperlipidemia, hypertension, diabetes mellitus and in other states known as risk factors. The ensuing inflammatory response is modulated by cytokines and growth factors, among them platelet-derived growth factor (PDGF), and monocyte chemoattractant protein-1 (MCP-1). In two independent studies, we demonstrated that mRNA levels for PDGF-A and -B and for MCP-1 are reduced after ingestion of n-3 fatty acids by human volunteers. This reduction persists after monocyte stimulation/differentiation by adherence. Moreover, the reduction is brought about only by dietary n-3 fatty acids and not by other classes of unsaturated fatty acids (n-6 or n-9). This appears to be one major mechanism of action of reduced progression/increased regression of established coronary artery disease by ingestion of 1.5 g/d n-3 fatty acids, as assessed by coronary angiography in a randomized placebo-controlled double-blind intervention study in 223 patients. The study was conducted according to "Good Clinical Practice," comprehensive rules regulating investigations with pharmaceutical compounds. Together, our investigations lend support to the importance of PDGF-A, PDGF-B, and MCP-1 in the pathogenesis of atherosclerosis, and the beneficial role of n-3 fatty acids therein.

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Atherosclerosis can be divided into two parts, the slow part of arterial lumen encroachment and the rapid part of lumen obstruction with sometimes catastrophic consequences. This review deals with the slow part, and the effect that is being exerted upon it by the two marine n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in humans.

According to the model of "response to injury" (1,2), the arterial endothelium is occasionally injured in hyperlipidemia, hypertension, diabetes mellitus, and in other states known as risk factors (1,3). Injured endothelial cells secrete less antithrombotic and more prothrombotic factors (2,4,5). Injured and activated endothelial cells also secrete cytokines and growth factors, leading to chemoattraction of mononuclear cells and immigration of monocytes/macrophages into the subendothelial layer (2,4). Macrophages in the intima then become lipid-rich foam cells and T-lymphocytes immigrate; together with other changes, a "fatty streak "develops (5). Proliferation of smooth muscle cells is then initiated, and a "fibrofatty lesion " results (6). "Advanced lesions " or atherosclerotic plaques are characterized by collagen, smooth muscle cells, immigrated mononuclear cells, and necrotic material (6–8). As a whole, this can be considered a chronic inflammatory process (2,7).

The cells involved in the formation of an atherosclerotic lesion (endothelial cells, smooth muscle cells, and mononuclear cells) communicate through a multitude of cytokines and growth factors (7,8), thus modulating the development of atherosclerosis. A central role is ascribed to platelet-derived growth factors-A and -B (PDGF-A and PDGF-B), and monocyte chemoattractant protein-1 (MCP-1) (2,4,7,8). PDGF induces expression of MCP-1 (9). Other activators of endothelial cells and mononuclear cells also regulate expression of PDGF and MCP-1 (8).

Epidemiology, mechanisms of action, and a number of studies in animal models have demonstrated an antiatherosclerotic potential of dietary n-3 fatty acids in terms of less arterial lumen encroachment (10–12). Previously, we demonstrated that dietary n-3 fatty acids reduced PDGF-A and -B mRNA steady state levels in unstimulated human mononuclear cells of volunteers (13). Against this background, we conducted the following two studies: (i) The first took place among healthy volunteers to test whether the reduction in PDGF-A and -B mRNA steady-state levels seen earlier (13) is limited to PDGF, is brought about only by n-3 fatty acids, or also by n-6 or n-9 fatty acids, and whether it persists after mild monocyte stimulation by adherence *ex vivo* (8). Other growth factors and cytokines investigated were MCP-1, heparin-bound epidermal growth factor (HB-EGF), and interleukin (IL)-10 (14). (ii) The second study took place in patients with coronary artery disease and examined the progression/regression of lesions found at coronary angiography (SCIMO, 15), an established intermediate end point for investigations in coronary atherosclerosis.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HB-EGF, heparin-bound epidermal growth factor; 12-HETE, 12-hydroxyeicosatetraenoic acid; IL, interleukin; LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein-1; 3n-PCR, nested, nonoverlapping nonradioactive reverse transcriptase (RT)-polymerase chain reaction; PDGF-A and PDGF-B, platelet-derived growth factors -A and -B.

## MATERIALS AND METHODS

*Volunteer study.* Volunteers ($n$ = 28) were evenly randomized to receive 7 g/d of n-3, n-6, or n-9 fatty acids (Biomedical Test Materials Program, Rockville, MD) or to serve as controls for 4 wk (14). For the quantitative analysis of mRNA levels in human peripheral blood cells, 3n-PCR, a nested, nonoverlapping nonradioactive reverse transcriptase-polymerase chain reaction (RT-PCR) was used (13,16). Experiments were performed in platelet-free preparations of human granulocytes or mononuclear cells, as assessed by light microscopy, the absence of the platelet 12-lipoxygenase product 12-hydroxyeicosatetraenoic acid (12-HETE) by HPLC, and the absence of EGF mRNA, a specific platelet marker (16). Quiescence of cells analyzed was confirmed by measurement of IL 1β (13,14). After suspension in a standard buffer supplemented with 5% serum from the respective donor at the respective time point, mononuclear cells were stimulated by 4 and 20 h adherence to polysterene (14). Personnel involved in analyses and data handling were unaware of the treatment allocation, i.e., "blinded" (14).

*Angiographic study (SCIMO, 15).* This was a randomized, double-blind, placebo-controlled, stratified, clinically controlled 2-yr trial, which was conducted according to the Guidelines for "Good Clinical Practice," a standard design for intervention trials investigating pharmaceutical compounds. Patients ($n$ = 223) with lesions detected at a coronary angiography and without a short number of exclusion criteria (tailored with the aim of losing few patients during the study) were recruited. Intervention consisted of six 1-g capsules/d for 3 mon followed by three for the subsequent 21 mon, containing either a placebo reflecting the fatty acid composition of the average European diet (17) or a 55% marine n-3 fatty acid concentrate. Patients were seen as outpatients during the study at 6-mon intervals. During a short hospital stay, angiograms were taken at the beginning and after 24 mon in a standardized manner. Films of angiograms were evaluated by an expert panel of three experienced cardiologists and rated according to a score system (18). Technically suitable angiograms with lesions considered to have changes were also evaluated using a computer-assisted quantitative angiography system, equivalent to other second-generation systems (secondary end point, ARRI, 19). Secondary end points were predefined cardiovascular events, e.g., sudden death, fatal or nonfatal myocardial infarction, congestive heart failure, and cerebrovascular events. Compliance was assessed by interrogation, capsule count, and by analysis of red cell phospholipid fatty acid composition.

## RESULTS

*Volunteer study.* Ingestion of n-6 or n-9 fatty acids left all variables investigated unaltered (except fatty acid compositions), as was the case in control volunteers. In quiescent mononuclear cells, dietary n-3 fatty acids downregulated mRNA steady-state levels of PDGF-A by 25 ± 10%, of PDGF-B by 31 ± 13%, and of MCP-1 by 40 ± 14%. HB-EGF of IL-10 mRNA steady-state levels were left unaltered by dietary n-3 fatty acids. The reductions in PDGF-A, PDGF-B, and MCP-1 mRNA steady-state levels persisted after stimulation by adherence for 4 or 20 h in a quantitatively similar way (14). We concluded that human gene expression for PDGF-A, -B, and MCP-1, factors thought relevant to atherosclerosis, is constitutive, is constant, and can be reduced only by dietary n-3 fatty acids in unstimulated and adherence-activated monocytes (14).

*SCIMO.* Among the patients, 112 were randomized to placebo, 111 to fish oil. A large number of baseline characteristics were evenly distributed. Meeting predefined noncardiovascular end points, and complaints about study medications, both resulting in early termination of the study, were evenly distributed in both patient groups. Among patients, 80 in the placebo group and 82 in the fish oil group underwent both angiograms, and the resulting pairs of films were evaluated for the primary endpoint. Analyses according to "intention to treat" were as follows. Of 80 pairs in the placebo group, 35 were considered changed in global score; of 82 in the fish oil group, 35 were considered changed in global score, slightly favoring the fish oil group ($P$ for changes in global score = 0.152). One pair in each group was considered unchanged in global score, because progression and regression were balanced. Coronary segments in the fish oil group showed less progression and more regression than did coronary segments in the placebo group. Of 48 segments with angiographic changes in the placebo group, 36 showed mild progression, 5 showed moderate progression, and 7 showed mild regression. Of 55 segments with angiographic changes in the fish oil group, 35 showed mild progression, 4 showed moderate progression, 14 showed mild regression, and 2 showed moderate regression ($P$ = 0.041). Analyses according to "clinical efficacy" and a number of other analyses by the expert panel underline the angiographic difference between placebo and fish oil, in favor of fish oil. Quantitative coronary angiography was possible on 29 pairs of films in the placebo group and 29 pairs of films in the fish oil group. In both groups, a loss in minimal luminal diameter occurred, which was slightly, but nonsignificantly larger in the placebo group. Cardiovascular events occurred in seven patients in the placebo group and in two in the fish oil group ($P$ = 0.10); other clinical parameters were not different. Interestingly, low density lipoprotein (LDL)-cholesterol levels tended to be higher in the fish oil group, at certain time points significantly so, whereas triglyceride levels were significantly lower during fish oil at most time points. The study medication was safe. Blinding of patients and investigators was maintained successfully. Noncompliance occurred in six placebo patients and in nine fish oil recipients. In contrast to our expectations, as assessed by ultrasound, no effect of dietary n-3 fatty acids was seen in the carotid arteries of the SCIMO patients (20). We concluded that dietary intake of n-3 fatty acids modestly mitigated the course of coronary atherosclerosis in humans.

## DISCUSSION

To our knowledge, investigations on the effects of diet or dietary fatty acids on gene expression *ex vivo* are few, whereas

a number of *in vitro* studies have been reported (21–24). Therefore, the precise mechanism(s) behind the reduced gene expression of PDGF-A and -B, and MCP-1 remain to be elucidated. Interestingly, reduction of mRNA levels by dietary n-3 fatty acids in quiescent cells translated into reduced mRNA levels after adherence in a quantitatively similar fashion. This limits slightly the area of research for mechanisms. However, mechanisms discernible *in vitro* are not necessarily operative (or important) *in* or *ex vivo*. Thus, elucidating and defining the underlying mechanism(s) will call for cumbersome volunteer studies.

SCIMO demonstrated a magnitude of effect of dietary n-3 fatty acids on the course of coronary atherosclerosis that is similar to the magnitude of effect seen in studies with a similar design, using other approaches such as cholesterol-lowering, lifestyle changes, or vigorous exercise (25). A smaller study with a less homogeneous patient population, using a different placebo, showed no effect of dietary n-3 fatty acids (26).

Based on "intention to treat," in only one of the two primary end points was statistical significance achieved. However, "clinical efficacy" and other analyses by the expert panel corroborate the conclusion that dietary n-3 fatty acids modestly mitigate the course of coronary atherosclerosis, as assessed by the coronary angiogram. Interestingly, the effect occurred in spite of increased LDL-cholesterol levels, essentially ruling out a dependence on conventional parameters of cholesterol metabolism, and at the same time, making it likely that the effect of n-3 fatty acids occurs at a broad range of LDL-cholesterol levels.

Clearly, the dose of n-3 fatty acids effective in the volunteer study (7 g/d) and the dose effective in SCIMO (1.5 g/d) are different. Therefore, a firm conclusion that reduced growth factor and gene expression are mechanisms of action responsible for the effect seen in SCIMO cannot be drawn. A study using the dose of SCIMO and an approach similar to the volunteer study seems necessary and is being conducted presently in our laboratory. Moreover, the importance of growth factors and cytokines other than PDGF-A and -B and MCP-1, believed to be involved in the pathogenesis of atherosclerosis, remains to be defined in specifically tailored *ex* and *in vivo* studies. All of this is complicated by the observation that in the same patients, n-3 fatty acids were effective in the coronary but not carotid arteries. Thus, the pathogenesis of atherosclerosis might differ in different vascular beds.

In summary, 7 g/d of dietary n-3, but not n-6 or n-9 fatty acids for 4 wk downregulated PDGF-A, PDGF-B, and MCP-1 mRNA levels measured *ex vivo* in mononuclear cells. After stimulation/differentiation of these cells by adherence, this downregulation persisted to a quantitatively similar degree. It is specific and selective for PDGF-A, PDGF-B, and MCP-1, which are thought to promote atherogenesis, but not for HB-EGF or IL1β. In a 2-yr randomized double-blind, placebo-controlled, clinically controlled coronary angiography study in 223 patients, 1.5 g/d dietary n-3 fatty acids reduced progression/increased regression of coronary lesions, as assessed by an expert panel of cardiologists. The two studies combined lend support to the importance of PDGF-A, PDGF-B, and MCP-1 in the pathogenesis of atherosclerosis, and the beneficial role of n-3 fatty acids therein. We hope that future work will more closely define a large number of aspects that remain unclear.

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# n-3 Fatty Acids and Revascularization Procedures

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**ABSTRACT:** Largely initiated by studies among Greenland Eskimos in the early 1970s, great attention has been given to the possible effects of the very long chain n-3 polyunsaturated fatty acids (PUFA) in a variety of cardiovascular disease states. A series of possibly positive effects on pathogenetic mechanisms in cardiovascular disease has evolved from laboratory studies in cell cultures and animals as well as in humans, focusing mainly on eicosanoid metabolism with reduced activities of platelets and leucocytes, reduced plasma triglycerides and, antiarrhythmic effects in the myocardium. A rationale for a positive effect of very long chain n-3 PUFA in the secondary prophylaxis after revascularization procedures obviously also exists. The positive clinical effects based on prospectively randomized trials are summarized as follows. After coronary artery bypass grafting (CABG), the SHOT study showed statistically significant reduction in angiographic vein graft occlusion in 610 patients after 1 yr with supplementation of 3.4 g/d of highly concentrated very long chain n-3 PUFA. The reduction in occlusion rates was significantly related to the change in the n-3 PUFA concentration in serum phospholipids during the study period with the occlusion rate in the upper quartile of such changes at only ~50% of that in the lower quartile. These results were also clearly related to the presence of angina pectoris and occurrence of myocardial infarction after 1 yr. Several studies were conducted in patients after percutaneous transluminal coronary angioplasty (PTCA). By 1993, two meta-analyses indicated a positive effect on the restenosis rate, a significant problem after otherwise successful PTCA. During the late 1990s, three large prospective randomized placebo-controlled angiographic studies were conducted with very long n-3 PUFA 5.1–8.0 g/d, all with completely negative results. Today, therefore, very long chain n-3 PUFA supplementation cannot be recommended to reduce the incidence of restenosis after PTCA. All studies were performed without stenting of the coronary lesion. In the very special revascularization procedure of heart transplantation, evolving hypertension and accelerated atherosclerosis have been major clinical problems. In other studies, positive effects by supplementation with very long chain n-3 PUFA (3.4–5.7 g/d) were obtained on the surrogate end points coronary vasoreactivity to acetylcholine and hypertension, respectively. On the basis of the presently available literature from clinical studies, recommendations for supplementation with very long chain n-3 PUFA can be given to patients after venous CABG (up to 3.4 g/d), and after heart transplantation (3.4–5.7 g/d) but not to patients after traditional PTCA. In fact, data from substudies suggested the possibility that large doses (5.1 g/d) of very long chain n-3 PUFA might be contraindicated because they induce a proinflammatory state in patients under oxidative stress.

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Largely initiated by the reports of Bang, Dyerberg, and coworkers in the 1970s that a low prevalence of coronary artery disease among Greenland Eskimos possibly was associated with their special diet rich in n-3 polyunsaturated fatty acids (PUFA) (1,2), great attention has been given to the influence of these very long chain PUFA on a variety of cardiovascular disease states.

In addition, a large number of laboratory studies in cell culture and animal models have pointed out a series of possible beneficial effects of very long chain n-3 PUFA on the pathogenetic mechanisms of cardiovascular disease, focusing mainly on eicosanoid metabolism with reduced activities of platelets and leucocytes, lowering of plasma triglycerides, and also antiarrhythmic effects in the myocardium.

The possibility of a rationale for positive effects in relation to various revascularization procedures is obviously present, and I will try to summarize the evidence that positive clinical effects of supplementation with very long chain n-3 PUFA can be expected.

Today the primary vascularization procedures include coronary artery bypass grafting (CABG) and percutaneous transluminal coronary angioplasty (PTCA) for coronary heart disease; I will focus mainly on these therapeutic approaches. In addition, I will include some interesting data obtained in the very special revascularization procedure of heart transplantation.

Starting with the last-mentioned data, Fleischhauer and coworkers in 1993 (3) elegantly demonstrated in a randomized study of heart transplant patients that supplementation of 5.7 g/d of very long chain n-3 PUFA normalized the pathologic reactivity of their coronary arteries to intracoronary injection of acetylcholine. Thus, endothelial-dependent vasodilatation was reestablished.

It is well known that heart transplant patients as a group become hypertensive (related to their cyclosporin therapy) and experience accelerated coronary atherosclerosis. In this connection, Andreassen and coworkers (4) in 1997 published their results from a randomized study of 28 heart transplant patients in which supplementation with 4 g/d of n-3 fatty acids significantly reduced the blood pressure compared with a group given corn oil. In fact, after 6 mon the systolic blood

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Abbreviations: CABG, coronary artery bypass grafting; CI, confidence interval; OR, odds ratio; PDGF, platelet-derived growth factor; PTCA, percutaneous transluminal coronary angioplasty; PUFA, polyunsaturated fatty acids.

pressure was lower than preoperatively in the n-3 fatty acid group compared with an increase of 17 mm Hg in the corn oil group. In the same period, the diastolic blood pressure had increased by 21 mm Hg in the corn oil group compared with only 10 mm Hg in the n-3 fatty acid group. Of course the data mentioned relate to surrogate end points, but might give a good indication of possible clinical effects for patients in this special group.

For the revascularization procedure of CABG, it is well recognized that up to 50% of patients experience occlusion of venous aortocoronary grafts by angiographic examination after 1 yr. The figures for occlusion of arterial grafting with the internal mammary artery are significantly lower, approaching ~15% after 5 yr.

The vein graft disease, with a high frequency of occlusion after 1 yr, is thought to be caused initially by thrombotic occlusion during the first weeks, and thereafter by a specialized intimal hyperplasia with possible secondary thrombotic occlusion. More traditional atherosclerotic lesions may be seen in vein grafts within the first postoperative year, but generally a period of >1 yr seems to be required for their development. Thus, processes of thrombosis, intimal hyperplasia, and traditional atherosclerosis seem to be involved in the pathophysiology of vein graft occlusion. Related to possible antiatherosclerotic effects of n-3 PUFA, both antithrombotic and antimitogenic mechanisms might be operative.

To date, only one large prospective randomized study on the effect of n-3 PUFA on the incidence of graft occlusion rates has been published. This is the so-called SHOT study by Eritsland and coworkers from our research group (5). In this study, 610 patients undergoing coronary artery bypass grafting were assigned randomly to either supplementation with 4 g/d of highly concentrated n-3 PUFA or a control group. Simultaneously, all patients by stratification received antithrombotic treatment with aspirin or warfarin. Both diet and serum phospholipid fatty acid profiles were monitored and the primary end point was 1-yr graft patency assessed by repeat angiography. The latter procedure was completed in 95% of the patients, and data on the serum phospholipid fatty acids indicated very good compliance with the study medication.

Vein graft occlusion rates per distal anastomoses were 27% in the n-3 PUFA group and 33% in the control group. That gives an odds ratio (OR) of 0.77 with a 95% confidence interval (CI) of 0.6–0.99 and a *P*-value of 0.034. When calculated on a patient basis, 43% in the n-3 PUFA group had one or more occluded vein grafts compared with 51% in the control group. That gives an OR of 0.72 with a 95% CI of 0.51–1.01 and a *P*-value of 0.05.

Interestingly, there was a significant trend to fewer patients with vein graft occlusions with increasing relative change in serum-phospholipid n-3 fatty acids during the study period (*P* for linear trend = 0.0037) (Fig. 1). No effect on a much lower occlusion rate (13.5%) was found for supplementation with n-3 PUFA for the internal mammary artery grafts.

From this study one could conclude that dietary supplementation with 4 g/d of highly concentrated n-3 PUFA

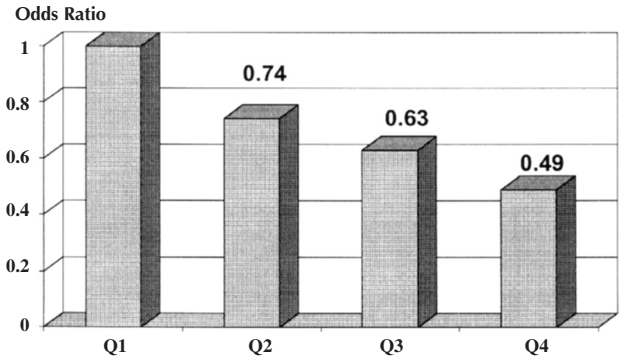


**FIG. 1.** Patients with vein graft occlusion in quartiles of the relative change in polyunsaturated n-3 fatty acids; $n = 524$ patients with vein shunts. *P* for trend = 0.0037 (5).

reduced the incidence of vein graft occlusion after 1 yr and that an inverse relation between relative change in serum phospholipid n-3 fatty acids and vein graft occlusions was observed.

Again, vein graft occlusion is a surrogate end point and the question arises about the clinical relevance of these data. The SHOT study was not designed to evaluate the clinical effects. However, evaluation of the clinical results showed that postoperative angina pectoris was significantly more often related to one or more vein graft occlusions which were present in 67.6% of patients with angina pectoris compared with 40.1% in patients with no postoperative angina ($P < 0.0001$) (6).

In larger prospective studies on CABG patients, there is also a clear correlation between long-term patency of venous aortocoronary grafts and morbidity and mortality after aortocoronary bypass grafting. Finally, it should be mentioned that in the SHOT study no differences in the prevalence of shunt occlusions were observed in the two stratified subgroups of antithrombotic treatment. Thus, it seems reasonable to advocate the supplementation of n-3 PUFA to patients after aortocoronary bypass grafting. According to the limited literature available, supplementation with 3–4 g/d n-3 PUFA concentrates seems reasonable.

The third procedure of revascularization that I will discuss is the potential effect of n-3 PUFA on the phenomenon of restenois after PTCA. This procedure was introduced by Gruentzig in 1977; in most cardiological centers, more patients with coronary heart disease are now being treated with PTCA than with aortocoronary bypass surgery for myocardial revascularization. Although the initial success rate with PTCA is high (~90%), restenosis occurs in up to 50% of the patients after 3–6 mon and obviously represents a large methodological problem and a therapeutic challenge today. The use of intracoronary stenting in parallel with PTCA has reduced the tendency to clinically important restenosis to ~50%. But restenosis remains an important threat to a lasting successful PTCA.

The process of restenosis should be looked on as a response to intimal damage brought about by repeated balloon pressures of ~10 atm. The restenosis process is composed of passive elastic vascular recoil, thrombus formation, and

mainly intimal smooth muscle cell proliferation, extracellular matrix formation, and vessel remodeling. The process includes the active participation of thrombogenic, mitogenic, and vasoactive factors, in which platelet aggregation, inflammation, and cell proliferation are major components. Special attention has been given to platelet-derived growth factor (PDGF) as an important factor for smooth muscle cell chemotaxis. Because n-3 PUFA are known to reduce platelet activation, downregulate leucocytic inflammatory activity, and reduce synthesis of PDGF, a solid rationale for a possible inhibitory effect on the process of restenosis is present.

From 1987 to 1992, seven clinical trials with n-3 PUFA were undertaken to evaluate the possible effect on restenosis after PTCA. The studies varied with respect to type and dosage of n-3 PUFA, time point of starting the supplementation, and diagnostic preciseness toward restenosis. In 1992 and 1993, two meta-analyses were published, the first by O'Connor and coworkers (7), the second by Gapinski and coworkers (8). On the basis of these trials, they concluded that there was a beneficial effect of n-3 PUFA on restenosis, with an OR of 0.71 (95% CI 0.54–0.94, $P = 0.016$) (7). In addition, Gapinski and coworkers (8) pointed out a possible dose-response effect based mainly on the studies with angiographic end-point evaluation. Both studies recommended larger prospective randomized studies to further establish the possible effect.

In the years from 1994 to 1999, three large, prospective placebo-controlled trials of n-3 PUFA in patients undergoing PTCA were published, i.e., Leaf *et al.* in *Circulation* in 1994 (9), Cairns *et al.* in *Circulation* in 1996 (10), and Johansen *et al.* in the *Journal of the American College of Cardiology* in 1999 (11). The studies were of equal size with patient numbers from 500 to 653; the dosage of n-3 PUFA was from 5.1 to 8.0 g/d; and all studies used corn oil as placebo. The n-3 PUFA supplementation was started from 7 to 14 d before the PTCA, and the presence of restenosis was evaluated by quantitative coronary angiography after 18 wk to 6 mon. There were no significant differences between the n-3 PUFA group and the placebo group with respect to restenosis.

In the study of Leaf and coworkers (9), 551 patients were randomized to receive 8.0 g/d of an ethyl ester formulation of n-3 PUFA or corn oil as placebo, starting 12–14 d before elective PTCA. At coronary angiography after 6 mon, 46% of the patients receiving corn oil met the criteria for restenosis (<50% final luminal diameter and >30% increase in narrowing at the stenosis site), compared with 52% in the n-3 PUFA group ($P = 0.37$). Thus, the restenosis rate was actually somewhat higher in the n-3 PUFA group, although this difference was not significant. In this study, two-thirds of the patients additionally received 200 mg α-tocopherol as antioxidant. The incidence of restenosis and the difference between the groups did not differ among patients receiving α-tocopherol and those who did not. They concluded that the supplement of 8 g/d of n-3 fatty acids failed to prevent the usual high rate of restenosis after PTCA.

In the EMPAR study by Cairns and coworkers from Canada (10), 653 patients were randomized to receive 5.4 g/d of n-3 PUFA in a triglyceride formulation or corn oil as placebo with a median start 6 d before elective PTCA and continued until angiography after 18 wk. Again, coronary angiography revealed equal frequency of restenosis defined as loss of >50% of the gain of luminal diameter achieved by PTCA. Thus, in the n-3 PUFA group, 46.5% of the patients fulfilled the criteria for restenosis compared with 44.7% in the placebo group. In this study, low-molecular-weight heparin was also tried in a $2 \times 2$ factorial design. The simultaneous testing of low-molecular-weight heparin did not influence these results. They concluded that there was no evidence for a clinically important reduction of restenosis by n-3 PUFA after PTCA in this trial.

Finally, in the CART study by Johansen and coworkers from our research group (11), 500 patients were randomly allocated to supplementation with 5.1 g/d of highly concentrated n-3 PUFA in an ethyl ester formulation or corn oil as placebo starting at least 2 wk before elective PTCA. Restenosis on quantitative coronary angiography after 6 mon was defined as >50% luminal stenosis or a late loss of at least 20% of diameter or an increase in stenosis of at least 0.7 mm. In this study, restenosis occurred in 45.9% of the patients in the n-3 PUFA group compared with 44.8% in the placebo group. That gives an OR of 1.05, a 95% CI of 0.69–1.59, and a *P*-value of 0.82. On the basis of treated stenoses, the restenosis rate was 40.6% in the n-3 PUFA group compared with 35.4% in the placebo group (OR 1.25, 95% CI 0.87–1.80, $P = 0.21$). In this study, we concluded that supplementation with 5.1 g/d n-3 PUFA for 6 mon initiated at least 2 wk before coronary angioplasty did not reduce the incidence of restenosis.

Given the results of these three large prospective randomized trials, the issue of n-3 PUFA supplementation to counteract restenosis after PTCA has come to a final result: It is not effective and should not be advocated for such use. It should be added that these trials were conducted before stenting became common in connection with PTCA. Today, most PTCA procedures are accompanied by stenting. The latter procedure has reduced the frequency of restenosis to about half that of PTCA alone, but the process of restenosis seems to be the same whether stenting is performed or not.

Finally, I would like to comment on the possibility of negative effects on patients with coronary artery disease with these high doses of highly concentrated n-3 PUFA. Thus, in the CART study, the clinical results, although not the primary aim of the study, seemed to be somewhat poorer in the n-3 PUFA group than in the placebo group. Thus, when angina pectoris was classified in the New York Heart Association classes after 6 mon, significantly more patients were in class 3 and 4 in the n-3 PUFA group than in the placebo group (22 vs. 13%, $P = 0.032$). In line with this observation, the use of betablockers and nitrates was more frequent in the n-3 PUFA group than in the placebo group (39 vs. 30% and 28 vs. 18%, respectively). Whether these observations are relevant to the recently described increase in soluble markers of inflammation after supplementation with high doses of highly concentrated n-3 PUFA (12) remains hypothetical, but obviously the possibility exists.

In summary, on the basis of the literature the following conclusions seem warranted: (i) supplementation with 3.4–5.7 g/d n-3 PUFA concentrate counteracts hypertension and normalizes coronary vasoreactivity to acetylcholine in heart transplantation patients; (ii) supplementation with 3.4 g/d n-3 PUFA concentrate counteracts occlusion of venous aortocoronary grafts 1 yr after by-pass operation; and (iii) supplementation with up to 8.0 g/d n-3 PUFA concentrate does not counteract restenosis 6 mon after PTCA.

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# The Electrophysiologic Basis for the Antiarrhythmic and Anticonvulsant Effects of n-3 Polyunsaturated Fatty Acids: Heart and Brain

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**ABSTRACT:** The n-3 polyunsaturated fatty acids (PUFA) have been shown to be antiarrhythmic in animals and probably in humans. PUFA stabilize the electrical activity of isolated cardiac myocytes by modulating sarcolemmal ion channels, so that a stronger electrical stimulus is required to elicit an action potential and the refractory period is markedly prolonged. Inhibition of voltage-dependent sodium currents, which initiate action potentials in excitable tissues, and of the L-type calcium currents, which initiate release of sarcoplasmic calcium stores, thus increasing cytosolic free calcium concentrations and activating the contractile proteins in myocytes, appears at present to be the probable major antiarrhythmic mechanisms of PUFA. Because the ion channels in neurons have channel proteins essentially homologous to those in the heart, the n-3 fatty acids would appear to be likely to affect the electrical activity in the brain in a manner similar to their effects in the heart, and accumulating evidence supports this notion. Evidence of important beneficial neurological effects of dietary n-3 PUFA are emerging with more likely to be discovered.

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After confirming earlier reports by McLennan *et al.* (1) that polyunsaturated fatty acids (PUFA), particularly of the n-3 class, prevent ischemia-induced malignant ventricular arrhythmias (2), we sought to learn the mechanism of their antiarrhythmic action. Working with Dr. Jing X. Kang, we adopted the cultured neonatal rat cardiac myocyte preparation for our studies because we could observe and record the contractile function of the isolated myocytes and the effects on their function of adding agents of interest to the medium bathing the myocytes. The beauty of this preparation is that the enzymatically separated neonatal myocytes can be cultured directly on microscope cover slips to which they adhere. By day 2 in culture, clumps of varying numbers of myocytes can be seen, and each syncytial cluster is beating spontaneously, rhythmically, and synchronously. With an inverted microscope, video camera with monitor, an edge monitor, and tracing recorder, we could observe and record the rate and amplitude of spontaneous contractions (3). We found that a variety of agents that can cause fatal arrhythmias in humans will cause an acceleration of the beating rate of the myocytes and then produce fibrillatory, asynchronous contractions mimicking fibrillation in the whole heart (4). Elevated perfusate ($Ca^{2+}$), the cardiac glycoside ouabain (3), catecholamines (5), thromboxane (4), lysophosphatidylcholine or acylcarnitine, and even the calcium ionophore A23187 (6) all caused the cultured myocytes to fibrillate. However, if low micromolar concentrations of the fish oil fatty acids eicosapentaenoic acid (20:5n-3, EPA) or docosahexaenoic acid (22:6n-3, DHA) were first added to the bathing medium of the myocyte, their beating rate would slow. But when the arrhythmogenic agent was added to the superfusate, no arrhythmias occurred. If the arrhythmic agent was added first and an arrhythmia was induced, then addition of the EPA or DHA to the superfusate within a few minutes stopped the arrhythmia in the continued presence of the cardiac toxin. Finally, addition of delipidated serum albumin, which has high-affinity binding sites for fatty acids will extract the free fatty acids from the cells and the fibrillation resumes (3).

These simple observations taught us two important things (3). First, they showed that the antiarrhythmic action of the n-3 PUFA required the fatty acids simply to partition (dissolve) into the lipophilic phospholipid acyl chains of the plasma membrane without covalent bonding to any constituent of the cell. Otherwise, the PUFA could not be extracted from the myocytes by the serum albumin. We had expected the PUFA to be incorporated rapidly into membrane phospholipids, but we have shown that once they are so incorporated they are no longer antiarrhythmic (7). Second, we found that only the free fatty acid is promptly arrhythmic. Administration of esterified PUFA, such as their ethyl esters or triglycerides, does not produce acute antiarrhythmic actions. In fact, the structure–function requirement for a molecule to be antiarrhythmic in the manner of these PUFA is simply a hydrophobic molecule with a free carboxyl group at one end of a long acyl chain or hydrocarbon and with two or more C=C unsaturated bonds (8).

By this time, we realized that the n-3 fatty acids must be affecting the basic automaticity/excitability of cardiac myocytes. Therefore, with Dr. Yong-Fu Xiao, we tested their effects on the electrophysiology of the cardiac myocytes. Two important effects were found (9). First, in the presence of these fatty acids (5–10 μM), a stronger electrical depolariz-

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Abbreviations: CNS, central nervous system; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HEK, human embryonic kidney; $IC_{50}$, 50% inhibitory concentration; PUFA, polyunsaturated fatty acids.

ing stimulus of 40–50% was required simply to elicit an action potential. This was because the presence of these PUFA caused a slight hyperpolarization of the resting or diastolic membrane potential, while at the same time moving the potential for the gating of the voltage-dependent $Na^+$ current to more positive values. Second, these fatty acids markedly prolonged the refractory period of the myocytes without any prolongation of duration of the action potential. These two electrophysiologic effects are important for the antiarrhythmic effects of PUFA.

To demonstrate that these fatty acids stabilize the cardiac myocytes electrically, a simple experiment was designed (6). While a continuous tracing of the regular spontaneous beating rate of the cultured neonatal rat cardiomyocytes was under way, the myocytes could be stimulated with an external voltage source at 15 V *via* two platinum electrodes immersed in the perfusion fluid at the two ends of the perfusion chamber. With this setup, it was easy to double the beating rate of the myocytes. When the external electrical stimulator was turned off, the contractions of the myocytes returned to their control beating rate. Adding the n-3 PUFA to the perfusate slowed the beating rate of the myocyte, but when the external electrical stimulus was again turned on at 15 V, the myocytes showed no response to the stimuli. At 20 V, the myocytes still did not respond; at 25 V, they did respond, but only to every other electrical stimulus. Now, after the addition of delipidated bovine serum albumin to the perfusing fluid and extraction of the free fatty acids from the myocytes, the beating rate again returned to its control rate. When the external electrical stimulator was again turned on at 15 V, the myocytes doubled their beating rate, just as they had before exposure to the n-3 PUFA. This experiment was done on isolated cardiac myocytes in the absence of humoral or neural regulation. When one considers that this is a direct action of the free n-3 fatty acids on every individual myocyte in the heart, one can appreciate what a potentially effective antiarrhythmic agent they are.

Because all electrical activity of excitable tissues results from the flow of charged ions through specific protein ion channels embedded in the plasma membranes of cells, we turned to an examination of the effects of PUFA on specific membrane ion currents. Dr. Xiao began his study with the effects of the fatty acids on the voltage-dependent $Na^+$ current, $I_{Na}$. PUFA inhibited the $I_{Na}$ in a concentration-dependent manner, with a 50% inhibitory concentration ($IC_{50}$) of 4.8 μM in neonatal rat cardiomyocytes (10), but only 0.51 ± 0.06 M in a human embryonic kidney (HEK) cell line, HEK293t, transiently expressing human myocardial sodium α-subunits, $hH1_\alpha$ (11). Inhibition occurred within seconds of application of the PUFA to the myocytes and was reversed as rapidly by the addition of delipidated bovine serum albumin to the perfusate. It was voltage dependent, but not use dependent, consistent with the lipophilic nature of PUFA (12). In both preparations, $I_{Na\alpha}$ in the rat cardiomyocyte and $I_{Na\alpha}$ in the human myocardial α-subunit (hH1) transiently expressed in HEK293t cells, PUFA caused a large voltage-dependent shift of the steady-state inactivation potential to more hyperpolarized values; the shift at $V_{1/2} = –19$ mV with 10 μM EPA in the neonatal rat cardiomyocyte and a further –27.8 mV with 5 μM EPA in the $hH1_\alpha$. There was no effect of PUFA on the activation of the $Na^+$ channels, only on the inactivated channel. PUFA prolonged the inactivated state of the $hH1_\alpha$ channels by speeding the transition from the activated to the inactivated state and retarding the slow inactivation phase of the channel. In more recent studies (13), the β1-subunit has been transiently coexpressed with the α-subunit in HEK293t cells to produce the $hH1_{\alpha\beta}$, and this shifted the steady-state inactivation potential to the right (to more depolarized potentials), returning the electrophysiology of the $hH1_\alpha$ channels back almost to identity with what we had observed for the neonatal rat cardiomyocytes. EPA was found to have no effect on the activation but only on the inactivation of the $Na^+$ currents, i.e., $I_{Na\alpha\beta}$, $I_{Na\alpha}$, and $I_{Na,rat}$. Consistent with the effects of these fatty acids solely on the inactivated state of the $hH1_{\alpha\beta}$ $Na^+$ channel is the finding that the binding or interaction of EPA to the inactivated state of the $Na^+$ channels displayed a 265-fold higher affinity for EPA than channels in the resting state of $hH1_{\alpha\beta}$ (13).

### *Current Hypothesis*

These effects of the n-3 PUFA (and DHA and α-linolenic acid do the same as EPA), we think, are pertinent to the antiarrhythmic actions of these fatty acids. Our current hypothesis (13) is that this voltage-dependent shift of the steady-state inactivation potential to more negative, hyperpolarizing voltages is important to the demonstrated antiarrythmic action of PUFA in ischemia-induced fatal arrhythmias. With a coronary thrombosis, there occurs a gradient of depolarizations of cardiomyocytes within the ischemic tissue. Cells in the central core of the ischemic tissue quickly depolarize and die due to lack of oxygen and metabolic substrates. Depolarization results from the dysfunctional state of Na,K-ATPase and the rise of interstitial $K^+$ concentrations in the ischemic tissue. But at the periphery of the ischemic zone, myocytes may be only partially depolarized. They become hyperexcitable because their resting membrane potentials become more positive, approaching the threshold for the gating of the fast $Na^+$ channel. Thus, any further small depolarizing stimulus (e.g., currents of injury) may elicit an action potential that may initiate an arrhythmia if it occurs out of phase with the electrical cycle of the heart. In the presence of the n-3 PUFA, however, a voltage-dependent shift of the steady-state inactivation potential to more hyperpolarized resting potentials occurs. The consequence of this voltage-dependent, hyperpolarizing shift is that the negative potential necessary to return these $Na^+$ channels from an inactive state to a closed resting, but activatable state, requires a physiologically unobtainable hyperpolarized resting membrane potential. Also these partially depolarized cardiomyocytes have $Na^+$ channels that can slip into "resting inactivation" from the closed resting state within milliseconds without eliciting an action potential (11). The

result of these two effects of the n-3 PUFA is that these partially depolarized myocytes are quickly eliminated from functioning, and their potential arrhythmic mischief is aborted. By contrast, myocytes in the nonischemic myocardium, with normal resting membrane potential, will not be so drastically affected by this voltage-dependent action of PUFA and will continue to function normally (13).

Disturbed regulation of cytosolic free calcium concentrations is another cause of malignant arrhythmias occurring in ischemia or resulting from a variety of cardiac toxins. Elevations of cytosolic calcium concentrations can result in tachyarrhythmias. The arrhythmias induced by some cardiac toxins mentioned (e.g., elevated extracellular $Ca^{2+}$, ouabain, or catecholamines) are examples of arrhythmias induced by excessive cytosolic $Ca^{2+}$ fluctuations. Such excessive cytosolic free $Ca^{2+}$ fluctuations can induce delayed after-potentials *in vivo*, which may trigger fatal arrhythmias if the after-potential occurs at a vulnerable moment in the electrical cycle of the heart. Because both current through the L-Ca gate ($I_{Ca,L}$) and sarcoplasmic reticulum release of $Ca^{2+}$ underlie many cardiac arrhythmias, together with Drs. A.M. Gomez and W.J. Lederer, the effects of PUFA on $I_{Ca,L}$ and $Ca^{2+}$ sparks were examined (14). Whole-cell voltage clamp techniques and confocal $Ca^{2+}$ imaging were used to determine the effects of PUFA on the voltage-gated L-type $Ca^{2+}$ current ($I_{Ca,L}$), elementary sarcoplasmic reticulum $Ca^{2+}$-release events ($Ca^{2+}$-sparks), and $[Ca^{2+}]_i$ transients in isolated adult rat ventricular myocytes. Extracellular application of EPA and the other antiarrhythmic PUFA, but not saturated or monounsaturated fatty acids produced a prompt and reversible concentration-dependent inhibition of $I_{Ca,L}$. The $IC_{50}$ of EPA was 0.8 μM for $I_{Ca,L}$ in neonatal rat heart cells and 2.1 μM in adult rat ventricular myocytes. Although the EPA-induced suppression of $I_{Ca,L}$ did not significantly alter the shape of the current-voltage relation, it did produce a small but significant negative shift of the steady-state inactivation curve ($\Delta V_{1/2}$ = –3 to –5 mV). The suppression of the $I_{Ca,L}$ by PUFA was voltage and time dependent but not use dependent. The effects of PUFA on $I_{Ca,L}$ resemble their effects on $I_{Na}$, except that the steady-state inactivation potentials for $I_{Ca,L}$ were shifted to the left to a much lesser degree.

When heart cells become "overloaded" with $Ca^{2+}$, they become arrhythmogenic and produce arrhythmogenic $I_{TI}$ currents and waves of elevated $[Ca^{2+}]_i$ that propagate within the heart cells. During the $Ca^{2+}$ overload, the ryanodine receptors (RyRs) also become more sensitive to the triggering process, producing an increased number of spontaneous $Ca^{2+}$ sparks and propagating waves of elevated $Ca^{2+}$, all of which can be viewed with the confocal microscope while measuring membrane current. Thus, it seems that our findings that the n-3 PUFA are potent inhibitors of $I_{Ca,L}$ and that this prevents the cytosolic $Ca^{2+}$ overload (14) appear to be the major mechanism by which the causes of these triggered arrhythmias evoked by ischemia or cardiac toxins are prevented by PUFA. Both repolarizing potassium currents, the initial fast outward current, $I_{to}$, and the slow, delayed rectifier current, $I_{ks}$, were also inhibited but less potently with $IC_{50}$ of 7.5 and 20 μM of DHA, respectively. The inward rectifier potassium current, $I_{k1}$, by contrast, was not affected by PUFA (Xiao, Y.-F. and Morgan, A.L., unpublished results). The net effect of inhibiting the two main repolarizing $K^+$ currents should result in prolongation of the action potential duration; however, the action potential was slightly but significantly shortened by PUFA. This action, together with the higher concentrations of PUFA required to inhibit these currents, makes us think that the effects of PUFA on $K^+$ currents do not play a significant role in their antiarrhythmic effects.

### *Effects of PUFA on the Brain*

Once we had found that the fatty acids affected the ion channels in the heart, the possibility that they likely affect the other excitable tissues, namely, skeletal muscles and the nervous system, was apparent. All excitable tissues utilize the same electrical signaling system generated by ionic currents through specific membrane ion channels, and these are highly conserved in voltage-dependent ion channels. It was the central nervous system (CNS) that interested us primarily. I turned to colleagues at the Epilepsy Institute at the University of Amsterdam, and Dr. Vreugdenhil and associates (15) performed whole-cell voltage-clamp experiments on isolated rat hippocampal CA1 neurons. They found the sodium and calcium channels in these neurons to be modulated by PUFA in a very similar way to what we had found in the heart but with the major effect being to prolong the inactivated state of these channels (15). A functional consequence of these electrophysiologic effects of PUFA in the CNS was then shown by Dr. R.A. Voskuyl *et al.* (16) at the University of Leiden in their rat model. They reported that the electrical threshold for generalized seizure activity was raised after the intravenous infusion of n-3 PUFA. Thus, an anticonvulsant action of PUFA occurs in the CNS. Once the modulation of the functions of cells of the nervous system has been demonstrated, we may expect other studies to report the effects of these fatty acids to modulate other important aspects of the nervous system and of behavior, for which we have mainly only descriptive explanations at present. As with the very rapid effects of the fatty acids on the heart, in the brain they also rapidly cross the blood–brain barrier and should reach all cells promptly (16).

### *Antiarrhythmic Clinical Benefits of n-3 PUFA*

Clinical evidence has been accumulating gradually, suggesting that the n-3 PUFA have antiarrhythmic effects in humans. There have been three encouraging secondary prevention trials that found suppression of sudden cardiac death in the experimental subjects ingesting n-3 fatty acids in their diet (17–19) and also one cohort-control study (20). Further work on the nervous system effects of these antiarrhythmic fatty acids may support our findings in the heart and, we hope, will be pursued by others.

In conclusion, it is apparent that there exists an influence on cardiac and other excitable tissues by common dietary fatty acids that has been largely overlooked. With ~250,000 sudden cardiac deaths annually in the United States, largely due to ventricular fibrillation, and millions more worldwide, there may be an enormous public health benefit from the practical application of this recent understanding. Initial reports suggest that n-3 PUFA are producing beneficial effects in the treatment of depression (21), bipolar, and other behavioral diseases (22). The knowledge that these fatty acids have direct physical effects on a fundamental property of the nervous system, namely, its electrical activity, should encourage further exploration of potential beneficial effects on brain functions both normal and pathologic. It seems likely that we are just scratching the surface of the potential health effects of these interesting dietary PUFA.

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# Myocardial Membrane Fatty Acids and the Antiarrhythmic Actions of Dietary Fish Oil in Animal Models

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**ABSTRACT:** Epidemiologic studies, animal studies, and more recently, clinical intervention trials all suggest a role for regular intake of dietary fish oil in reducing cardiovascular morbidity and mortality. Prevention of cardiac arrhythmias and sudden death is demonstrable at fish or fish oil intakes that have little or no effect on blood pressure or plasma lipids. In animals, dietary intake of fish oil [containing both eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)] selectively increases myocardial membrane phospholipid content of DHA, whereas low dose consumption of purified fatty acids shows antiarrhythmic effects of DHA but not EPA. Ventricular fibrillation induced under many conditions, including ischemia, reperfusion, and electrical stimulation, and even arrhythmias induced *in vitro* with no circulating fatty acids are prevented by prior dietary consumption of fish oil. The preferential accumulation of DHA in myocardial cell membranes, its association with arrhythmia prevention, and the selective ability of pure DHA to prevent ventricular fibrillation all point to DHA as the active component of fish oil. The antiarrhythmic effect of dietary fish oil appears to depend on the accumulation of DHA in myocardial cell membranes.

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Epidemiologic studies suggest that regular fish consumption may have a role to play in reducing cardiovascular disease morbidity and mortality. It is widely regarded that the long-chain n-3 fatty acids in fish oil are the active nutrients. A variety of cardiovascular effects attributable to fish oils and n-3 fatty acids could contribute, including reduction of blood pressure, antiplatelet actions, and lowering of circulating triglyceride concentrations. However, regular fish or fish oil consumption by patients who have survived a myocardial infarction was shown in 1989 to reduce mortality without attenuating blood pressure, plasma lipids, or new cardiac events (1). Reducing dietary fat or increasing fiber intake was ineffective, suggesting a specific role of the n-3 polyunsaturated fatty acids (PUFA). Postinfarction patients represent a cardiovascular disease population considered to be at very high risk of sudden cardiac death. The findings of the Diet and Reinfarction Trial (DART) (1), when considered in the light of contemporary animal studies (2), suggested a novel alternative mechanism for fish oil in reducing cardiovascular disease mortality, i.e., preventing fatal cardiac arrhythmias.

Publication of the DART results coincided with or preceded a series of clinical trials of class I antiarrhythmic drugs in similar, high-risk, postinfarction patients (3,4). Unlike fish oil, the antiarrhythmic drugs not only failed to prevent fatal arrhythmias, but the trials were halted early because of an increased incidence of sudden cardiac death in the treated groups compared with the placebo groups. More recently, the Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto Miocardico (GISSI) Prevenzione trial, a randomized controlled trial of >11,000 postinfarction patients, found a reduced incidence of sudden death after low-dose fish oil supplementation, thus confirming the earlier DART results and providing further support for a possible antiarrhythmic effect of fish oil (5). Sudden cardiac death from ventricular fibrillation remains a serious problem in medicine. This report reviews the data from animal studies that may provide evidence for or against an antiarrhythmic effect of regular fish oil consumption.

## *Fish Oil and Cardiac Arrhythmia*

Numerous studies demonstrate that dietary lipids can both influence the phospholipid fatty acid composition of myocardial cells and modulate cardiac arrhythmia vulnerability. Diets rich in PUFA of plant (n-6) or marine (n-3) origin reduce the incidence of fatal cardiac arrhythmias generated by acute regional myocardial ischemia in rats (6–9), by electrical stimulation and myocardial ischemia in the marmoset monkey (10), and by calcium or isoprenaline in isolated myocardial tissue (11,12). However, a comparison of the effects of fish oils and dietary vegetable oils indicates an enhanced antiarrhythmic effect of dietary fish oils beyond that attributable to their PUFA content alone (Fig. 1) (13). Fish oils typically contain 30–40% PUFA, mainly as eicosapentaenoic acid (EPA, 20:5n-3) or docosahexaenoic acid (DHA, 22:6n-3); yet without exception, they provide equal or greater antiarrhythmic protection than do equivalent dietary intakes of polyunsaturated vegetable oil, typically containing in excess of 60% PUFA as linoleic acid (18:2n-6) (2,13,14). The difference between the two fatty acid families is more decisively demonstrated in studies in which animals consumed different levels

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Abbreviations: DART, Diet and Reinfarction Trial; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acids.

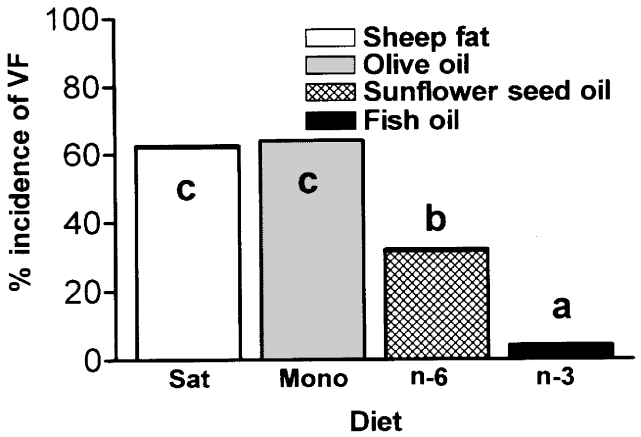


**FIG. 1.** Incidence of ventricular fibrillation (VF) during either 15 min of myocardial ischemia and subsequent reperfusion or during reperfusion after 5 min ischemia in rats after 6 wk of consuming a diet containing 10% sheep perirenal fat (open column, sat), olive oil (grey column, mono), sunflower seed oil (hatched column, n-6), or fish oil (closed column, n-3). Columns not sharing a common letter are significantly different ($\chi^2$, $P < 0.05$, $n = 24–25$).

of n-3 and n-6 PUFA within diets of equal total PUFA content. Marmoset monkeys consuming a diet containing n-3 PUFA required significantly higher threshold stimulation current in comparison to those consuming an n-6 diet to induce ventricular fibrillation electrically under both control and ischemic conditions or when vulnerability was further enhanced by β-adrenergic stimulation (15). In rats subjected to a similar dietary regimen, the antiarrhythmic effect of fish oil was maintained after the n-3 content was diluted by blending with a highly saturated animal fat; the antiarrhythmic effect of n-6 PUFA-rich vegetable oil was lost, however, after blending with the animal fat (16). Dietary fish oil, but not corn oil, also protects against fatal ventricular fibrillation induced by acute administration of a high dose of catecholamines in rats (17). Therefore, a specific antiarrhythmic effect of n-3 fatty acids is proposed, which can be demonstrated in a nonhuman primate, the marmoset monkey, as well as in the commonly used rat models of arrhythmia.

### *Fish Oil and Membrane Fatty Acids*

Modulation of dietary fat intake has marked effects on the fatty acid composition of myocardial cell membranes (18). The phospholipids that make up the myocardial cell membranes are highly responsive to the presence or absence of PUFA of the n-6 and n-3 families. Fish oils used in dietary studies vary considerably in their composition, both in respect to the total n-3 fatty acid content and in the relative concentrations of EPA and DHA (2,13–17,19–23). Studies consistently show that regardless of the type of fish oil used, whether it is high EPA or high DHA fish oil, DHA is the fatty acid that accumulates in myocardial phospholipids after fish oil consumption with very little change in EPA. It accumulates in exchange for monounsaturated oleic acid (18:1n-9) as well as the n-6 PUFA, linoleic acid (18:2n-6) and arachidonic acid (20:4 n-6) (Table 1).

The fatty acid profile of the cell membrane, although directly influenced by the fatty acid composition of the diet, is subject to some regulatory action. Thus, after 6 wk of consumption of a fish oil diet the plasma fatty acids varied from those in the diet, and the profile of heart phospholipid fatty acids differed markedly from those of both the plasma and the diet (Fig. 2). A diet containing 28% of the fat as EPA and 12% as DHA produces rat myocardial membranes containing <5% EPA and >20% DHA. A fish oil containing 12% EPA and 28% DHA can produce myocardial EPA levels of <1% and DHA levels >30%, whereas consumption of a fish oil–free diet will result in undetectable EPA levels and <10% DHA. Thus, the heart, like the brain, has the capacity to accumulate and retain high levels of DHA (21).

Although early studies often fed animals for 12 mon or more, the age of the animal is an important consideration because young control animals are less prone to fatal arrhythmias than are older animals (24). In adult rats, significant alterations in membrane composition occur within 2 d of fish oil feeding; in contrast, it takes 7–14 d of feeding to achieve maximal incorporation of EPA or DHA into the myocardial membrane of adult rats, whereas significant antiarrhythmic effects are achieved only after 7 d or more of feeding (Table

**TABLE 1**
**Major Unsaturated Fatty Acids in Myocardium of Rats Fed Olive Oil (control) for 4 wk or Olive Oil for 4 wk, Then Fish Oil for 2, 4, 7, or 14 d[a,b]**

| | 18:1n-9 OA | 18:2n-6 LA | 20:4n-6 AA | 20:5n-3 EPA | 22:6n-3 DHA | % rats with VF[c] |
|---|---|---|---|---|---|---|
| Control | 10.6 ± 0.2 | 17.0 ± 0.8 | 24.9 ± 0.3 | ND | 10.3 ± 0.4 | 58 |
| 2 d | 6.3 ± 0.9 | 13.3 ± 0.7 | 21.9 ± 0.9 | 0.44 ± 0.02 | 19.2 ± 1.5 | |
| 4 d | 5.8 ± 0.9 | 12.1 ± 0.9 | 18.4 ± 0.6 | 0.55 ± 0.06 | 24.1 ± 1.2 | 45 |
| 7 d | 3.4 ± 0.1 | 9.8 ± 0.2 | 15.8 ± 0.4 | 0.71 ± 0.01 | 30.8 ± 0.3 | 25[d] |
| 14 d | 4.5 ± 0.3 | 7.7 ± 0.3 | 16.1 ± 0.5 | 0.67 ± 0.01 | 30.5 ± 0.8 | |

[a]Data represent means ± SEM of individual fatty acids as a percentage of total fatty acid content ($n = 5$/group).
[b]OA, oleic acid (18:1n-9); LA, linoleic acid (18:2n-6); AA, arachidonic acid (20:4n-6); EPA, eicosapentaenoic acid (20:5n-3); and DHA, docosahexaenoic acid (22:6n-3).
[c]The percentage of rats that developed ventricular fibrillation (VF) during acute myocardial ischemia in control group or after 4 or 7 d fish oil feeding.
[d]$P < 0.05$ vs. control, $\chi^2$ ($n = 12$ per group).

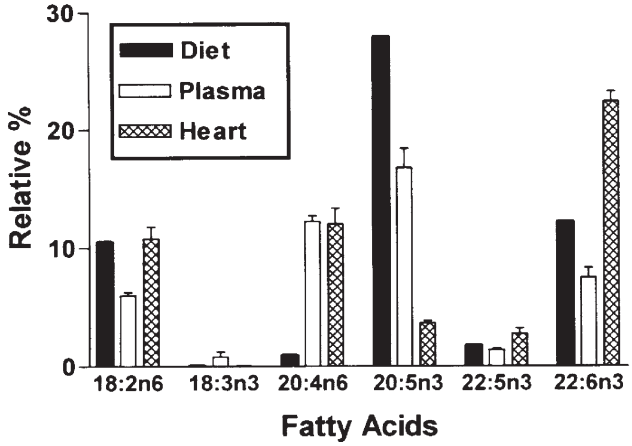


**FIG. 2.** Polyunsaturated fatty acid composition of the rat diet (open columns), plasma phospholipid (closed columns), or myocardial phospholipid (hatched columns) in rats after 6 wk fish oil consumption. Bars represent the mean ± SEM, n = 5.

1). Direct comparison of DHA and EPA shows that when rats are fed pure n-3 fatty acids at levels of <0.5% of the diet by weight, DHA is potently antiarrhythmic, whereas EPA is not (25). Under these conditions, consumption of pure EPA is associated with only a small elevation in myocardial DHA levels and an even smaller increase in myocardial EPA. Combining the data from many studies, there is an apparent linear relationship between the dietary intake of DHA and its accumulation in the myocardium (Fig. 3). Studies to date suggest that in rats, a myocardial membrane DHA composition >18% is required for antiarrhythmic effects during myocardial ischemia, whereas <16% is not protective.

In conclusion, in view of the wide variety of both fish oil diets and animal models used in published studies, the results permit unequivocal interpretation that dietary fish oil fatty acids are antiarrhythmic and in particular prevent ventricular

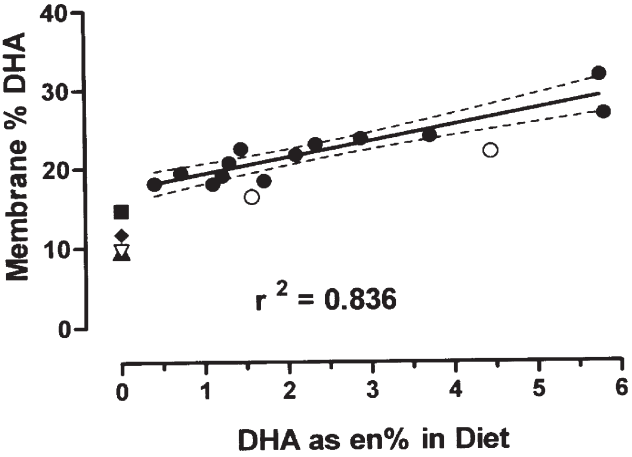


**FIG. 3.** Relationship between dietary intake of docosahexaenoic acid (DHA) as percentage of energy (en%) in the diet and myocardial phospholipid fatty acid composition of rats (closed symbols) or marmoset monkeys (open symbols) fed fish oil or pure DHA- (circles), eicosapentaenoic acid (EPA)- (square), or canola oil– (diamond) supplemented diets or control diets (triangles). Feeding periods of fish oil diets ranged from 5 to 52 wk. Line represents linear line of best fit ± 95% confidence limits for rats fed fish oil or DHA.

fibrillation believed to be so often responsible for sudden cardiac death in humans. The preferential accumulation of DHA in myocardial cell membranes, the association of its accumulation with arrhythmia prevention, and the selective ability of pure DHA to prevent ventricular fibrillation all point to DHA as the active component of dietary fish oil. The antiarrhythmic effect of dietary fish oil observed *in vivo* appears to depend on the myocardial membrane accumulation of DHA.

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# n-3 Fatty Acids, Heart Rate Variability, and Sudden Cardiac Death

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**ABSTRACT:** Sudden cardiac death (SCD) is a major cause of mortality in Western countries. Furthermore, SCD is often the first manifestation of coronary artery disease, making it difficult to prevent. Heart rate variability (HRV), which can be determined by extended recording of the heart rate by 24-h Holter monitoring, has been shown to be one of the best predictors of the risk of SCD. There is increasing evidence from animal experiments and clinical trials in humans that n-3 fatty acids reduce the risk of SCD. We have studied the effect of n-3 fatty acids on HRV and present data clearly showing that n-3 fatty acids increase HRV. This adds further to the hypothesis that an increased intake of n-3 fatty acids may reduce the risk of SCD.

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This chapter describes the possible protective effect of marine n-3 polyunsaturated fatty acids (PUFA) against sudden cardiac death (SCD) in humans. First, some facts are given regarding SCD, stressing the serious effect of this major problem. Then predictors of SCD will be addressed with emphasis on heart rate variability (HRV). Finally, human studies showing a beneficial effect of n-3 PUFA on HRV will be introduced together with a brief review of studies showing a reduced risk of SCD in humans with a modest intake of n-3 PUFA.

## *Sudden Cardiac Death*

SCD accounts for >300,000 deaths annually in the United States (1). If the definition of SCD is restricted to death within 2 h from onset of symptoms, 12% of all natural deaths have been classified as sudden and >80% of these death are due to cardiac disease (2). Thus, diffuse coronary atherosclerosis constitutes the most common underlying substrate for SCD.

Despite this significant reduction in cardiovascular mortality rate during the past decades, the proportion of patients suffering from SCD has remained constant. Because SCD is responsible for ~50% of the mortality from cardiovascular disease in Western countries (3), attention has been paid to identify persons at risk. However, such identification is flawed by the fact that SCD is often the first manifestation of coronary heart disease (CHD). In other words, although it may be possible to identify high-risk patients and implant cardiodefibrillators, these patients constitute only a very small percentage of the total number at risk for SCD. Therefore, a substantial further reduction in death from acute coronary events can be achieved only from measures preventing death before arrival at the hospital or by reducing the incidence of CHD.

## *Risk Factors for SCD with Emphasis on HRV*

Gender is a major risk factor for SCD because 75% of SCD occur in men. Other traditional risk factors are age, hypertension and left ventricular hypertrophy, hypercholesterolemia, glucose intolerance and diabetes, decreased vital capacity, smoking, and heart rate. Many of these factors have been identified from the Framingham Study (4).

A major independent predictor of SCD is left ventricular dysfunction (5). Also, the presence of ventricular arrhythmias such as premature ventricular depolarizations (PVD) predicts SCD in patients with CHD especially if PVD exceed 10/h in 24-h Holter recordings (6). Other electrocardiogram (ECG) analyses used in predicting SCD are QT prolongation, QT dispersion (7), signal-averaged ECG, and heart rate turbulence (8).

A decreased 24-h HRV is one of the strongest independent predictors for SCD and arrhythmic events (9–11), and HRV is likely the ideal parameter with which to assess intervention therapies because it is stable and free of any placebo effects (11). HRV is considered to represent a noninvasive tool with which to assess cardiac autonomic tone at the level of the sinus node and, thus, indices of HRV provide a window onto autonomic modulation of the heart. Increased HRV normally reflects increased parasympathetic tone, whereas a decreased HRV may reflect a sympathetic predominance.

In population studies, decreased HRV has had predictive value for mortality among healthy adults. It is a well-established risk factor for arrhythmic events and mortality among postmyocardial infarction (MI) patients, and many interventions with drugs leading to increased HRV are also associated with better survival rates (12).

There are many mechanisms precipitating SCD. However, activation of the sympathetic nervous system is essential, leading to a decreased HRV, an increase in sympathetic tone,

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Abbreviations: CHD, coronary heart disease; CRF, chronic renal failure; DART, Diet and Reinfarction Trial; ECG, electrocardiogram; HRV, heart rate variability; MI, myocardial infarction; PUFA, polyunsaturated fatty acids; PVD, premature ventricular depolarizations; SCD, sudden cardiac death; SDNN, standard deviation of all normal RR intervals during 24 h.

increasing blood pressure, shear forces, heart rate, platelet aggregation, blood viscosity, and lowering of the ventricular fibrillation threshold. Such changes increase the likelihood of plaque rupture or erosion and platelet aggregation, resulting in ischemic or electrical SCD (13). Increasing evidence suggests that sympathetic hyperactivity during the abrupt rupture of a vulnerable plaque resulting in thrombotic occlusion of a coronary artery favors the genesis of life-threatening ventricular tachyarrhythmias, whereas vagal activation exerts an antiarrhythmic effect (14). The importance of a decreased HRV in post-MI patients has been further substantiated in the recent ATRAMI trial, which included >1200 post-MI patients. HRV was found to carry significant independent information regarding prediction of death (10). Furthermore, apparently healthy subjects subsequently suffering SCD have a decreased HRV (15).

### *Effects of n-3 PUFA on the Risk of SCD in Humans*

The Diet and Reinfarction Trial (DART) was the first study with death as an end point, which suggested an antiarrhythmic effect of n-3 PUFA and thereby a protection against SCD (16). In this trial men with a previous MI who were advised to eat fatty fish at least twice a week had a 29% reduction in mortality after 2 yr compared with patients not given this dietary advice. The survival curves for the two groups separated quite early during the followup, which argued against an antiatherosclerotic effect of n-3 PUFA, and the increased incidence of nonfatal MI in the group given fish advice argued against an antithrombotic effect. On the basis of the emerging evidence for an antiarrhythmic effect of n-3 PUFA in animals, such an effect was suggested as an explanation of the DART results.

A population-based case-control study with 334 cases of primary cardiac arrest and 493 controls found that dietary intake of n-3 PUFA from seafood equal to one fatty fish meal per week was associated with a 50% reduction in the risk of primary cardiac arrest compared with no intake of n-3 PUFA (17). Furthermore, in a subgroup of patients, the levels of n-3 PUFA in red blood cells were measured and the authors divided the subjects into quartiles according to the content of n-3 PUFA in the cell membranes. In comparing the subjects from the lowest quartile with those from the highest quartile, a 70% reduction in the risk of SCD was observed. Thus, there was clearly a decrease in the risk of SCD with increasing cellular levels of n-3 PUFA.

In line with this, the results from the U.S. Physicians' Health Study in which 20,551 male physicians were followed for up to 11 yr showed that consumption of fish once a week was associated with a 52% reduction in the risk of SCD compared with men who consumed fish less than monthly (18). A more frequent intake of fish did not reduce the risk of SCD further.

In a trial from India, 360 patients with suspected MI were randomized to daily supplementation with ~1.1 g of n-3 PUFA, 2.9 g of α-linolenic acid, or placebo (19). Cardiac events were significantly reduced after 1 yr with both active treatments compared with placebo, but cardiac deaths were reduced only in the fish oil group. These patients were not entirely comparable to post-MI patients in Western countries with respect to standard medical treatment because only a very small fraction of these Indian patients received thrombolytic therapy, and the randomization failed to balance the proportion of diabetics and the extent of coronary disease among groups.

Recently, the large GISSI Prevenzione trial including more than 11,000 post-MI patients was published (20). Nearly 3,000 of these patients were given one fish oil capsule daily (0.85 g of n-3 PUFA) for 3.5 yr and compared with a control group of equal size. n-3 PUFA significantly reduced overall mortality and cardiovascular mortality. Moreover, n-3 PUFA supplementation resulted in a striking 45% reduction in the incidence of SCD compared with controls. As in the DART trial, the survival curves in the GISSI Prevenzione trial concerning the n-3 PUFA group controls separated quite early and the difference became significant after only 90 d (Marchioli, R., personal communication). This argues for an antiarrhythmic effect of n-3 PUFA caused by the incorporation of n-3 PUFA into cardiac cell membranes.

Overall, these studies demonstrate that dietary intake of n-3 PUFA clearly is associated with a reduction in SCD. This again points to an antiarrhythmic effect of n-3 PUFA in humans, although such an effect is not addressed directly in these studies with hard end points.

### *n-3 PUFA and the Risk of SCD Assessed by HRV*

In a series of human studies, the potential antiarrhythmic effect of n-3 PUFA has been addressed by means of 24-h HRV. High-risk patients ($n = 55$) with a previous MI and left ventricular dysfunction (ventricular ejection fraction <40%) were randomized to either 5.2 g of PUFA daily or control for 12 wk (21). At baseline, a positive significant correlation ($r = 0.30$, $P < 0.05$) was found between levels of n-3 fatty acids in cell membranes (platelets) and HRV (22). Furthermore, patients who had at least one fish meal per week tended to have a higher HRV than patients not eating fish. After dietary intervention with n-3 PUFA, the important HRV parameter SDNN [standard deviation of all normal RR intervals during 24 h] increased significantly from 115 ms at baseline to 124 ms ($P = 0.01$) after n-3 PUFA supplementation, strongly indicating an antiarrhythmic effect of n-3 PUFA (21). Post-MI patients with the lowest HRV were at the highest risk of SCD, and if the patients with a baseline SDNN < 100 ms were analyzed separately, SDNN increased from 85 ± 16 to 102 ± 21 ms after supplementation with n-3 PUFA ($P = 0.006$). Thus, in this high-risk subgroup, the effect of n-3 PUFA on SDNN was most pronounced.

Patients with chronic renal failure (CRF) are also at high risk of malignant ventricular arrhythmias. In 29 CRF patients, a strong positive association between granulocyte levels of n-3 PUFA and HRV was found, indicating a possible antiarrhythmic effect of n-3 PUFA also in these patients (23).

The relationship between the dose of n-3 PUFA and effect on HRV in healthy subjects has also been investigated (24). Healthy volunteers ($n$ = 60) were allocated to 2.0 g of n-3 PUFA, 6.6 g of n-3 PUFA acids daily, or control. The dietary supplementation was given for 12 wk. At baseline, there was a highly significant positive correlation between marine n-3 fatty acid levels in cell membranes and HRV in men, whereas this association was absent among women. Figure 1 shows the correlation between the docosahexaenoic acid content in platelets and SDNN in men. After dietary intervention, a significant dose-dependent increase in HRV was observed among the men and no effect on HRV was seen in women. The increase in HRV was most pronounced for healthy men with a low baseline HRV. In Table 1, HRV indices are given before and after dietary supplementation with n-3 PUFA for those men belonging to the lowest tertile with respect to SDNN at baseline. Thus, this study showed that even in healthy men, it is possible to increase HRV. This may be of importance because an attenuated HRV in healthy subjects increases the risk of SCD later in life (15).

Recently, HRV was examined in 295 patients referred for coronary angiography (25). HRV was positively associated with the content of n-3 PUFA in both cell membranes and adipose tissue. Furthermore, multiple regression analysis revealed that n-3 PUFA–related factors independently correlated with HRV. An accompanying Editorial stated that, given the safety and low cost of implementing a recommendation for a modest amount of fish in the diet, adequate dietary fish intake has a significant role to play in the primary and secondary prevention of out-of-hospital SCD (26).

Also, in patients with type 1 diabetes mellitus, a significant positive correlation between HRV and n-3 PUFA in cell membranes was observed (27), suggesting that n-3 PUFA supplementation might be beneficial in these patients at high risk of SCD.

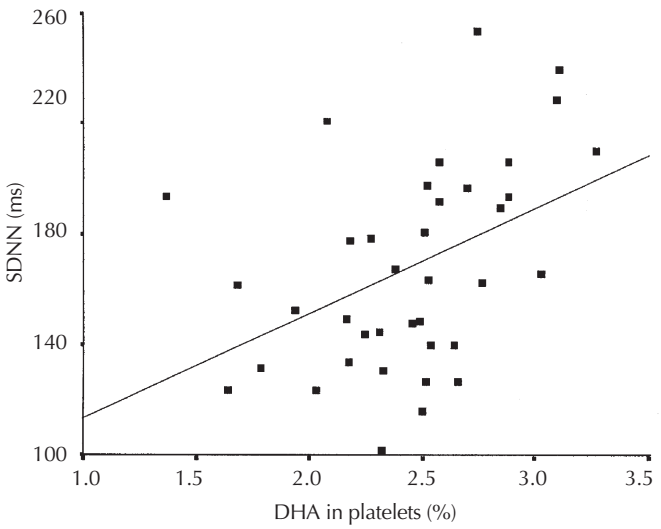


**FIG. 1.** The relation between the level of docosahexaenoic acid (DHA) in platelets and the heart rate variability parameter SDNN (standard deviation of all normal RR intervals during the 24-h recording) in healthy men; $r = 0.48$, $P < 0.01$.

**TABLE 1**
**Heart Rate Variability Indices Before and After 12 wk of Dietary Supplementation with n-3 PUFA (2.0 or 6.6 g) in Healthy Men with a Low Baseline SDNN[a,b]**

| | Before n-3 PUFA | After n-3 PUFA | P-value |
|---|---|---|---|
| RR (ms) | 751 (67) | 796 (93) | 0.03 |
| SDNN (ms) | 130 (9) | 142 (20) | 0.03 |
| SDANNindex (ms) | 120 (18) | 134 (27) | 0.04 |
| RMSSD (ms) | 26 (7) | 32 (9) | 0.01 |
| PNN50 (%) | 6 (5) | 10 (6) | 0.03 |

[a]Values are means (SD).
[b]Abbreviations: RR, mean of all normal RR intervals during the 24-h recording; SDNN, standard deviation of all normal RR intervals in the entire 24-h recording; SDANNindex, the standard deviation of the mean RR intervals measured in successive 5-min periods; RMSSD, the square root of the mean of the sum of the squares of differences between adjacent intervals; pNN50, the percentage of successive RR interval differences ≥50 ms.

In conclusion, the close association between cellular levels of n-3 PUFA and HRV in humans and the fact that dietary supplementation with n-3 PUFA increases HRV support an antiarrhythmic effect of n-3 PUFA in humans, and may explain in part the ability of dietary n-3 PUFA to reduce the risk of SCD. Further intervention studies are warranted in patients with ischemic heart disease as well as in other high-risk groups such as patients with diabetes mellitus.

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# Efficacy of n-3 Polyunsaturated Fatty Acids After Myocardial Infarction: Results of GISSI-Prevenzione Trial

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**ABSTRACT:** Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio (GISSI)-Prevenzione was conceived as a population, pragmatic trial on patients with recent myocardial infarctions conducted in the framework of the Italian public health system. In GISSI-Prevenzione, patients were invited to follow Mediterranean dietary habits, and were treated with up-to-date preventive pharmacological interventions. Long-term n-3 PUFA (1 g daily) but not vitamin E (300 mg daily,) was beneficial for death and for combined death, nonfatal myocardial infarction, and stroke. All the benefit, however, was attributable to the decrease in risk for overall, cardiovascular, cardiac, coronary, and sudden death.

At variance with the orientation of a scientific scenario largely dominated by the "cholesterol–heart hypothesis," GISSI-Prevenzione results indicate n-3 PUFA (virtually devoid of any cholesterol-lowering effect) as a relevant pharmacological treatment for secondary prevention after myocardial infarction.

As to the relevance and comparability of GISSI-Prevenzione results, up to 5.7 lives could be saved every 1000 patients with previous myocardial infarction treated with n-3 PUFA (1 g daily) per year. Such a result is comparable to that observed in the Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) trial, where 5.2 lives could be saved per 1000 hypercholesterolemic, coronary heart disease patients treated with pravastatin for 1 yr.

The choice of a relatively low-dose regimen (1-g capsule daily) more acceptable for long-term treatment in a population of patients following Mediterranean dietary habits, and the pattern of effects seen in GISSI-Prevenzione (namely, reduction of overall mortality with no decrease in the rate of nonfatal myocardial infarction) all strongly suggest that n-3 PUFA treatment should be considered a recommended new component of secondary prevention. The importance of this combined/additive effect is further suggested by the analyses of the interplay between diet and n-3 PUFA: There is an interesting direct correlation between size of the effect and "correctness" of background diets. It can be anticipated that a conceptual barrier must be overcome: A "dietary drug" should be added to "dietary advice," which remains fundamental to allow this statement to become true in clinical practice.

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The importance of n-3 polyunsaturated fatty acids (PUFA) in human health was noted 20 years ago by Bang and co-workers (1) who suggested that it was the high n-3 fatty acid content in Eskimo's diets that accounted for the low rate of ischemic heart disease. Some years later, Kromann and Green (2) reported on the low mortality of Greenland Inuit from ischemic heart disease and other chronic diseases. Since then, many studies have been carried out on the role on n-3 PUFA in health and disease and in growth and development; most of the studies however, have focused on the prevention and management of cardiovascular disease (3–17).

From the wealth of information made available by studies that explored and supported the antiatherogenic and antithrombotic effects of n-3 PUFA, it now appears that these fatty acids are able to stabilize myocardial membranes electrically, reducing susceptibility to ventricular dysrhythmias and consequently the risk of sudden death (18–31). The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione trial (32–34) was conceived at the beginning of the last decade as a large-scale, population-based clinical trial of patients with recent myocardial infarction, aimed at testing the effectiveness of the administration of the following: (i) n-3 PUFA, which are virtually devoid of any cholesterol-lowering effect, and have also been suspected of increasing the susceptibility of low density lipoprotein particles to oxidative phenomena (35); and (ii) vitamin E, a promising antioxidant substance, sustained by a wealth of experimental and epidemiologic data (36–55). The purpose of this paper was to provide updated data on the GISSI-Prevenzione trial and help clarify some methodological issues raised by the study.

## PATIENTS AND METHODS

### *Study Design*

The study design has been described previously (33,34). Briefly, patients with recent (3 mon) myocardial infarction were enrolled. Eligible patients had no known contraindica-

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Abbreviations: ACE, angiotensin-converting enzyme; CHD, coronary heart disease; CI, confidence interval; DART, Diet and Reinfarction Trial; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GISSI, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico; PUFA, polyunsaturated fatty acids.

tions to the dietary supplements, were able to provide informed written consent, and had no unfavorable short-term outlook (e.g., overt congestive heart failure or cancer). Age limits were not defined; the clinicians' decision to include elderly subjects in the study depended merely on the expectation of potential benefits in the light of the patient's clinical condition.

A multicenter, open-label design, in which patients were randomly allocated to four treatment groups was adopted. In the absence of evidence for preferred doses of treatments, we decided on daily doses of n-3 PUFA as one gelatine capsule containing 850–882 mg eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as ethyl esters, and 300 mg vitamin E, given as one capsule of synthetic α-tocopherol; these doses used existing available formulations to help maintain compliance in patients already receiving many other long-term treatments. Patients were randomly assigned n-3 PUFA alone ($n$ = 2835), vitamin E alone ($n$ = 2830), n-3 PUFA and vitamin E combined ($n$ = 2830), or no supplement (control, $n$ = 2828). Patients were asked to adhere to recommended preventive treatments, i.e., aspirin, β-blockers, and inhibitors of angiotensin-converting enzyme (ACE) (statins were not supported by definitive data on efficacy when the trial was started). The treatment assigned had to be continued until the end of follow-up. As an "open" study, it was recommended that participating cardiologists provide the same care and treatment attitude for all patients, regardless of their allocation.

Trial procedures were planned to mimic as closely as possible the routine care after a myocardial infarction. Follow-up visits were scheduled at 6, 12, 18, 30, and 42 mon; these included clinical assessment and the administration of a food-frequency questionnaire. The primary combined efficacy end points were the following: the cumulative rate of all-cause death, nonfatal myocardial infarction, and nonfatal stroke; and the cumulative rate of cardiovascular death, nonfatal myocardial infarction, and nonfatal stroke. We did secondary analyses for each component of the primary end points and for the main causes of death. Subgroup analyses for women and patients aged >70 yr were prespecified in the protocol. The validation of the clinical events included in the primary end points was assured by an *ad hoc* committee of expert cardiologists and neurologists who were not aware of patients' treatment assignment.

*Revision of the database.* After the main results of GISSI-Prevenzione were published (34), more updated information was retrieved through the investigators, census offices, and hospital records. The availability of more information on clinical events allowed further completion of the validation of events included in the main end point. In particular, various unknown causes of death, previously included in the item "other deaths," could be correctly attributed and validated, whereas some nonfatal major cardiovascular events were rejected. Finally, a careful check of the database was performed and at the end of the revision, a patient erroneously randomized twice by a center was identified and eliminated from the analysis.

*Statistical methods.* According to the protocol, follow-up data were right-censored at 42 mon, when follow-up information on the vital status of patients was available for 99.9% of the population through clinical visits or census offices. The analysis was done by intention to treat and according to the main strategy defined in the protocol, i.e., a four-way analysis of efficacy of n-3 PUFA supplements, vitamin E supplements, and the combined treatments compared with controls, as well as the efficacy of the combined treatment compared with individual interventions. According to the possibility of

**TABLE 1**
**Baseline Characteristics of Randomized Patients[a]**

| | | All ($n$ = 11323) |
|---|---|---|
| Male/female | | 9,658 (85.3%)/ 1,665 (14.7%) |
| Age (y) | ≤50 | 2,324 (20.5%) |
| | 51–60 | 3,395 (30.0%) |
| | 61–70 | 3,756 (33.2%) |
| | 71–80 | 1,627 (14.4%) |
| | >80 | 221 (2.0%) |
| Time from AMI to randomization (d) | <10 | 2,964 (26.2%) |
| | 10–15 | 2,604 (23.0%) |
| | 16–30 | 2,577 (22.8%) |
| | ≥30 | 3,178 (28.1%) |
| Secondary diagnoses | | |
| Arterial hypertension | | 4,026 (35.6%) |
| Diabetes mellitus | | 1,683 (14.9%) |
| Nonsmokers | | 2,499 (22.2%) |
| Ex-smokers | | 3,935 (35.0%) |
| Smokers | | 4,808 (42.8%) |
| Body mass index > 30 kg/m$^2$ | | 1,644 (14.7%) |
| Previous myocardial infarction | | 1,357 (12.1%) |
| Claudication | | 501 (4.4%) |
| Angina grade (CCVS) | | |
| No angina | | 6,729 (60.9%) |
| No limitation (I) | | 3,596 (32.6%) |
| Slight limitation (II) | | 509 (4.6%) |
| Severe limitation (III)/at rest (IV) | | 209 (1.9%) |
| Dyspnea grade (NYHA) | | |
| No dyspnea | | 3,829 (34.1%) |
| No limitation (I) | | 6,252 (55.7%) |
| Dyspnea on normal/mild exertion (II–III) | | 1,136 (10.1%) |
| Ejection fraction | ≤0.30 | 249 (2.6%) |
| | 0.31–0.40 | 1,071 (11.1%) |
| | >0.40 | 8,319 (86.3%) |
| Premature ventricular beats | >10/h | 1,068 (13.5%) |
| Previous sustained ventricular tachycardia | | 73 (0.9%) |
| Ventricular arrhythmias | | 1,876 (23.5%) |
| Positive exercise-stress test | | 2,137 (28.9%) |
| Mean (SD) characteristics | | |
| Age | | 59.3 (10.6) |
| Time since diagnosis of AMI (d) | | 25.1 (20.9) |
| Body mass index (kg/m$^2$) | | 26.5 (3.7) |
| Ejection fraction | | 52.6 (10.6) |
| Total blood cholesterol (mg/dL) | | 210.8 (42.1) |
| LDL cholesterol (mg/dL) | | 137.4 (38.0) |
| HDL cholesterol (mg/dL) | | 41.5 (11.5) |
| Triglycerides (mg/dL) | | 162.0 (85.6) |

[a]AMI, acute myocardial infarction; CCVS, Canadian Cardiovascular Society; NYHA, New York Heart Association; LDL, low density lipoprotein; HDL, high density lipoprotein. In some sections, numbers do not add up because of missing values.

**TABLE 2**
**Dietary Habits and Main Therapeutic Interventions at Baseline and During Study[a]**

| | All ($n$ = 11323) |
|---|---|
| Dietary habits | |
| Fish (1 time/wk) | |
| Baseline | 8,212 (73.2%) |
| 6 mon | 8,616 (86.3%) |
| 42 mon | 6,558 (87.7%) |
| Fruit (once daily) | |
| Baseline | 9,009 (80.3%) |
| 6 mon | 8,679 (87.3%) |
| 42 mon | 6,546 (88.0%) |
| Fresh vegetables (once daily) | |
| Baseline | 4,460 (39.7%) |
| 6 mon | 5,303 (53.1%) |
| 42 mon | 4,085 (54.5%) |
| Olive oil (regularly) | |
| Baseline | 8,258 (73.6%) |
| 6 mon | 7,939 (79.6%) |
| 42 mon | 6,161 (82.5%) |
| Pharmacological therapy | |
| Antiplatelet drugs | |
| Baseline | 10,309 (91.0%) |
| 6 mon | 9,098 (87.8%) |
| 42 mon | 6,699 (82.9%) |
| Angiotensin-converting enzyme inhibitors | |
| Baseline | 5,280 (46.9%) |
| 6 mon | 4,235 (40.9%) |
| 42 mon | 3,162 (39.1%) |
| β-Blockers | |
| Baseline | 4,986 (44.3%) |
| 6 mon | 4,272 (41.2%) |
| 42 mon | 3,108 (38.4%) |
| Cholesterol-lowering drugs | |
| Baseline | 534 (4.7%) |
| 6 mon | 3,109 (28.6%) |
| 42 mon | 3,934 (45.5%) |
| Revascularization procedures[b] | |
| CABG or PTCA | |
| Baseline | 560 (5.0%) |
| 6 mon | 1,783 (15.8%) |
| 42 mon | 2,723 (24.0%) |

[a]CABG, coronary artery bypass; PTCA, percutaneous transluminal coronary angioplasty. In some sections numbers do not add up because of missing values. Patients alive at baseline, $n$ = 11,323; 6 mon, $n$ = 11,089; and 42 mon, $n$ = 10,081.
[b]Number and percentage of patients revascularized during the study are cumulative.

conducting a two-way factorial analysis as well as of exploring interaction between treatments, we fit multivariate models including the two experimental treatments and the interaction term. If significant, the latter indicates effect modification when the two treatments are given together (56–58). All $P$-values are from two-sided tests for significance.

## RESULTS

The characteristics of GISSI-Prevenzione patients are shown in Tables 1 and 2. Subjects in GISSI Prevenzione appeared to be a relatively low-risk population of acute myocardial infarction survivors recruited early after the index event, i.e., 50% of subjects were recruited within 16 d after myocardial infarction, mean age (± SD) was 59 ± 11 yr; 16% were >70 yr old; 15% were women; and 14% had an ejection fraction ≤40%. Total blood cholesterol levels were almost normally distributed at recruitment with a mean of 211 ± 42 mg/100 mL. Arterial hypertension, diabetes mellitus, and claudication were present in 36, 15, and 4% of patients, respectively; 43% of patients smoked before the index events and only one third of them were still smoking thereafter.

Patients recruited into the study received life style recommendations, and up to date preventive interventions. At the end of the study, in addition to the drugs tested in the trial, antiplatelet drugs, β-blockers, ACE-inhibitors, and lipid-lowering drugs were prescribed to 83, 38, 39, and 46% of patients, respectively. Finally, 5% of patients had coronary artery bypass graft or angioplasty procedures before recruitment and a total of 24% of patients had been revascularized at the end of the study.

### *The Four-Way Analysis*

The full profile of the effects of n-3 PUFA is summarized in Table 3. In the four-way analysis, the 15% relative decrease in risk for the combined primary end point of death, nonfatal myocardial infarction, and nonfatal stroke [95% confidence interval (CI), 2–6, $P$ = 0.022] and the decrease in risk for the other combined end point of cardiovascular death, nonfatal myocardial infarction, and nonfatal stroke (20%, 95% CI, 6–32, $P$ = 0.006) were significant. Analyses of the individual components of the main end point showed that the decrease in mortality (20% of total deaths, 30% of cardiovascular deaths, and 44% of sudden deaths) obtained with n-3 PUFA accounted for all of the benefit seen in the combined end point. There was no difference across the treatment groups for nonfatal cardiovascular events. The results for the combined treatment compared with controls on the primary combined end point and on total mortality were consistent with those obtained with n-3 PUFA alone. No increased benefit was apparent when the rate of the combined end point of death, nonfatal myocardial infarction, and nonfatal stroke among patients receiving n-3 PUFA plus vitamin E was compared with that of the group receiving n-3 PUFA alone or with that of patients treated with vitamin E alone.

Treatment with n-3 PUFA alone significantly lowered the risk of total coronary heart disease (CHD) events (0.78 [0.65–0.94], $P$ = 0.008), whereas the risk reduction observed either with vitamin E alone or with the combined treatment was not significant. No change in fatal plus nonfatal stroke was found for any tested treatment. No change in the patients' risk of undergoing coronary artery bypass surgery or angioplasty was observed for n-3 PUFA or for vitamin E treatment. Patients receiving vitamin E and controls did not differ significantly when data were analyzed for the combined end point and for total mortality.

**TABLE 3**
**Overall Efficacy Profile of n-3 PUFA, Vitamin E, and n-3 PUFA + Vitamin E Treatments[a,b]**

| | Rate of events | | | | Four-way analysis | | | Two-way analysis with interaction term | Two-way analysis without interaction term |
|---|---|---|---|---|---|---|---|---|---|
| | n-3 PUFA ($n$ = 2835) | Vitamin E ($n$ = 2830) | n-3 PUFA plus vitamin E ($n$ = 2830) | Control ($n$ = 2828) | n-3 PUFA relative risk (95% CI) | Vitamin E relative risk (95% CI) | n-3 PUFA + vitamin E relative risk (95% CI) | n-3 PUFA relative risk (95% CI) | n-3 PUFA relative risk (95% CI) |
| Main endpoints | | | | | | | | | |
| Death, nonfatal MI, and nonfatal stroke | 358 (12.6%) | 376 (13.3%) | 360 (12.7%) | 419 (14.8%) | 0.85 (0.74–0.98) | 0.89 (0.78–1.03) | 0.85 (0.74–0.98) | 0.85 (0.74–0.98) | 0.90 (0.81–0.99) |
| CVD death, nonfatal MI, and nonfatal stroke | 266 (9.4%) | 291 (10.3%) | 290 (10.3%) | 330 (11.7%) | 0.80 (0.68–0.94) | 0.88 (0.75–1.03) | 0.87 (0.74–1.02) | 0.80 (0.68–0.94)* | 0.89 (0.79–0.99) |
| Secondary analyses | | | | | | | | | |
| All fatal events | 239 (8.4%) | 255 (9.0%) | 238 (8.4%) | 299 (10.6%) | 0.79 (0.67–0.94) | 0.85 (0.72–1.00) | 0.79 (0.67–0.94) | 0.79 (0.67–0.94) | 0.85 (0.76–0.97) |
| CVD death | 144 (5.1%) | 166 (5.9%) | 166 (5.9%) | 204 (7.2%) | 0.70 (0.56–0.86) | 0.81 (0.66–0.99) | 0.81 (0.66–0.99) | 0.70 (0.56–0.86)** | 0.83 (0.72–0.97) |
| Cardiac death | 113 (4.0%) | 134 (4.7%) | 134 (4.7%) | 172 (6.1%) | 0.65 (0.51–0.82) | 0.78 (0.62–0.97) | 0.77 (0.62–0.97) | 0.65 (0.51–0.82)** | 0.80 (0.68–0.95) |
| Coronary death | 101 (3.6%) | 112 (4.0%) | 108 (3.8%) | 146 (5.2%) | 0.68 (0.53–0.88) | 0.76 (0.60–0.98) | 0.73 (0.57–0.94) | 0.68 (0.53–0.88)** | 0.80 (0.67–0.97) |
| Sudden death | 52 (1.8%) | 62 (2.2%) | 59 (2.1%) | 92 (3.3%) | 0.56 (0.40–0.79) | 0.67 (0.49–0.93) | 0.64 (0.46–0.88) | 0.56 (0.40–0.79)** | 0.72 (0.56–0.91) |
| Other death | 95 (3.3%) | 89 (3.1%) | 72 (2.5%) | 95 (3.3%) | 0.99 (0.74–1.31) | 0.93 (0.70–1.24) | 0.78 (0.55–1.02) | 0.99 (0.74–1.31) | 0.90 (0.73–1.11) |
| Unknown cause | 35 (1.2%) | 34 (1.2%) | 24 (0.9%) | 51 (1.8%) | 0.68 (0.44–1.04) | 0.66 (0.43–1.02) | 0.47 (0.29–0.76) | 0.47 (0.29–0.76) | 0.69 (0.49–0.96) |
| Non-CVD death | 60 (2.1%) | 55 (1.9%) | 48 (1.7%) | 44 (1.6%) | 1.35 (0.91–1.98) | 1.24 (0.84–1.85) | 1.08 (0.72–1.63) | 1.35 (0.91–1.98) | 1.08 (0.82–1.42) |
| Nonfatal CVD events | 138 (4.9%) | 144 (5.1%) | 141 (5.0%) | 139 (4.9%) | 0.98 (0.78–1.24) | 1.03 (0.82–1.30) | 1.01 (0.80–1.27) | 0.98 (0.78–1.24) | 0.98 (0.83–1.15) |
| Nonfatal MI | 104 (3.7%) | 120 (4.2%) | 119 (4.2%) | 113 (4.0%) | 0.91 (0.70–1.18) | 1.06 (0.82–1.37) | 1.05 (0.81–1.35) | 0.91 (0.70–1.18) | 0.95 (0.79–1.14) |
| Nonfatal stroke | 37 (1.3%) | 27 (1.0%) | 25 (0.9%) | 30 (1.1%) | 1.22 (0.75–1.98) | 0.90 (0.53–1.51) | 0.83 (0.49–1.40) | 1.22 (0.75–1.98) | 1.08 (0.75–1.55) |
| Subsidiary analyses | | | | | | | | | |
| CHD death and nonfatal MI | 198 (7.0%) | 224 (7.9%) | 220 (7.8%) | 251 (8.9%) | 0.78 (0.65–0.94) | 0.89 (0.74–1.06) | 0.87 (0.73–1.04) | 0.78 (0.65–0.94)* | 0.87 (0.77–0.99) |
| Fatal and nonfatal stroke | 50 (1.8%) | 37 (1.3%) | 42 (1.5%) | 40 (1.4%) | 1.24 (0.82–1.87) | 0.92 (0.59–1.44) | 1.04 (0.67–1.60) | 1.24 (0.82–1.87) | 1.19 (0.88–1.60) |
| PTCA or CABG | 588 (20.7%) | 538 (19.0%) | 584 (20.6%) | 575 (20.3%) | 1.01 (0.90–1.14) | 0.93 (0.82–1.04) | 1.02 (0.90–1.14) | 1.01 (0.90–1.14) | 1.005 (0.97–1.14) |

[a]Relative risk, calculated by Cox regression analysis.
[b]Abbreviations: CVD, cardiovascular disease; MI, myocardial infarction; CHD, coronary heart disease; PTCA, coronary angioplasty; CABG, coronary artery bypass graft.
[c]Patients with two or more events of different types appear more than once in columns but only once in rows.
*$P < 0.10$; **$P < 0.05$ for the interaction term exploring the effects of the two treatment combined.

### *The Two-Way Analysis*

As stated in the protocol, GISSI-Prevenzione was originally conceived and designed as a four-arm clinical trial aimed at recruiting 12,000 patients to allow the comparison of four groups (i.e., n-3 PUFA, vitamin E, and their combination, vs. a control group with standard treatment only) of 3000 patients each over a 3-yr follow-up. The GISSI-Prevenzione design also allowed analyses to be conducted according to the factorial approach (two-way) with or without adjustment for potential interaction between tested interventions. We decided to formally present both analyses (four- and two-way), to allow readers to compare them and judge for themselves the reliability of a statistical analysis of a factorial design when complete independence of either treatment effects or outcomes is not assured.

The two-way analysis adjusted for interaction gave exactly the same results as the four-way analysis and clearly modified all of the point estimates of n-3 PUFA effects on the various study outcomes that were observed in the two-way analysis without adjustment for interaction. The difference between the two-way analyses including or excluding the interaction term reflects the effect modification due to patients receiving both experimental treatments. The interaction term almost reached significance for cardiovascular mortality plus nonfatal myocardial infarction and stroke, and reached the formal level of significance for cardiovascular, cardiac, and sudden death. Such results clearly indicate the need of appropriately allowing for interaction and confounding in the analyses as well as the correctness of the four-way analysis on the basis of both the *a priori* expectations and *post hoc* facts.

## DISCUSSION

GISSI-Prevenzione was a secondary prevention, pragmatic, population trial aimed at assessing the effect of promising treatments on top of accepted preventive interventions such as modification of lifestyle and dietary habits, and pharmacologic therapy (antiplatelets, β-blockers, ACE-inhibitors), in the framework of the clinical practice of a country-wide network of hospitals within the Italian national public health service. The size of the network (about half of the Italian cardiology departments) and of the recruited population, the pragmatism of

the study design aimed at not interfering with clinical practice, and the widespread use of effective preventive interventions assure the transferability of its results to patients with myocardial infarction. In addition to the results of the Diet and Reinfarction Trial (DART), several epidemiologic studies [e.g., Multiple Risk Factor Intervention Trial (MRFIT), Western Electric Study, Zutphen study] suggested that even a low dietary fish intake was associated with a clear reduction of cardiovascular risk in non-Mediterranean countries compared with no intake. GISSI-Prevenzione further extended this finding, i.e., n-3 PUFA, administered as a drug in a population exposed to recommended and Mediterranean dietary habits (also including fish) and to preventive pharmacologic interventions, were able to lower the risk of death in patients with recent myocardial infarction. It is worth noting, in addition, that the results of preliminary subgroup analyses suggest no interaction among correctness of diet, n-3 PUFA administration, and risk of death (data not shown). If any, a somewhat greater, nonsignificant effect was observed in n-3 PUFA–treated subjects with better dietary habits or higher fish intake.

In terms of the number of subjects needed to be treated to save a life, this lower risk of death corresponds to 46.7 (95% CI, 27.2–162.4) subjects to be treated for 3.5 yr or to 163.4 (95% CI, 95.4–568.3) subjects to be treated per year. The latter estimates compare favorably with the results of recent clinical trials, if the correct benefit per year is calculated. For instance, the benefit of pravastatin in the Long-term Intervention with Provastatin in Ischaemic Disease (LIPID) trial was 32.3 (95% CI, 22.4–57.7) subjects to be treated for 6.1 y or 197.0 (95% CI, 136.6–353.0) subjects to be treated per year (59). Such estimates underline the need to consider the duration of follow-up in comparing absolute measures of benefit of different trials. At variance with relative measures of effect, absolute measures of effect are greatly influenced by both the duration of follow-up and the background absolute risk of recruited populations (the latter can be estimated correctly from the rate of events per year in the control group).

GISSI-Prevenzione results were consistent with existing scientific evidence. Several prospective cohort studies found an inverse relationship between n-3 PUFA from fish intake and CHD death. The Zutphen study found that men with low or no fish intake had a higher rate of CHD compared with men who consumed fish one or more times per week (7). The 30-y follow-up in the Western Electric Study, in the Multiple Risk Factor Interventional trial and in the Honolulu Heart Program confirmed this finding (8–10). According to the per-protocol four-way analysis of GISSI-Prevenzione, both primary combined end points were significantly lowered (two-tailed $P < 0.0179$ and $< 0.006$) with n-3 PUFA treatment (by 16 and 20%, respectively). In addition, total mortality (i.e., the most important end point) was 21% lower in patients receiving n-3 PUFA treatment (two-tailed $P = 0.0064$). It is worth underlining that mortality can hardly be considered a "secondary" outcome in any clinical trial. Finally, cardiovascular, cardiac, coronary, and sudden death were significantly decreased by n-3 PUFA treatment.

The results obtained with n-3 PUFA are consistent with those of the DART trial (14). They found a 29% decrease over 2 y in overall mortality in men who ate fatty fish twice a week, with no decrease in the rate of nonfatal myocardial infarction. In addition to the results of the DART trial, the Lyon Diet Heart study (11,15) and the Indian trial by Singh and colleagues (12,13) strongly suggest a protective effect of n-3 PUFA. In the 20,551 male patients of the U.S. Physicians' Health Study, regular fish consumption (≥1 fish meal/wk) conferred a 52% risk-adjusted reduction in sudden deaths compared with fish intake <1 time/mo (6). As in GISSI-Prevenzione, a significant reduction of total mortality paralleled by no modification of nonfatal myocardial infarction was observed. In the U.S. Health Professionals Study a nonsignificant reduction of non-fatal myocardial infarction and CHD death was observed (5). Siscovick *et al.* (25) reported that little or no fish intake (or low n-3 PUFA blood levels) was associated with an increased risk of sudden cardiac death compared with regular fish consumption. However, not all epidemiologic studies have documented benefits from fish intake. In the Alpha-Tocopherol, Beta Carotene cancer study, a nonsignificant excess of risk-adjusted CHD was found in the highest quintile of intake of n-3 PUFA (2.5 g/d) compared with the lowest (0.9 g/d). This is not the only puzzling result of this study, however; the saturated fatty acid intake was also associated with a significantly lower risk of CHD death (60). A recent European study was unable to show that increased levels of adipose tissue DHA were cardioprotective (61). In contrast, Seidelin *et al.* (62) showed an inverse association between adipose tissue DHA levels and coronary artery stenosis.

At the inception of the GISSI-Prevenzione study, information on the antiarrhythmic effects of n-3 PUFA was available, albeit limited to some animal experiments. n-3 PUFA treatment was supposed to act through a modification of the mechanisms related to thrombosis, inflammation, endothelial dysfunction, and atherosclerosis, with the true mechanism of action not known. The triglyceride-lowering effect was not considered important at the dose of n-3 PUFA used in the study and in a mixed population comprised mainly of normolipidemic subjects. During the course of the study, however, new information became available strongly supporting an antidysrhythmic effects of n-3 PUFA, especially for preventing fatal ischemia-induced malignant ventricular rhythms (20). Consistent with the results of the DART trial and with the wealth of epidemiologic and experimental evidence on the antiarrhythmic effects of n-3 PUFA, the nonsignificant results of GISSI-Prevenzione on nonfatal myocardial infarctions along with the significant results on total mortality and sudden death, all strongly suggest an effect of n-3 PUFA on electrical stability of cell membranes.

As to the study design, several recent clinical trials were designed as factorial trials and therefore analyzed according to the two-way approach (63–65). The Physicians' Health Study, for instance, was a correctly designed factorial trial, i.e., aspirin was supposed to act through its antiplatelet activ-

ity on the risk of thrombotic events, whereas β-carotene was supposed to act as an antioxidant to lower the risk of cancer (56,57). Therefore both the outcome measures and the hypothetical biological effects of the treatments were completely independent in this trial. A factorial design allows a clinical trial to include substantially fewer patients than would be required to test two hypotheses separately. Such optimization, however, has a price. The price is to forgo testing the efficacy of the two combined treatments and to aim only at assessing the independent effect of the two treatments. The latter issue, however, is particularly important. In fact, the following two statistical requirements must be fulfilled to test two treatments with a factorial design: (i) the two treatments must have independent effects (i.e., their mechanisms of action should be different and independent of each other); and (ii) the outcome measures (i.e., end points) should be different and independent. If both requirements were fulfilled in GISSI-Prevenzione, then a total of 6900 patients would have been required according to pretrial expectations to carry out a $2 \times 2$ factorial clinical trial with the same power ($1\text{-}\beta = 98\%$ and $= 0.05$). The independence of the outcomes, however, was not fulfilled in GISSI-Prevenzione, whereas the independence of pharmacologic mechanisms of actions known when the study was started (see below) was at least questionable.

Because the main outcome measure of GISSI-Prevenzione (total mortality plus nonfatal myocardial infarction and nonfatal stroke) was significantly lowered according to both statistical approaches, the difference between the 4- and 2-way analyses is only a quantitative issue with respect to the size of effect observed. The size of the benefit, in fact, was greater in the four-way than in the two-way analysis. The four-way analysis, which avoids the possible interference of the interaction of effects between treatments, should be seen as the per-protocol approach showing the "true" results.

In conclusion, GISSI-Prevenzione was conceived as a population-based, pragmatic trial on patients with recent myocardial infarction conducted in the framework of the Italian public health system. Long-term n-3 PUFA (1 g/d), but not vitamin E (300 mg/d), was beneficial for death and for combined death, nonfatal myocardial infarction, and stroke. All of the benefit, however, was attributable to the decrease in risk for overall, cardiovascular, cardiac, coronary, and sudden death. Up to 5.7 lives could be saved every 1000 patients with previous myocardial infarction treated with n-3 PUFA (1 g/d) each year. It is worth noting that n-3 PUFA therapy was given to patients following Mediterranean dietary habits, which are characterized by a high intake of fruit, vegetables, olive oil, and with almost 90 and 55% of subjects consuming fish at least 1 and 2 times/wk, respectively. This issue is particularly important, because dietary recommendations given to GISSI-Prevenzione patients were remarkably similar to the dietary guidelines recently proposed by the American Heart Association for reducing the risk of cardiovascular disease (66). At variance from the orientation of a scientific scenario largely dominated by the "cholesterol-heart hypothesis," GISSI-Prevenzione results indicate that n-3 PUFA (virtually devoid of any cholesterol-lowering effect) represent a relevant pharmacologic treatment for secondary prevention after myocardial infarction. Treatment with n-3 PUFA should be considered a recommended new component of secondary prevention. It can be anticipated that a conceptual barrier must be overcome, i.e., a "dietary drug" should be added to "dietary advice," which remains fundamental to allow this statement to become true in clinical practice (67).

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# n-3 Polyunsaturated Fatty Acids and Cardiovascular Diseases: To Whom, How Much, Preparations

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**ABSTRACT:** An expert round table discussion on the relationship between intake of n-3 polyunsaturated fatty acids (PUFA) mainly of marine sources and coronary heart disease at the 34th Annual Scientific Meeting of European Society for Clinical Investigation came to the following conclusions:

1. Consumption of 1–2 fish meals/wk is associated with reduced coronary heart disease (CHD) mortality.
2. Patients who have experienced myocardial infarction have decreased risk of total, cardiovascular, coronary, and sudden death by drug treatment with 1 g/d of ethylesters of n-3 PUFA, mainly as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The effect is present irrespective of high or low traditional fish intake or simultaneous intake of other drugs for secondary CHD prevention. n-3 PUFA may also be given as fatty fish or triglyceride concentrates.
3. Patients who have experienced coronary artery bypass surgery with venous grafts may reduce graft occlusion rates by administration of 4 g/d of n-3 PUFA.
4. Patients with moderate hypertension may reduce blood pressure by administration of 4 g/d of n-3 PUFA.
5. After heart transplantation, 4 g/d of n-3 PUFA may protect against development of hypertension.
6. Patients with dyslipidemia and or postprandial hyperlipemia may reduce their coronary risk profile by administration of 1–4 g/d of marine n-3 PUFA. The combination with statins seems to be a potent alternative in these patients.
7. There is growing evidence that daily intake of up to 1 energy% of nutrients from plant n-3 PUFA (α-linolenic acid) may decrease the risk for myocardial infarction and death in patients with CHD. This paper summarizes the conclusions of an expert panel on the relationship between n-3 PUFA and CHD. The objectives for the experts were to formulate scientifically sound conclusions on the effects of fish in the diet and the administration of marine n-3 PUFA, mainly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and eventually of plant n-3 PUFA, α-linolenic acid (ALA, 18:3n-3), on primary and secondary prevention of CHD. Fish in the diet should be considered as part of a healthy diet low in saturated fats for everybody, whereas additional administration of n-3 PUFA concentrates could be given to specific groups of patients. This workshop was organized on the basis of questions sent to the participants beforehand, on brief introductions by the participants, and finally on discussion and analysis by a group of ~40 international scientists in the fields of nutrition, cardiology, epidemiology, lipidology, and thrombosis.

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### *Fish and Coronary Heart Disease*

To highlight the benefit of fish intake in low- as well as high-risk populations, cross-sectional studies on Inuits and Danes and later different Japanese populations have suggested that fish consumption is protective against coronary heart disease (CHD) (1,2). In a recent review of all prospective cohort studies examining the relationship between fish intake and CHD mortality and including >115,000 individuals, the authors concluded that fish consumption is not associated with a lower rate of CHD mortality in low-risk populations, whereas fish consumption at 40–60 g daily is associated with markedly reduced CHD mortality in high-risk populations (3). The most striking observation was the high CHD mortality in people not eating fish at all and the significant reduction in those with an average fish intake of 1–2 meals per week. The mechanisms related to this effect of fish consumption are not clear; however, epidemiologic and clinical studies suggest that antiarrhythmic effects from the consumption of fish and marine n-3 polyunsaturated fatty acids (PUFA) may be of significance (4,5). No inverse association between fish intake and fatal CHD was found in other prospective studies (3). This may have been related to a higher overall fish intake by subjects in these studies. However, confounding factors related to nutritional and genetic variables may be involved.

Clinical, secondary prevention trials including dietary supplementation of n-3 PUFA from marine sources *via* two meals of fatty fish per week or a Mediterranean, α-linolenic acid–rich diet showed a decrease in the risk of myocardial infarction and death (6,7). The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI) Prevenzione Trial, including >11,000 patients with recent myocardial infarction (MI), showed a preventive effect both on total and cardiovascular, coronary, and sudden death, with n-3 PUFA treatment (1 g/d) given on top of Mediterranean dietary habits and currently recommended secondary prevention treatments (8). Special attention should be given to patients after coro-

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Abbreviations used: CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA. eicosapentaenoic acid; GISSI, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico; MI, myocardial infarction; PUFA, polyunsaturated fatty acids.

nary artery bypass (CAB) surgery. Graft occlusion is a common complication, and there are indications that vein graft occlusion frequency may be reduced by n-3 PUFA (9).

### *n-3 PUFA and Cardiovascular Risk Factors*

In a series of intervention studies, it has been well documented that administration of n-3 fatty acids has beneficial effects on patients with moderate hypertension, on patients with hypertension developing after heart transplantation, and on patients with dyslipidemia and severe postprandial hyperlipemia, all independent risk factors for developing CHD (10–12). The effects on these risk factors, as well as effects on platelet endothelial cell interaction and myocardial cells (14,15), may explain the clinical effects of n-3 PUFA and may also point out subgroups of the population for whom supplementation of n-3 PUFA on a regular basis should be considered.

### *To Whom?*

On the basis of present knowledge we recommend the following as candidates for administration of long-chain n-3 PUFA: (i) patients with myocardial infarction; (ii) patients with hypertriglyceridemia; (iii) and patients with CAB vein grafts, moderate hypertension, decreased heart rate variability, increased platelet reactivity, and after heart transplantation should also be considered for treatment.

### *How Much Should Be Given?*

The initial studies suggesting a relationship between n-3 PUFA and a low incidence of CHD were performed in population groups with diets that contained very high amounts of n-3 PUFA and were also low in saturated and high in monounsaturated fatty acids. In addition, the diets were usually low in n-6 PUFA. The prospective cohort studies have documented that in the Western world with a "traditional" Western diet, the most striking effects have been observed in high-risk populations with an intake of fish equal to 1–2 meals of fatty fish per week (~0.6 g/d).

Both prospective, randomized secondary prevention studies based on increased intake of fatty fish in addition to other healthy dietary habits, or supplementation of the diet with n-3 PUFA, seem to indicate that ~1 g/d of these fatty acids is beneficial (6,8). However, no larger secondary prevention studies using higher dosages have been performed. Subgroup analysis of the GISSI Prevenzione Study (8) showed that the effect of n-3 PUFA supplementation is present irrespective of currently prescribed pharmacologic treatments for secondary prevention after MI. Similarly, the effect was present in subjects eating no fish and in those consuming ≥2 fish meals/wk (R.M.).

With regard to α-linolenic acid, the Lyon Heart Study (7) included 0.61 energy% of this acid in the diet. Supplementation of both plant and marine n-3 PUFA may be required due to their different physiologic effects (for review see Ref. 15). On the basis of nutritional studies, a recommendation for α-linolenic acid of 1 energy% has been suggested. However, more studies are required to evaluate the optimal daily intake with regard to cardiopreventive effects.

Most studies that have evaluated the effect of marine n-3 PUFA on cardiovascular risk factors such as hypertension, dyslipidemia, and postprandial hyperlipemia have shown effects of a daily administration of 3–4 g PUFA (EPA 49%, DHA 35%).

### *Which Preparations?*

There was general agreement in the group that fish should be the first choice as a source for marine n-3 PUFA, including at least two fish meals per week for everyone as part of normal, healthy dietary habits. Administration of n-3 PUFA preparations given at a daily dosage of 1–4 g should be considered in patients post-MI and to other patient groups for whom the use of n-3 PUFA is indicated, especially if the patient is not able to eat rich fish meals.

Because consumption of n-3 PUFA has been associated with a higher susceptibility to oxidation of low density lipoproteins, it is likely that use of concentrated n-3 PUFA products should be accompanied by adequate amounts of antioxidants such as α-tocopherol (16).

In conclusion, in population groups with a high risk for CHD, there is good scientific evidence to recommend intake of n-3 PUFA both from plant and marine sources as part of otherwise healthy dietary habits. One–two fatty fish meals per week is recommended. In patients with coronary heart disease or a risk profile that includes hypertension or hypertriglyceridemia for CHD, higher dosages up to ~4 g/d of marine n-3 PUFA should be used. Of total energy, 1 energy% should come from α-linolenic acid.

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